

Published in final edited form as:

J Neurosci Res. 2007 June ; 85(8): 1724–1733.

Increases in Expression of 14-3-3 Eta and 14-3-3 Zeta Transcripts during Neuroprotection Induced by Δ^9 -Tetrahydrocannabinol in AF5 Cells

Jia Chen¹, Chun-Ting Lee¹, Stacie L. Errico¹, Kevin G. Becker², and William J. Freed¹

¹Development and Plasticity Section, Cellular Neurobiology Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, Baltimore, Maryland ²Gene Expression and Genomics Unit, Intramural Research Program, National Institute on Aging, National Institutes of Health, Department of Health and Human Services, Baltimore, Maryland

Abstract

The molecular mechanisms involved in NMDA-induced cell death and Δ^9 -tetrahydrocannabinol (THC)-induced neuroprotection were investigated *in vitro* using an AF5 neural progenitor cell line model. By microarray analysis, Ywhah, CK1, Hsp60, Pdc4 and Pdc7 were identified as being strongly regulated by both NMDA toxicity and THC neuroprotection. The 14-3-3 eta (14-3-3 η ; gene symbol Ywhah) and 14-3-3 zeta (14-3-3 ζ ; gene symbol Ywhaz) transcripts were decreased by NMDA treatment, and increased by THC treatment prior to NMDA, as measured by cDNA microarray analysis and quantitative real-time RT-PCR. Other 14-3-3 isoforms were unchanged. While up-regulation of 14-3-3 ζ expression was observed 30 min after treatment with THC plus NMDA, down-regulation by NMDA alone was not seen until 16 hr after treatment. By Western blotting, THC increased 14-3-3 protein only in cells that were also treated with NMDA. Over expression of 14-3-3 η or 14-3-3 ζ by transient plasmid transfection increased 14-3-3 protein levels, and decreased NMDA-induced cell death. These data suggest that increases in 14-3-3 proteins mediate THC-induced neuroprotection under conditions of NMDA-induced cellular stress.

Keywords

NMDA; 14-3-3 proteins; cannabinoids; neuroprotection; microarray

The 14-3-3 proteins were first identified in 1967 (Moore and Perez, 1967). It is well-established that 14-3-3 proteins, which consists of seven isoforms in human cells ($\beta, \gamma, \zeta, \sigma, \epsilon, \eta, \tau$) (Martin et al., 1993), play crucial roles in many biological processes including control of cell proliferation, response of cells to DNA damage, prevention of apoptosis, regulation of chromatin structure, and gene expression. 14-3-3 proteins carry out these effects through binding, and modulating the activity of, a host of signaling proteins. Particularly high levels of 14-3-3 proteins are present in the brain (Martin et al., 1993).

One important function of 14-3-3 proteins is to support cell survival. 14-3-3 proteins, as anti-apoptotic factors in cells, interact with a number of apoptosis regulatory proteins, such as Bad (Datta et al., 2000), FKHL1 (Brunet et al., 1999), and apoptosis signal-regulating kinase 1

(ASK1) (Zhang et al., 1999). Through such direct interactions, 14-3-3 proteins also regulate the function of other pathways, including the JNK and p38 MAPK signaling pathways (Ichijo et al., 1997). 14-3-3 proteins, in general, seem to promote cell survival by binding and sequestering proteins which otherwise activate signaling pathways that are involved in initiating apoptosis.

There are many studies demonstrating effects of 14-3-3 proteins on cell death and apoptosis. For example, elevation of 14-3-3 γ has been shown to protect astrocytes from ischemic injury (X. Chen et al., 2005). Inhibiting the function of 14-3-3 proteins, such as by expression of 14-3-3 inhibitor peptides (Masters et al., 2002), or expression of mutant forms of 14-3-3 (Xing et al., 2000), is sufficient to induce apoptosis. Difopein, a peptide which specifically inhibits 14-3-3 protein-protein interactions, initiates apoptosis by itself, and enhances the toxicity of cisplatin (Masters and Fu, 2001). Therefore, regulating expression of 14-3-3 proteins may provide molecular targets that can be used to protect cells from apoptosis.

