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THE ROLE OF NUCLEAR FACTORS OF ACTIVATED T-CELLS (NFAT)
IN NEURONAL DEATH

By

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M.S., Agricultural University of Warsaw, 2001

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THE ROLE OF NUCLEAR FACTORS OF ACTIVATED T-CELLS (NFAT)
IN NEURONAL DEATH

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ABSTRACT

The Role of Nuclear Factors of Activated T-cells (NFAT) in Neuronal Death

Agata M. Habas

November 29, 2004

Nuclear Factor of Activated T-cells (NFAT) is a calcium-responsive transcription factor that orchestrates immune response and cardiovascular development. NFAT is also present in neurons and glia from the Central Nervous System (CNS) where it participates in calcium signaling. In physiological conditions, neuronal NFAT may contribute to the plasticity-related transcriptional regulation. On the other hand, excessive activation of calcium signaling and the subsequent overactivation of NFAT may contribute to neuronal death. To explore the possibility of NFAT-involvement in neuronal death I have determined which components of NFAT signaling are expressed in cultured rat hippocampal or cortical neurons. I found the presence of mRNAs for NFATc1, c3, c4 and c5. I also found that in primary neurons, NFAT transcriptional activity is increased by KCl-mediated depolarization or by chemical trophic deprivation induced by a PI3K/Akt signaling inhibitor LY294002. This transcriptional activity may be blocked by calcineurin inhibitors: cyclosporin A and FK506 and ERK1/2 pathway inhibitor, U0126. Interestingly, overexpression of NFATc4 potentiated apoptosis induced by trophic deprivation in cortical neurons. In summary, these data support the idea that excessive activation of NFAT may produce neuronal death following trophic deprivation. In future, I plan to study the mechanism of NFAT involvement in neuronal death.

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CHAPTER I

INTRODUCTION

The cells of a multicellular organism are members of a highly organized community in which the number of cells is regulated by the proliferation or the rate of cell death. Mature neurons are non-proliferating cells so their number is regulated mostly by cell death. Neurons can undergo cellular suicide by a process known as programmed cell death or apoptosis. Cell death plays a very important role during development and also in adult organism. Mouse paws for example, are sculpted by cell death during embryonic development (Wood et al., 2000; Alberts et al., 2002). When a tadpole changes into a frog, the cells in the tail die, and the tail, which is not needed in the frog disappears (Wood et al., 2000; Alberts et al., 2002). In the developing nervous system, up to half or more of the nerve cells normally die soon after they are formed (Wood et al., 2000; Alberts et al., 2002). In this case, cell death adjusts the number of nerve cells to match the number of target cells that require innervation (Wood et al., 2000; Alberts et al., 2002). In adult tissues, cell death balances cell division (Alberts et al., 2002). If the physiological cell death is inhibited, cells normally destined to die, survive, leading to autoimmune conditions and cancer (Li and Yuan, 1999; Alberts et al., 2002). On the other hand, excessive cell death may contribute to several pathological conditions, including stroke and neurodegenerative diseases (Fig. 1) (Putcha and Johnson, 2004). Neurons may die in pathological conditions in diseases including Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, several forms of cerebellar degeneration, spinal muscular atrophy, and Alzheimer's disease (Hutchins and Barger, 1998; Sastry and Rao, 2000; Yuan et al., 2003). Several factors, such as oxidative stress, excitatory toxicity, inappropriate Ca^{2+} homeostasis,

mitochondrial dysfunction, and lack of sufficient supply of neurotrophic factors, have been shown to contribute to cell death (Sastry and Rao, 2000).

Figure 1. Regulation of homeostasis. Some diseases characterized by defective cell death (left) or excessive cell death (right).

Neuronal cell death may be divided into at least 2 types: apoptosis and necrosis (Petersen et al., 1999; Sastry and Rao, 2000; Habas et al., 2001). Necrosis is a passive process, mostly seen in pathologies and is generally pro-inflammatory (Raff et al., 1993; Habas et al., 2001; Kanduc et al., 2002). It is characterized by early swelling of intracellular organelles followed by swelling and loss of the plasma and nuclear membrane integrity (Petersen et al., 1999; Sastry and Rao, 2000). Apoptosis is a response to a specific pro-apoptotic signal or weak damage that involves specific biochemical events frequently including *de novo* gene expression in a dying cell (Raff et al., 1993; Vaux, 1993; Habas et al., 2001).

The characteristics of apoptosis include cytoplasmic condensation, nuclear pyknosis, chromatin condensation, DNA fragmentation, cell rounding, membrane blebbing, cytoskeletal collapse, and formation of membrane bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or neighboring cells (Sastry and Rao, 2000; Yuan et al., 2003) (Table 1). Apoptotic neuronal death may play an im-

portant role in several pathologies in CNS. Defects in the control of apoptosis leading to excessive programmed cell death may contribute to stroke, ischemia, Traumatic Brain Injury (TBI), Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD) (Choi, 1996; Ekshyyan and Aw, 2004; Gibson, 2001; Behl, 2000; Martin, 2001).

Apoptosis is a process that requires active cellular events (Pittman et al., 1993; Kanduc et al., 2002). Therefore, apoptosis takes longer to kill than the passive necrosis and may provide a better therapeutic window to perform neuro-protective manipulations (Kanduc et al., 2002).

Programmed cell death may be inhibited either by activation of anti-apoptotic or by inhibition of pro-apoptotic signaling pathway.

Table 1 Comparative features of necrosis and apoptosis (Sastry and Rao, 2000).

Necrosis	Apoptosis
Altered membrane permeability; Potassium loss; sodium entry; fall in membrane potential	Cytosol condensation
Swelling of all cytoplasmic compartments	Generally intact organelles
Destruction of mitochondria and other organelles	Protruberances from cell surface separate to form apoptotic bodies
Depletion of cellular energy (ATP)	No depletion of cellular energy
Lowered macromolecular synthesis	Macromolecular synthesis activation is required
Affects tracts of contiguous cells	Affects scattered individual cells
Loose aggregates of chromatin	Highly condensed granular aggregates of chromatin

There are two major pathways leading to apoptotic death. They both employ activation of caspases and proteases. The first pathway can be initiated by DNA damage or trophic deprivation (TD). The death signal is sent through signaling molecules including p53 or JNK to mitochondria from where cytochrome c is released to the cytoplasm (Polster et al., 2004; Harada and Grant, 2003). The key regulators of cy-

cytochrome c release are proteins from Bcl-2 family including anti-apoptotic members like Bcl-2, Bcl-X_L, Bag-1 and members that induce apoptosis: Bax, Bad and Bid. Cytochrome c leads to formation of an active complex composed of a protein Apaf-1 and a protease caspase-9. In this complex, caspase-9 becomes active and stimulates other caspases including caspase-3, -6 and -7 that execute apoptosis (Fig. 2A) (Green and Reed, 1998; Robertson and Orrenius, 2000; Tsujimoto and Shimizu, 2000).

The second mechanism of programmed cell death includes pro-apoptotic signaling molecules known as death receptors such as p75 NGF receptor, TNFR1, Fas/CD95/Apo-1, TRAIL (TNF-related apoptosis inducing ligands), DR4, and DR5 (Krammer, 2000; Nagane et al., 2000). After binding to their ligands, death receptors activate caspase 8 or 10. This is followed by the activation of caspases -3, -6 and -7 (Fig. 2B). Therefore, in both apoptotic pathways, programmed cell death occurs via caspase-mediated cleavage of multiple proteins leading to DNA fragmentation, destruction of cellular structures and formation of apoptotic bodies (Thornberry and Lazebnik, 1998; Tsujimoto and Shimizu, 2000; Habas et al., 2001).

Figure 2. Various pathways of apoptosis. A. Mitochondrial pathway of apoptosis, B. Death receptor pathway of apoptosis.

In summary, programmed cell death is needed for the proper function of each organism. Apoptosis is an important factor in various diseases. Both increased or

decreased apoptosis may result in pathologies. Therefore, identification of apoptotic regulation may provide new opportunities for therapies against such disorders as cancer or neurodegeneration.

Damaging stimuli in the nervous system

In the nervous system, various damaging stimuli activate signaling pathways that trigger cell death including apoptosis. Calcium influx, oxidative stress, DNA damage and TD are thought to be important cell death triggers in neurodegenerative diseases, traumatic injuries, stroke or ischemia (Simonian and Coyle, 1996; Mason et al., 1999; Petersen et al., 1999; Ghatan et al., 2000; Hugon et al., 2000; Lewen et al., 2001; Mattson, 2003).

It is known that intracellular Ca^{2+} overload can trigger either necrotic or apoptotic cell death (Orrenius and Nicotera, 1994; Vajda, 2002). Development of strategies that will block apoptotic and non-apoptotic death induced by Ca^{2+} influx is important as it may lead to the effective neuroprotection. Several effects of Ca^{2+} are mediated by Ca^{2+} -binding proteins (e.g. calmodulin or calcineurin) (Orrenius and Nicotera, 1994). Cell death caused by calcium overload may be induced by the excessive stimulation of glutamate receptors. Two classes of glutamate receptors have been identified: (1) ionotropic glutamate receptors, which are ligand gated ion channels, and (2) metabotropic glutamate receptors, which are known as G-protein coupled receptors (Uings and Farrow, 2000). Excessive stimulation of the ion channels of N-methyl D-aspartate subtype of ionotropic glutamate receptors (NMDA receptors) leads to robust increase of Ca^{2+} load inside the cell and necrotic or apoptotic cell death (Orrenius and Nicotera, 1994; Vajda, 2002). On the other hand, moderate calcium influx through NMDA receptors plays a key role in neuronal development, survival and plasticity (Ghosh and Greenberg, 1995).

Neurons require the presence of survival factors to suppress cell death. Therefore,

TD induces neuronal apoptosis, which in CNS may play a significant role in neurodegenerative diseases, fetal alcohol syndrome and neurotrauma (Hetman and Xia, 2000; McIntosh et al., 1998; Bhave et al., 1999). It may also play a role in cell death during development of the nervous system (Li et al., 2001). A number of survival factors for neurons have been identified including serum, insulin like growth factor-1, neurotrophins and NMDA. These factors activate the phosphatidylinositol-3 kinase (PI3K) pathway, which use one of several signal transduction pathways implicated in the survival of neurons (Hetman and Xia, 2000). In primary neuronal culture, TD may be induced by serum withdrawal or by inhibition of PI3K pathway using its chemical inhibitor, LY294002 (Hetman and Xia, 2000).

The role of transcription factors in neuronal death

Apoptotic signaling in neurons leads to activation of killer gene expression that is mediated by several transcription factors like p53 or Forkhead box transcription factor, class O (FOXO) (Morrison and Kinoshita, 2000; Brunet et al., 2001). FOXO induces death receptors like Fas and FasL, or TRAIL (TNF-related apoptosis inducing ligands), Tumor Necrosis Factor-alpha (TNF α) and its receptor (Brunet et al., 2001). Another transcription factor HIF (Hypoxia Inducible Factor) and p53, which are activated after oxidative stress, promote transactivation of pro-apoptotic bcl-2 family members leading to neuronal death (Halterman and Federoff, 1999). NF κ B (Nuclear Factor κ B) is a transcription factor, which is activated in response to inflammation or oxidative stress. NF κ B may serve a dual role in stimulating pathways that protect neuronal cells from death by activation of its target genes, Bcl-2 or Bcl-x and also in stimulating pathways that promote cell death by induction of TRAIL and other death receptors (Tamatani et al., 2000; Wajant, 2004). CCAAT enhancer binding proteins (c/EBPs) are transcription factors that play a role in cell survival in non-neuronal cells, while in neurons they promote death (Cortes-Canteli et al., 2002;

Marshall et al., 2003). Nuclear Factor of Activated T-cells (NFAT) is a transcription factor that contributes to lymphocyte death. It does so by regulation of killer genes including FasL, Tumor Necrosis Factor-alpha (TNF α), and cyclooxygenase-2 (Cox2) in lymphocytes (Lenzlinger et al., 2002; Humar et al., 2004). On the other hand, it is known that FasL, TNF α and Cox2 can induce neuronal death and their increased expression accompanies ischemia, TBI, stroke and some neurodegenerative diseases like AD or PD (Venters et al., 2000; Cernak et al., 2001; Facchinetti et al., 2002; Xiang et al., 2002). Therefore, it is possible that activation of NFAT contributes to neuronal loss that may play an important role in different neuronal diseases.

Nuclear Factors of Activated T-cells (NFAT)

NFAT is a family of transcription factors in which several members are activated in response to calcium. The NFAT signaling pathway was first described in lymphocytes as a pathway transducing signals from the polymorphic T-cell receptor to genes that coordinate an immune response (Graef et al., 2001). Several NFAT members are also necessary for the formation of the cardio-vascular system including morphogenesis of vertebrate heart valves. On the other hand, their pathological activation may produce cardiac hypertrophy (Graef et al., 2001; Crabtree and Olson, 2002).

Table 2 List of NFAT proteins (Hogan et al., 2003).

Protein	Other names	Regulation
NFATc1	NFAT2, NFATc	Ca ²⁺ /Calcineurin
NFATc2	NFAT1, NFATp	Ca ²⁺ /Calcineurin
NFATc3	NFAT4, NFATx	Ca ²⁺ /Calcineurin
NFATc4	NFAT3	Ca ²⁺ /Calcineurin
NFATc5	TonEBP	Osmotic stress
		Integrin (α 6 β 4) activation

Five NFAT genes NFATc1-4 and NFATc5, have been identified (known also as

NFAT1-4 and NFAT5; see table 2) (Bushdid et al., 2003; Hogan et al., 2003). The primordial family member is NFATc5, the only NFAT-related protein represented in the *Drosophila* genome. NFATc5 is identical to TonEBP (tonicity element binding protein), a transcription factor crucial for cellular responses to hypertonic stress (Fig. 3B) (Hogan et al., 2003). NFATc cytoplasmic subunits require other transcription factors for DNA binding, including AP1, MEF2, GATA4 generally referred to as nuclear partners (NFATn) (Fig. 3A). The nuclear components of NFAT transcription complexes are often regulated by the protein kinase C (PKC) and Ras/MAPK (Mitogen-Activated Protein Kinase) pathways (Graef et al., 2003).