A few studies have shown that 14-3-3 expression can be dynamically regulated (McGowan et al., 1999; van Hemert et al., 2001). The 14-3-3 η (YWHAH) promoter has been mapped and contains potential AP-2-like, AP-3-like, Oct-6, and SP-1 binding sequences, suggesting that 14-3-3 η expression is potentially responsive to cAMP and other cellular signals (Muratake et al., 1996). Consistent with this possibility, 14-3-3 η expression is increased by methamphetamine in human astrogloma cells (Muratake et al., 1995). X.Q. Chen and coworkers (2005) showed that ischemia upregulates 14-3-3 γ expression in cortical astrocytes. Nevertheless, there are relatively few reports of conditions under which expression of 14-3-3 proteins are altered by external stimuli, such as by stimulation with excitatory amino acids. Similarly, there are very few known conditions or experimental models in which changes in the expression of 14-3-3 proteins, *per se*, initiate, promote or inhibit apoptosis.

In the present study, we employed a cell line, AF5, which is sensitive to excitotoxic cell death induced by N-methyl-D-aspartate (NMDA) in high concentrations, to screen for gene expression changes following NMDA treatment. Δ^9 -tetrahydrocannabinol (THC), which has a neuroprotective effect in this model (Chen et al., 2005b), was used to identify transcripts for which a change in expression was reversed under a neuroprotective condition. Decreased expression of two forms of 14-3-3, 14-3-3 η (Ywhah) and 14-3-3 ζ (Ywhaz), was caused by toxic concentrations of NMDA, and reversed to above control levels by treatment with THC. In order to test the hypothesis that 14-3-3 η and 14-3-3 ζ are capable of mediating the neuroprotective effect of THC, AF5 cells were transfected with plasmids encoding 14-3-3 η or 14-3-3 ζ . Both plasmids increased overall 14-3-3 protein levels and partially blocked NMDA toxicity, while control plasmids were ineffective. Therefore, changes in the level of expression of 14-3-3 η and 14-3-3 ζ may play an important role in the survival-promoting effect of THC in AF5 cells during NMDA-induced excitotoxicity.

MATERIALS AND METHODS

Chemicals and Materials

N-Methyl-D-aspartate (NMDA) and Δ^9 -THC were obtained from Sigma (St. Louis, MO). (R)-(+)-WIN 55,212-2 mesylate salt and (E)-Capsaicin were from Tocris Cookson (Ellisville, MO). RNA STAT-60 was purchased from TEL-TEST (Friendswood, TX). TURBO DNA Free kit and 18S-ribosomal RNA were from Ambion (Austin, TX). Reverse transcription reagents and non-radioactive cytotoxicity assay kit were purchased from Promega (Madison, WI). The DyNAmo SYBR Green qPCR kit was obtained from New England Biolabs (Beverly, MA). The ECL chemiluminescence kit was purchased from Amersham Biosciences (Piscataway, NJ). FuGENE 6 transfection reagent was obtained from Roche Applied Science (Indianapolis, IN).

Cell Culture, Plasmids, and DNA Transfection

The AF5 rat mesencephalic cell line which was immortalized using a fragment of the SV40 large T antigen gene known as T155g, has been described previously (Truckenmiller et al., 1998, 2002). Cells were grown in DMEM/F12 with 10% fetal bovine serum. 14-3-3 vectors, recombinant hexahistidine (HA)-14-3-3 ζ and its mutant derivative HA-14-3-3 ζ K49E (Zhang et al., 1997) and His-tagged 14-3-3 η (Subramanian et al., 2004) were graciously provided by Dr. Haian Fu (Emory University, Atlanta, GA). Pc3/hrGFP was a kind gift from Dr. Stephen Dewhurst (University of Rochester School of Medicine and Dentistry, Rochester, NY). pCDNA 3.1 and pDEST26, the control vectors for 14-3-3 ζ , 14-3-3 ζ K49E and 14-3-3 η were purchased from Invitrogen Corporation (Carlsbad, CA). AF5 cells were plated on 12-well plates at 1×10^5 cells/ml. Cells were transiently transfected with the appropriate vector (0.5 μ g DNA/ml) using the FuGENE 6 transfection reagent according to the manufacturer's instructions.