Figure 3. Activation of transcription by NFAT. A. NFATc1-c4 components are dephosphorylated and translocated to the nucleus where they bind to the DNA and induce several genes like IL2, IL3, IL4, IL5, IFN γ , TNF α , FasL, CD40L, GM-CSF. To activate transcription, they require nuclear partners (NFATn) including AP1, GATA4 or MEF2. B. NFATc5 is activated and localized to the nucleus where it binds to the DNA and induces osmotically regulated genes.

NFATs contain both the nuclear localization sequence (NLS) and the nuclear export sequence (NES). (Okamura et al., 2000; Crabtree and Olson, 2002). Whether NLS or NES is masked depends on the phosphorylation state of specific serine residues in the regulatory domain (Crabtree and Olson, 2002; Hogan et al., 2003). Phosphorylation of these serine residues exposes NES whereas dephosphorylation exposes NLS (Crabtree and Olson, 2002; Hogan et al., 2003). In resting cells, the NLS of the cytoplasmic NFAT is masked due to phosphorylation on these serine residues. In

stimulated cells, an increase of intracellular calcium ions activates Ca^{2+} -dependent Ser/Thr phosphatase, calcineurin (CaN), which then dephosphorylates the masking residues. Consequently, NLS is exposed and NFAT can be carried into the nucleus by the importin α/β . Inside the nucleus, NFAT may be re-phosphorylated by protein kinases, including Ser/Thr kinase, Glycogen Synthase Kinase 3 β (GSK-3 β), Thr/Tyr (Threonine/Tyrosine) MAP kinases, p38, cAMP-dependent protein kinase (PKA) and Jun N-terminal kinase (JNK), exposing its NES and be exported by the exportin (Fig. 4) (Crabtree and Olson, 2002).

Figure 4. Regulation of NFAT translocation. NFATc1-c4 components are dephosphorylated by calcineurin and translocated to the nucleus by the importin; however, phosphorylation of NFAT by kinases activates its translocation to the cytoplasm by the exportin.

In resting T cells, NFAT is phosphorylated and localized in the cytoplasm. After cell stimulation, it is dephosphorylated by CaN and translocated to the nucleus where it binds to the DNA (Murphy and Hughes, 2002). It binds to promoter regions and induces expression of several genes including cytokines, such as interleukin (IL)2, IL3, IL4, IL5, IL8, IL13, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ , and tumor necrosis factor (TNF) α , and cell surface receptors, such as CD40L, CTLA-4, and FasL (Masuda et al., 1998; Kiani et al., 2000; Humar et al., 2004). NFAT plays a critical role in the coordinate induction of expression of these genes (Masuda et al., 1998).

NFATc5 has distinct characteristics from those of NFATc1-c4. First, the DNA-binding domain does not bind with Fos or Jun to form NFAT:AP1 heteromeric transcription factor. Second, NFATc5 lacks the conserved regulatory domains of the calcium-regulated NFATs and its subcellular distribution is not dependent on calcineurin (Macian et al., 2001). It has been reported that NFATc5 is responsible for cellular responses to osmotic stress. In osmotically-stressed cells, NFATc5 expression is increased and it is localized to the nucleus (Lopez-Rodriguez et al., 1999). Moreover, NFATc5 binding elements are present in the regulatory regions of a variety of osmotically-regulated genes including aldose reductase or sodium-myoinositol transporter (Macian et al., 2001).

NFAT family members were also found in astrocytes and hippocampal neurons (Graef et al., 1999; Graef et al., 2001; Jones et al., 2003). The activation of NFAT-dependent transcription in neurons requires Ca^{2+} influx via L-type voltage-sensitive calcium channel. Neuronal NFAT transcriptional complexes bind to inositol 1, 4, 5-triphosphate receptor, type 1 (IP3R1) promoter and are likely to be one of the transcription factors that regulate activity-dependent expression of this receptor during development (Graef et al., 2001). The role of NFAT in the central nervous system (CNS) is not well characterized. So far, it was shown that the NFATc4 isoform is involved in neurotrophin-mediated synaptic plasticity and memory formation (Graef et al., 1999; Groth and Mermelstein, 2003). Furthermore, it is known that neurotrophins and netrins stimulate calcineurin-dependent nuclear localization of NFATc4 and activation of NFAT-mediated gene transcription in cultured neurons leading to outgrowth of embryonic axons. Sensory and commissural neurons lacking calcineurin function or NFATc2, c3 and c4 are unable to respond to neurotrophins or netrin-1 with efficient axonal outgrowth (Graef et al., 2003). So far, the role of NFAT in neuronal survival is not known but it was shown that NFAT is involved in cell survival in cardiomyocytes (Pu et al., 2003). NFATc3 and NFATc4 are required for cardiac development and

proper mitochondrial function (Bushdid et al., 2003). Moreover, NFAT transcription factors are involved in hypertrophy, but they are also critical survival factors that inhibit cardiomyocyte apoptosis induced by calcineurin activation (Pu et al., 2003). It has been also shown that NFATc1 and NFATc2 are involved in cell death in lymphocytes (Humar et al., 2004; Cernal et al., 2001; Venters et al., 2000a; 2000b; Xiang et al., 2002). Moreover, NFAT is involved in activation of various killer genes including FasL, TNF α and Cox2, which cause death in neurons. Therefore, I decided to check whether NFAT is involved in neuronal apoptosis.

Possible involvement of NFAT in neuropathologies

Graef and co-workers reported that NFAT activity is present in neurons and is induced by calcium (Graef et al., 1999). So far, it was shown that NFAT is an important mediator of T-cell apoptosis (Linette et al., 1996; Srivastava et al., 1999). Moreover, Dr. Michal Hetman found that cortical neurons overexpressing NFATc4 undergo apoptosis (Fig. 5) while cells receiving a dominant negative mutant form of NFATc4 are partially protected from cell death induced by TD (Fig. 6) (unpublished observation). Consequently, I hypothesize that excessive NFAT activation may play a crucial role in neuronal death in neurological diseases.