NMDA and Reagent Treatment

Cell cultures (2 days *in vitro*) were washed with Mg²⁺, Ca²⁺-free Dulbecco's phosphate buffered saline (DPBS) solution once and maintained in various concentrations (5 mM, 7.5 mM and 10 mM) of NMDA in HBSS without calcium and magnesium, containing 5 μ M glycine at 37°C for 30 min. The medium was then replaced by DMEM/F12 medium without serum and cultures were returned to the incubator and maintained at 37°C for an additional 16–18 hr.

For cannabinoid treatment, the cells were first incubated with cannabinoids (WIN 55,212-2 or THC) in serum-free DMEM/F12 for 6 hr, and the media were changed to 7.5 mM NMDA in HBSS for 30 min. For capsaicin treatments, the cells were first incubated with capsaicin alone for 30 min, and the media were then changed to media containing NMDA. After exposure, cells were cultured in fresh serum-free DMEM/F12 media for 16–18 hr.

cDNA Microarray

Total RNA was extracted from AF5 cells using RNA STAT-60 kit according to the manufacturer's instructions. cDNA microarray analysis was performed using a mouse developmental cDNA microarray containing 15k clones derived from early Kargul libraries (Tanaka et al., 2000; Kargul et al., 2001; Noailles et al., 2003). cDNA probes were prepared by reverse transcription with [³³P]dCTP from 5 μ g total RNA obtained from each sample. cDNA probes were purified by using Biospin P-30 spin columns (BioRad, Hercules, CA). Array membranes were prehybridized in 4 ml microhybridization buffer (Research Genetics, Huntsville, AL) in a rotating hybridization oven at 55 °C for 2 hr, and then heat-denatured cDNA probes were applied to the microarrays for 18 hr at 55 °C with rotation. The microarray membranes were sequentially washed in 2 \times SSC containing 0.1% SDS for 5 min twice at room temperature, and in 2 \times SSC containing 0.1% SDS for 20 min twice at 65 °C. The microarrays were exposed to low energy phosphorimager screens (Molecular Dynamics, Sunnyvale, CA) for 5 days and scanned with a Phosphorimager Storm 860 system (Molecular Dynamics) at 50 μ m resolution. ImageQuant software (Molecular Dynamics) was used for image analysis.

Microarray Data Analysis

Z score transformation was employed to compare the array data between different treatments (Cheadle et al., 2003). Z score transformation allows analyzing array data independent of the original pixel intensities and can be used in the calculation of p values for significance estimates. To calculate gene expression changes after cannabinoids or capsaicin treatments, Z scores were converted to Z ratios, which represent fold-like changes for each gene. Statistical analysis was based on an increase or decrease of at least 2.0, and $p < 0.05$.

Quantitative Real Time RT-PCR

Total RNA was isolated (RNA STAT-60 kit, Tel-Test Inc) and first-strand cDNA was synthesized (Promega) as described previously (Chen et al., 2005b) using 2 μ g of total RNA from the same samples used for the microarray analysis in a 20 μ l reaction. qPCR reactions were carried out using the DNA Engine Opticon Fluorescence Detection System (MJ Research, Waltham, MA) using DyNAmo SYBR Green qPCR kits according to the manufacturer's instructions. The primer sequences and size of the PCR product for 14-3-3 η was 5' CTTAGCCAAACAAGCCTTCG -3'; 5'-ATCTGAATAGCTGTGCTGCC-3', 278 bp, and for 14-3-3 ζ was 5'-CACAGCAAGCATACCAAGAA-3'; 5'-AGAATGAGGCAGACAAAGGT-3', 336bp. The results were analyzed using Opticon software. Relative expression was calibrated by normalizing to 18S-ribosomal RNA.