Figure 5. Overexpression of NFATc4 induces neuronal apoptosis. (A) Neurons were transfected for 48 hours with pBJ-NFATc4 wt and the β -galactosidase expression plasmid pON260 (0.6 μ g + 0.2 μ g of plasmid DNA/well). Expression of recombinant proteins was studied by immunostaining (green - HA epitope tag of NFATc4, red - β -galactosidase). Morphology in transfected cells was verified by staining with Hoechst 33258. Transfected cells were identified by immunostaining for β -gal and Hoechst staining identified whether the cell was dead or alive. (B) NFAT-mediated transcription was assayed 48 hours after transfection of NFAT-Luc reporter (0.12 μ g/well) + pBJ5 empty cloning vector or wt NFATc4 or a dominant negative NFATc4 mutant, C-delta (0.48 μ g/well). EF1LucZ was added to normalize for transfection efficiency. (C) Neurons were transfected using calcium phosphate method with pBJ5 empty cloning vector, pBJ5-NFATc4 wt or pBJ5-NFATc4C-delta and co-transfected with β -galactosidase expression plasmid, pON260 (0.4 μ g NFATc4 + 2 μ g pON260 of plasmid DNA/2 millions cells in a 35 mm dish). Neuronal apoptosis in transfected cells were identified by immunostaining for β -gal and by Hoechst 33258 staining. In (B) and (C) $n = 3$ in triplicate determinations, the error bars are SEMs.

Figure 6. Inhibition of NFATc-driven transcription by NFATc4-delta reduces neuronal apoptosis induced by trophic deprivation. Neurons were transfected with NFAT-Luc reporter (0.12 g/well) + pBJ5 empty cloning vector or a dominant negative pBJ5-NFATc4 mutant, C-delta (0.48 g/well). EF1LacZ was added to normalize for transfection efficiency. Twenty four hour-apoptotic treatments were initiated 48 hours after transfection. LY, LY294002 (30 μ M), 0%S, medium without serum. Results for $n = 2$ (duplicate determinations in 1 representative experiment), the error bars are SEMs.

To set the stage for further testing of this possibility, I studied expression of various NFATc family members in rat primary cortical and hippocampal neurons and characterized NFATc-mediated transcriptional response in these systems.

CHAPTER II

METHODS

Plasmids

Dominant-negative mutant of NFATc4 was generated by Dr. Hetman through deletion of the C-terminal DNA binding domain starting at amino acid 439 of human cDNA for NFATc4. The mutant was then cloned N-terminally to the HA tag in pBJ5 mammalian expression vector. Expression plasmid for wt and constitutive active NFATc4 are described (Graef et al., 1999; Neal and Clipstone, 2003; Schubert et al., 2003). Expression plasmid for wt NFATc1 is described (Beals et al., 1997).

Drugs

Pharmacological agents that were used are commercially available and were dissolved using appropriate solvents. KCl (30 mM; VWR) was dissolved in H₂O; LY294002 (30 μ M; Sigma) was dissolved in DMSO; CsA (1 μ g/ml; Bedford Laboratories) was dissolved in 0.9% sodium chloride; FK506 (200 ng/ml; A.G. Scientific, Inc.) was dissolved in DMSO; U0126 (40 μ M; Sigma) was dissolved in DMSO.

Antibodies

Anti- β gal (1:500, rabbit; Calbiochem) and anti-HA (1:80, mouse; Novus biologicals) were used for immunostaining as primary antibodies. Texas Red-conjugated goat antibody to rabbit IgG (1:100; Calbiochem) or for double staining additional secondary antibody FITC-conjugated goat antibody to mouse IgG (1:100; Calbiochem), was

used for immunostaining as secondary antibodies.

Cell culture

Primary culture of cortical and hippocampal neurons were prepared from newborn Sprague-Dawley rats according to the established protocols (Furshpan and Potter, 1989; Bading and Greenberg, 1991). Culture medium was Basal Medium Eigel (BME) supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, Ut), 35mM glucose, 1mM L-glutamine, 100 U/ml of penicillin, 0.1 mg/ml streptomycin. Cytosine arabinoside (AraC) (2.5 μ M) was added to cultures on the second day after seeding (DIV 2) to inhibit the proliferation of non-neuronal cells. AraC at this concentration does not affect neuronal survival (Hetman et al., 1999). Cortical neurons were seeded in concentration of 2 million cells/35 mm plate or 0.5 million cells/well on 24-well plate. Hippocampal neurons were seeded in concentration of 0.6 million of cells/35 mm plate or 0.15 million of cells/well on 24-well plate. Using this procedure, the cultures consist of about 80-90% MAP-2 positive neurons and about 10% of GFAP positive astrocytic cells at DIV5 (Hetman et al., 1999a). All experiments were performed at *in vitro* days 6 or 7, when the newborn-derived neurons are susceptible to oxidative stress, excitotoxicity, DNA damage agents or TD (Hetman et al., 1999; Hetman et al., 2002). Both cortex and hippocampus ensure enough cells to perform biochemical analysis such as promoter assays, western blots or electromobility shift assays.

RT-PCR assay

RNA for RT-PCR was isolated from neurons using the SV Total RNA Isolation Kit (Promega). cDNA synthesis was done using a AMV cDNA synthesis kit from Invitrogen. Primers, designed using OligoExplorer freeware from University of Koupio,

as follows:

NFATc1 sense-aacgccctgaccaccgatag, asense-gctgccttcggtctcatagtg;

NFATc2 sense-ctgccagcttcattttcgacacct, asense-ttgaggcatccatgagaacagca;

NFATc3 sense-gtagagactgacatcccttg, asense-atctgtagatttattggcttttc;

NFATc4 sense-tgccaacattgactgtgctgga; asense-acaccactttagagtctggcagga;

NFATc5 sense-gccaaagcacctcactatg, asense-gcaggagttgtatttcg.

PCR was run using standard protocols and MJ Research thermal cycler. Reaction product was analyzed by agarose gel electrophoresis.

Transfection of neurons

Neurons were transfected using Lipofectamine 2000 reagent (Invitrogen) at DIV3-4 according to the established protocols (Hetman et al., 2002). Medium from 24-wells plates was collected and replaced by transfection media - Basal Medium Eagle (BME, BioWhittaker) supplemented with 35mM Glucose, 1mM L-glutamine, 100 U/ml of peniciline and 0.1 mg/ml streptomycine. Transfection mixture contained 100 µl per well of BME, 0.6 µg per well DNA and 1.5 µl lipofectamine 2000 per well (Invitrogen). First, 50 µl of BME was mixed with lipofectamine in one eppendorf, another 50 µl of BME was mixed with DNA in another eppendorf and then the two mixtures were mixed together and incubated 20-60 minutes in room temperature before adding to the cells. Cells were transfected with 100 µl of transfection mixture per well and were incubated 2 hours at 37°C under a water saturated atmosphere of 95% ambient air and 6.5% CO₂. Transfection medium was then removed and replaced by conditioned medium collected from the cells 2 hours earlier that was supplemented with 1g/ml glucose and centrifuged in 1000 rpm by 10 minutes in room temperature in order to get rid of dead cells. Cells transfected with plasmid conjugated with EGFP could be seen live or after fixation without immunostaining.

Immunostaining

Cortical neurons plated on 24-wells plates were co-transfected using Lipofectamine 2000 (Invitrogen) with NFATc4 wt, with NFATc4 tagged with hemagglutinin (HA) or sr α together with pON260. Two days after transfection, cells were treated with drugs (LY294002, 30 μ M or serum deprivation) then fixed or untreated cells were fixed with 4% para-formaldehyde in PBS. After washing with PBST (phosphate buffered saline - PBS, Triton 0.1%), cells were permeabilized with 0.5% NP40 diluted in PBS for 30 minutes, then all unspecific sites were blocked with 2.5% BSA, 2.5% horse serum diluted in PBST overnight. Next, primary antibody, that was either anti- β gal (1:500, rabbit) or anti-gal (1:500, rabbit) mixed with anti-HA (1:80, mouse), was added to the cells and was incubated for 2 hours in room temperature. Then, cells were washed 3 times with PBST and secondary antibody was added that was Texas Red-conjugated goat antibody to rabbit IgG (1:100) or for double staining additional secondary antibody FITC-conjugated goat antibody to mouse IgG (1:100) was used and cells were incubated with these antibodies 1 hour in room temperature. Then, cells were washed with PBST and additionally stained with Hoechst 33258 to visualize the nuclei.

Reporter assay

Cortical neurons plated on 24-well plates were transfected using Lipofectamine 2000 (Invitrogen) with 0.2 μ g/well NFATc-Luc (Graef et al., 1999) and 0.125 μ g/well EF1 α .LacZ DNA for normalization of transfection efficiency. Two days after transfection, cells were treated with 30mM KCl and incubated for 24 hours. Then, cells were lysed with 100 μ l/well of reporter lysis buffer (Promega) and activities of luciferase or β -galactosidase were measured as described using Orion II Luminometer and Promega reagents (β -galactosidase Enzyme Assay System with Reporter Lysis Buffer and Luciferase Assay System). The reporter gene luciferase activity was nor-

malized to β -galactosidase activity expressed as the fold induction relative to the control.

Serum deprivation

Serum deprivation was performed as described previously (Hetman et al., 1999). Briefly, cells were washed twice with serum-free BME and incubated in serum-free BME supplemented with 35 mM glucose, 1 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 mM cytosine arabinoside (all from Sigma). Control cells were washed similarly and then incubated for matched time points in serum-containing conditioned medium.

CHAPTER III

RESULTS

To identify which members of NFAT family are present in neurons, I performed RT-PCR assay using NFAT-specific primers and cDNA from primary cortical or hippocampal neurons or from P1 brain. As a positive control, I used cDNA from newborn rat tissues including heart, spleen and thymus. It is known that in these tissues several NFATc family members are expressed. My results indicate that NFATc1, NFATc3, NFATc4 and NFATc5 are present in mRNAs isolated from cultured cortical and hippocampal neurons (Fig. 7).

Figure 7. Expression of NFAT family members in cultured neurons. Half μ g of DNAs and 2 μ M specific primers were used for each PCR reaction. For NFATc1 the number of cycles was 29, for NFATc2, NFATc3, NFATc4 and NFATc5 - 32 cycles. Equal amounts of template were further confirmed by PCR with GAPDH-specific primers. Tissues were isolated from newborn rats, neuronal cDNA was prepared from cells 6 days after plating (DIV6). PCR products were analyzed by agarose electrophoresis.

Then, I tested whether NFAT-mediated transcriptional activity is present in primary cortical neurons. I transfected cortical neurons with a plasmid construct that

contains a luciferase reporter gene under control of an NFAT promoter. Three days after transfection, I stimulated cells for 20 hours with 30mM KCl. Luciferase activity in neurons transfected with the reporter was 2000 times higher than in untransfected cells. Interestingly, NFAT-mediated transcription was further stimulated with KCl exceeding the control values by 2.5 times. It suggests that NFAT activity is present in cortical neurons and is increased by calcium influx (Fig. 8A).

Figure 8. Calcineurin and MAPK pathways dependent activation of NFAT-mediated transcription in cortical neurons by KCl. (A) Cells were transfected with NFAT-Luc reporter (0.2 μ g plasmid DNA/well in a 24-well plate) and EF1lacZ plasmid (0.125 μ g plasmid DNA/well). Three days after transfection, cells were treated with 30 mM KCl for 24h. Neurons were lysed and luciferase activity was measured. (B), (C) Cells were transfected in (C) with pBJ5-NFATc1 wt (0.1 μ g plasmid DNA/well) or in (B) with pBJ5-NFATc4 wt (0.1 μ g plasmid DNA/well) and with NFAT-Luc (0.2 μ g plasmid DNA/well) reporter and EF1lacZ (0.125 μ g plasmid DNA/well) plasmid to normalize for transfection efficiency. After 72h cells were pretreated with cyclosporin A, CsA (1 μ g/ml) and FK506, FK (0.2 μ g/ml) or with U0126 (40 μ M) for 30 min. and then were treated with 25 mM KCl. After another 20h, cells were lysed and luciferase activity was determined. In (A) $n = 3$ (triplicate determinations in 1 representative experiment), in (B) and (C) $n = 6$ (triplicate determinations from 2 representative experiments), the error bars are SEMs.

Next, I overexpressed pBJ5-NFATc4 wt or pBJ5-NFATc1 wt isoforms in cortical neurons. The transfection efficiency using Lipofectamine ranged from 0.1 to 10% of neurons. Three days after transfection, I stimulated cells with 25 mM KCl in the presence or absence of calcineurin's inhibitors: Cyclosporin A (CsA) and FK506 (FK) or MAPK pathway blocker, U0126. I noticed that after overexpression of NFATc4 wt, NFAT-mediated transcription was increased above 150 times by KCl. Similarly, after overexpression of NFATc1 wt, KCl induced NFAT-mediated transcription, which was increased above 40 times. Interestingly, this phenomenon was blocked in both cases by calcineurin inhibitors and by MAPK pathway inhibitor (Fig. 8B and 8C). These data suggest that in cortical neurons, both calcineurin and MAPK pathways are involved in KCl response activating NFAT-mediated transcription.

It is known that in non-neuronal cells, activation of NFAT is regulated by Ca^{2+} influx leading to translocation of NFAT from the cytoplasm to the nucleus where it activates gene transcription. To test this phenomenon, I transfected cells with pNFATc4 EGFP fusion protein and stimulated cells with 25 mM KCl two days after transfection. My data indicate that in the absence of KCl, NFATc was present in pericarial cytosol and in the processes. In the presence of KCl, NFATc was translocated to the nucleus (Fig. 9). These data show that NFAT can be translocated to the nucleus upon Ca^{2+} influx.

Figure 9. Translocation of NFATc. Cortical neurons were transfected with pNFATc4-EGFP and 48 hours later were treated with 25 mM KCl. Green staining indicates localization of NFATc4-EGFP fusion protein that is translocated to the nucleus after KCl treatment.

To check if proapoptotic stimulation with LY294002, which is a chemical inhibitor of the main trophic support signaling pathway, phosphatidylinositol-3-kinase (PI3K) can increase NFAT driven transcription, cortical neurons were co-transfected with NFAT-Luc reporter, pBJ5NFATc4 wt and EF1lacZ. Three days after transfection, cells were treated with LY294002 in the presence or absence of calcineurin's inhibitors, CsA and FK or MAPK pathway blocker, U0126. Cells treated with vehicle were used as a control. LY294002 induced almost 10-fold increase in NFAT-mediated transcription, which was blocked by CsA + FK and by U0126 (Fig. 10). These results suggest that NFATc4-mediated transcription is activated by the inhibitor of PI3K via calcineurin and MAPK pathways.

Figure 10. Activation of NFATc4-mediated transcription by LY via calcineurin and MAPK pathways. Cells were transfected with pBJ5-NFATc4 wt (0.1 mg plasmid DNA/well) and with NFAT-Luc (0.2 mg plasmid DNA/well) reporter and EF1lacZ (0.125 mg plasmid DNA/well) plasmid to normalize for transfection efficiency. After 72h, cells were pretreated with cyclosporin A, CsA (1 mg/ml) and FK506, FK (0.2 mg/ml) or with U0126 (40 mM) for 30 min. and then were treated with inhibitor of PI3K, LY294002 (30 mM). After 20h, cells were lysed and luciferase activity was determined. Results for $n = 3$ (triplicate determinations in 1 representative experiment), the error bars are SEMs.

In order to test a possible NFAT involvement in apoptosis, I co-transfected cortical neurons with NFATc4 wt or empty vector (sr α) in the presence of β -galactosidase expression plasmid that was a marker for the transfection. Two days after transfection, apoptosis was induced by serum deprivation and LY294002 treatment. After 24 hours

of incubation, cells were fixed and immunostaining were performed. The number of transfected apoptotic cells was scored (Fig. 11). Expression of NFATc4 wt was visible in cortical neurons after immunostaining. Data show that expression of NFATc4 wt was sufficient to enhance neuronal death induced by both serum deprivation and LY294002 treatment from 20-25 to 40%.

Figure 11. NFATc4 wt magnifies cell death in cortical neurons after trophic deprivation. (A) Neurons were transfected for 48 hours with pBJ-NFATc4 wt and the β -galactosidase expression plasmid, pON260 (0.6 μ g + 0.2 μ g of plasmid DNA/well). Expression of recombinant proteins was studied by immunostaining (green - HA epitope tag of NFATc4, red - β -galactosidase). Morphology in transfected cells was verified by staining with Hoechst 33258. Transfected cells were identified by immunostaining for β -gal and Hoechst staining identified whether the cell was dead or alive. (B) Cortical neurons were transfected with empty vector, Sr α (0.4 μ g of DNA/well), pBJ-NFATc4 dominant negative mutant or pBJ-NFATc4 wt (0.2 μ g of plasmid DNA/well) and the β -galactosidase expression plasmid, pON260 (0.2 μ g DNA/well). Two days after transfection, cells were treated with serum deprivation or LY294002. The effectiveness of transfection was studied by immunostaining with β -galactosidase. The morphology of transfected cells was verified by staining with Hoechst 33258. The number of dead cells as counted using a microscope. Results for $n = 3$ (triplicate determinations in 1 representative experiment), the error bars are SEMs.

In summary, my data suggest that NFAT is present in primary neuronal cultures. Moreover, its transcriptional activity is induced by calcium influx or TD. In both cases, calcineurin and MAPK pathways are required for the activation. Furthermore, overexpression of NFATc4 wt enhances apoptosis induced by TD in neuronal culture.

CHAPTER IV

DISCUSSION

I found that several members of the NFATc family are expressed in postnatal hippocampal and cortical neuronal cultures. Furthermore, I demonstrated that NFAT could mediate transcription response in these cells following KCl stimulation. I also found that, in KCl treated cells, calcineurin and MAPK pathways were required for NFAT-driven transcription and that it was accompanied by NFATc translocation to the nucleus. Interestingly, I observed activation of NFAT-mediated transcription following LY294002 treatment. This activation may involve calcineurin and MAPK pathways. In addition, LY294002 treatment or TD induced more apoptosis in cortical neurons that overexpressed NFATc4 wt. Therefore, I propose that in neurons, NFAT may be involved in TD-induced apoptosis.

Using RT-PCR, the expression of NFATc1, NFATc3, NFATc4 and NFATc5 was found in primary cortical and hippocampal neurons. So far, NFATc expression was shown in non-neuronal cells including lymphocytes, natural killer cells and astrocytes (Lyakh et al., 1997; Viola et al., 1998; Jones et al., 2003). In hippocampal neurons, expression of only one NFATc isoform, NFATc4, has been reported (Graef et al., 1999). My data suggest that it is also present in cortical neurons. Expression of other NFATc isoforms including NFATc1, NFATc3 and NFATc5 in neurons is described for the first time in this thesis. Cortical and hippocampal neurons are not pure neuronal cultures. These cultures may also contain astrocytes. The culture protocol procedure that was used in this research was reported to result in primary cortical cultures consisting of 90% of neurons and 10% astrocytes (Hetman et al., 1999). Therefore, immunocytochemistry or in situ hybridization is needed to confirm that

NFATc1, c3, c4 and c5 are indeed expressed in neurons. NFATc2, which was not expressed in neurons, may play an important role in other cell types. For example, in lymphocytes, its expression leads to cell death (Peng et al., 2001). However, lack of NFATc2 expression in neurons suggests that other NFATc isoforms are responsible for the role that NFATc2 plays in other cell types. It suggests that NFATc isoforms may activate different genes in various cell types.

Importantly, the various isoforms of NFAT family may mediate diverse responses in the CNS as NFATc1 and NFATc3 are regulated by Ca^{2+} /calcineurin pathway while NFATc5 is regulated by osmotic stress (Hogan et al., 2003). My data indicate that non-toxic, membrane depolarizing concentration of KCl activated endogenous NFAT in cortical neurons. Moreover, NFAT-mediated transcriptional activity was induced by KCl in cells overexpressing NFATc1 wt and NFATc4 wt. Inhibition of calcineurin or MAPK pathways blocked the induction. Similarly, NFAT activation by KCl that required calcineurin has been shown previously in hippocampal neurons and that K^{+} depolarization acts specifically through L-type Ca^{2+} channels (Graef et al., 1999). My data show that KCl induced NFAT-dependent transcription was not blocked by inhibition of NMDA receptors, which are another major Ca^{2+} channel in neurons (data not shown). Therefore, in cortical neurons, activation of NFAT-mediated transcription by calcium influx is likely mediated by L-type Ca^{2+} channels.

It is known that Ras/MAPK pathway is activated after membrane depolarization, which leads to calcium influx through L-type Ca^{2+} channels in PC12 cell lines, in cortical primary cultured neurons and in cerebellar granule neurons (Bading and Greenberg, 1991; Rosen et al., 1994; Borodinsky and Fisman, 1998). My data show inhibition of NFAT-mediated transcription activity after blocking the ERK1/2 pathway. Therefore, it suggests that ERK1/2 participates in NFAT activation by calcium influx. In many cell types including lymphocytes or cardiomyocytes, Ras/MAP kinase signals facilitate NFAT-dependent transcription (Crabtree and Olson, 2002).

My results provide the first report suggesting ERK involvement in NFAT activation in neurons. It suggests that neuronal signaling machinery, which regulates NFAT, is similar to that of lymphocytes and cardiomyocytes, which required both calcineurin and ERK to activate NFAT (Genot et al., 1996).

My data indicate that in cortical neurons NFATc4-mediated transcription is induced by LY294002 treatment. Interestingly, the NFATc4-driven transcription activation triggered by LY294002 required calcineurin and MAPK pathways. It is known that in primary neurons LY294002 blocks the main trophic support signaling pathway by inhibition of PI3K, which in consequence activates GSK3 β (Hetman et al., 2000). This activation may inhibit NFATc (Graef et al., 1999). At the moment, it is unclear how NFATc escapes this inhibition in LY-treated neurons. Some possibilities may include: (i) activation of stress-activated JNK pathway by TD (Xia et al., 1995), or (ii) activation of TNF α R by inhibition of PI3K that leads to calcium influx and therefore, calcineurin activation (Wang et al., 2002). It is known that JNK pathway is activated by TD (Xia et al., 1995; Le-Niculescu et al., 1999), which modulates activity of AP-1, leading to induction of FasL and cell death in neurons (Le-Niculescu et al., 1999; Raoul et al., 1999). Activation of AP1 by JNK may counteract the inhibitory regulation of NFAT by GSK3 β resulting in activation of NFAT-driven transcription. Another possibility is that TD triggers calcineurin activation that can antagonize increased NFATc phosphorylation by GSK3 β . Indeed, calcium signaling may be activated by stimuli active during TD. For instance, in colon cancer cells, TNF α , which is also a NFAT target gene, activated TNF α R leading to calcium influx (Wang et al., 2002).

The role of NFAT activation by TD or by LY294002 treatment in neurons is unclear. An interesting possibility is that NFAT is involved in TD-triggered cell death. Interestingly, NFAT was shown to play a role in cell death of lymphocytes (Srivastava et al., 1999; Youn et al., 2000). The unpublished data from Dr. Hetman's laboratory

suggests involvement of NFATc4 in neuronal death (see Fig. 5 in the introduction). For instance, overexpression of NFATc4 wt increased number of apoptotic cells, while inhibition of NFAT by a dominant negative mutant, had no effect on cell death. This result suggests that the active NFATc4 isoform may be a pro-death factor in neurons. Moreover, I found enhancement of cell death in neurons overexpressing NFATc4 after TD. This observation indicates that NFATc4 may be a stress-activated transcription factor, which together with other transcription factors including FOXO, mediates cell death in neurons. NFATc4 may bind to DNA and NFATn components forming a complex like they do in non-neuronal cells to subsequently activate NFAT target genes including killer genes like FasL, Cox2 or TNF α that were implicated in neuronal death (Venters et al., 2000a, b; Cernak et al., 2001; Facchinetti et al., 2002; Xiang et al., 2002).

Other transcription factors were also shown to trigger various cellular responses depending on the activating stimulus. In neurons, contribution of NF κ B to pro- and anti-apoptotic events has been reported. For instance, TNF α is able to protect primary hippocampal neurons exposed to excitotoxic and apoptotic insults (Cheng et al., 1994; Barger et al., 1995) via NF κ B-mediated expression of several anti-apoptotic genes including an antioxidant defense enzyme, manganese superoxide dismutase (MnSOD), and cell death inhibitors Bcl-2, Bcl-xL and IAPs (Dixon et al., 1997; Mattson et al., 2000). Pro-apoptotic role of NF κ B has been shown by activation of NMDA receptor by the excitotoxin quinolinic acid, which triggers NF κ B activation and increase of pro-apoptotic effectors of NF κ B activity: p53 and c-myc expression (Qin et al., 1999). NF κ B-dependent activation of other killer genes including CD95 was shown during neuronal death caused by ischemia (Schneider et al., 1999).

Other transcription factors also act in similar ways leading either to cell death or survival. AP1 is an example of a transcription factor whose induction results in activation of various genes, such as apoptosis enhancers, FasL or Bim, or apoptosis

inhibitors including Bcl3 (Shaulian and Karin, 2002). The regulation and function of transcription factors playing dual roles are highly complex. These transcription factors are also important regulators of many aspects of the cellular response. Their differential activation can result in the regulation of different target genes through differences in the DNA-binding affinity. Interactions with various DNA-binding proteins, often resulting in cooperative DNA-binding, can determine which genes become activated by transcription factors. Moreover, coactivator and co-repressor complexes are often required for the transcription factors function. Therefore, transcription factors working together and operating as a part of a network of coordinately regulated DNA-binding proteins determine the gene expression required for the response to a particular cellular stimulus (Neil et al., 2004).

Figure 12. Activation of NFAT in cortical neurons. NFAT is activated by KCl or TD via calcineurin and MAPK pathways leading to NFAT nuclear translocation where it binds to DNA and its nuclear partners, NFATn including AP1, GATA4 or MEF2. Formation of such complex induces activation of transcription.

Similarly, NFAT can play differential roles in neurons. Depending on pro-survival or pro-death stimuli that activated NFAT, it may transactivate distinct sets of genes producing either cell survival or death. It is the balance between pro-apoptotic and anti-apoptotic target genes that will determine whether the final outcome will be cell survival or cell death and that balance may be set at different points for various cell

types (Shaulian and Karin, 2002). Also, different isoforms of NFAT may be selectively activated by different stimuli to regulate either survival or killer genes. For instance, in lymphocytes, activation of NFATc1 and NFATc2 isoforms leads to cell death (Peng et al., 2001) while activation of NFATc3 plays a pro-survival role (Oukka et al., 1998). Therefore, in neurons, various isoforms of NFAT can have opposite effects on cell survival.

In the work presented here I report that NFAT is expressed in cortical and hippocampal neurons. Furthermore, I found activation of NFAT-mediated transcription by pro-survival KCl and pro-apoptotic LY294002 treatment. Both stimuli activated NFAT via calcineurin and MAPK pathways. Apoptosis induced by serum deprivation and LY294002 treatment was increased in NFATc4 wt overexpressing neurons suggesting that NFAT may regulate killer genes (Fig. 12). Therefore, these results indicate the importance of NFAT as a transcription factor playing a role in the regulation of a balance between pro-apoptotic and anti-apoptotic target genes. Further studies are needed to identify the role of NFAT signaling in trophic deprived or trophic supported neurons.

Future directions

It is known that when NFATc is activated, it is translocated to the nucleus where it binds to its nuclear partners and to DNA (Crabtree and Olson, 2002; Neal and Clipstone, 2001). Therefore, I plan to use Electromobility Shift Assay (EMSA) to study DNA binding activity to NFAT-response elements in nuclear proteins isolated from cortical and hippocampal neurons treated with LY294002, serum withdrawal or KCl (Lyakh et al., 1997). Lysates will be collected at different times after treatment. To identify DNA binding components of NFAT, an Electromobility Supershift Assay (ESA) with specific antibodies against NFAT nuclear partners including AP1, GATA4 and MEF2 will be used. Similarly, antibodies against NFATc family members will

be used to identify which members are involved in NFAT-mediated transcription. An alternative approach for these methods might be Chromatin Immunoprecipitation assay (ChIP), which can use antibodies to precipitate DNA bound by transcription factors. Moreover, in contradistinction to EMSA, it can be used *in vivo*, providing a powerful tool to unravel the basic mechanism in gene expression.

Data obtained in our laboratory show that NFATc4 mediates apoptosis in cortical neurons. Therefore, I will study neuroprotective effect after NFAT blocking. I showed that NFAT activation is calcineurin and MAPK pathways-mediated, so I will identify whether calcineurin inhibitors, cyclosporin A and FK506 or MAPK blocker, U0126 protect against TD or LY294002-induced neuronal apoptosis mediated by NFATc4. Because cyclosporin A additionally inhibits the mitochondrial permeability transition pore and is neuroprotective (Scheff and Sullivan, 1999; Santos and Schauwecker, 2003), first I will investigate whether FK506 blocks NFAT activation and translocation alone. Subsequently, I will test whether FK506 blocks neuronal apoptosis. Next, I will verify if NFAT-induced neuronal apoptosis is isoform-specific. I will achieve that by overexpressing neurons with other NFAT wt isoforms in the presence of β -galactosidase expression plasmid, which will be exposed to TD. I will use pBJ5-NFATc4 delta mutant as an NFAT blocker, because it blocks all other NFAT isoforms. I will use empty vector pBJ5 as a control. After various time points, I will fix cells, then do immunostaining and verify apoptosis in correlation to transfected cells. In addition, lentiviral vectors will be used to transduce neurons with NFAT wt, ca, dominant negative forms or VIVIT (Naldini et al., 1996). Dominant negative mutants of NFATc4 inhibit all members of the NFATc family (NFATc1-4). It is generated by deletion of the C-terminal DNA binding domain and blocks the binding of NFAT to DNA (Chow et al., 1999). VIVIT blocks NFATc1-4 interactions with the phosphatase, calcineurin, which mediates NFATc activation through Ca^{2+} influx. It is a high-affinity calcineurin-docking peptide, which was selected from combinatorial

peptide libraries based on the calcineurin-docking motif of NFAT (Aramburu et al., 1999). Lentiviral vectors transduction gives high efficiencies (90%), so MTT assays will be used for verification of neuronal survival.

I hypothesize that NFAT activation, which is calcineurin and MAPK-mediated can be opposed by p38 or PKA kinases. I plan to test regulation of NFAT by p38 and PKA using MKK3 and/or MKK6 ca mutants to activate p38, forskolin and Sp-cAMP to activate PKA. These tools will be used to test whether activation of p38 and PKA can decrease NFAT-driven transcription in neurons. To assess the possible involvement of p38 or PKA in regulating NFAT-induced apoptosis, I will express NFATc wt and treat cortical or hippocampal neurons with p38 or PKA activators. SB202190 or SB 203580 (p38 inhibitors) and H89 or Rp-cAMP (inhibitors of PKA) will be used to study the contribution of p38 and PKA to NFAT-mediated apoptosis that will be assessed by immuno- and bisbenzimidine staining. If p38 and PKA are involved in regulation of NFATc, their activation should block NFAT nuclear translocation and inhibit neuronal apoptosis. Inhibition of these kinases should abolish the protective effect of p38 or PKA activators. Activation of NFAT leads to transcriptional activation of various genes including FasL, TNF α and Cox2, which are implicated in neuronal death (Humar et al., 2004; Cernak et al., 2001; Venters et al., 2000a; 2000b; Xiang et al., 2002). Therefore, I plan to evaluate possible involvement of FasL, Cox2 and TNF α in NFAT-induced neuronal death. In order to study expression of these factors, protein lysates will be collected from neurons treated with LY294002 or trophic withdrawal and westerns will be performed. If expression of these proteins increases after NFAT activation, TNF α and FasL receptors and Cox2 will be blocked to assess whether they may contribute to NFAT-mediated neuronal death.

This project may result in novel neuroprotective strategies in neuronal diseases like traumatic brain injury, spinal cord injury, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke or ischemia.

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ABBREVIATIONS

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AraC	cytosine arabinoside
BCS	bovine calf serum
BME	basal medium eagle
β -gal	β -galactosidase
CaN	calcineurin
CD40L	CD40 ligand
C/EBPs	CCAAT enhancer binding proteins
ChIP	chromatin immunoprecipitation assay
CNS	central nervous system
Cox-2	cyclooxygenase-2
CPDD	cisplatin
CPT	camptothecin
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CsA	cyclosporin A
DIV	days in vitro
DMSO	dimethyl sulfoxide
dn	dominant negative
EGFP	enhanced green fluorescent protein
EMSA	electromobility shift assay
ESA	electromobility supershift assay
FasL	Fas ligand

FK	FK506
FOXO	forkhead box transcription factor
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK-3 β	glycogen synthase kinase-3 β
HA	hemagglutinin
HD	huntingtin disease
HIF	hypoxia inducible factor
HNE	4-hydroxynonenal
IFN γ	interferon γ
IgG	immunoglobulin G
IL	interleukin
IP3R1	inositol 1, 4, 5-triphosphate receptor, type 1
JNK	jun N-terminal kinase
LB	lysis buffer
LY	LY294002
MAP-2	microtubule associated protein 2
MAPK	mitogen activated protein kinase
MEF-2	myocyte enhancer factor-2
MMS	methyl methanesulfonate
NES	nuclear export sequence
NFAT	nuclear factors of activated T-cells
NFATn	nuclear partners of NFAT
NF κ B	nuclear factor κ B
NGF	nerve growth factor
NLS	nuclear localization sequences
NMDA	N-methyl D-aspartate

PBS	phosphate buffered saline
PBST	PBS with triton-X 100
PCD	programmed cell death
PCR	polymerase chain reaction
PD	Parkinson's disease
PI3K	phosphatidylinositol-3 kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-PCR
SCI	spinal cord injury
SNP	sodium nitroprusside
TBI	traumatic brain injury
TBST	tris buffered saline with tween
TD	trophic deprivation
Thr/Tyr	threonin/tyrosine
TNF α	tumor necrosis factor α
TNFR1	receptor 1 of tumor necrosis factor
TonEBP	tonicity element binding protein
TRAL	TNF-related apoptosis inducing ligands
wt	wild type

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