Western Blot Analysis and Antibodies

Anti-14-3-3 β (sc-629) antibody, which is broadly reactive among 14-3-3 protein family members, was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and α -tubulin antibody (T5168) was obtained from Sigma (St. Louis, MO). Western blotting was performed as previously described (Chen et al., 2005b). The primary antibody concentrations were 1:1000 for anti-14-3-3 β and 1:2000 for anti- α -tubulin. Following incubation with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1: 1000 to 1: 2000, Sigma, St. Louis, MO), immunoreactive bands were visualized by ECL, quantitated by volume densitometry using NIH image software, and normalized to α -tubulin. Data shown are means \pm SEM.

Measurement of LDH Activity

The release of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium was used as an index of cell death. After exposure to NMDA or control medium, the medium was collected and assayed for LDH activity using a non-radioactive cytotoxicity assay kit from Promega according to the manufacturer's protocol. LDH activity is measured by formation of nicotinamide adenine dinucleotide, resulting in the conversion of an iodonitro tetrazolium into a red formazan product, which was measured with a microtiter plate reader at an absorption wavelength of 490 nm. Results are presented as a percentage of control values.

Statistical Analysis

All cell culture experiments were conducted independently three to six times for each treatment group. Statistical analyses were performed using GraphPad InStat (V 3.05). Data were analyzed by one-way ANOVA with *post-hoc* Tukey multiple comparison tests. In all cases, differences were considered to be significant when $p < 0.05$. Data are presented as means \pm S.E.M.

RESULTS

Microarray Analysis

We employed cDNA microarrays to profile gene expression changes in AF5 cells 16 hr post-stimulation in cells treated with (a) NMDA alone, or with (b) THC, (c) WIN 55,212-2, and (d) capsaicin prior to NMDA exposure. A total of 768 transcripts, accounting for 5.1% of the total 15k genes in the array, were increased or decreased at least 2.0-fold ($p < 0.05$) in AF5 cells exposed to 7.5 mM of NMDA as compared to untreated control cells. Of these 768 transcripts, 50 transcripts were decreased by NMDA treatment, while 718 transcripts were increased (Fig. 1A). Pretreatment with THC prior to NMDA exposure reversed the decreased gene expression which was seen after NMDA treatment for 13 of the 50 decreased transcripts. Pretreatment with THC prior to NMDA exposure reversed the increased gene expression seen after NMDA treatment for 82 of the 718 increased transcripts (Fig. 1). The complete list of transcripts up-

regulated by THC combined with NMDA, and 32 selected examples of the 82 transcripts decreased by THC combined with NMDA are shown in Table 1 and Table 2.

One of the 13 transcripts decreased by NMDA and reversed by THC was Ywhah, or 14-3-3 η (Table 3). Of the seven known isoforms of 14-3-3 transcript, five are represented in the microarray. These are Ywhaq, Ywhag, Ywhae, Ywhab and Ywhah. Table 3 lists the changes in expression of these five 14-3-3 transcripts by NMDA or by NMDA plus cannabinoids or capsaicin. Among these five 14-3-3 isoforms, there were no significant changes except in Ywhah (14-3-3 η). Expression of Ywhah was down-regulated by 2.14-fold after NMDA exposure, while THC treatment in combination with NMDA caused a 3.08-fold increase, to levels above that of the control condition. WIN 55,212-2, another cannabinoid receptor agonist which does not induce neuroprotection in this model and capsaicin, an antioxidant which is a less effective neuroprotective agent than THC, produced smaller and non-significant changes in Ywhah expression after NMDA exposure. No changes in Ywhah were observed when cells were exposed to cannabinoids or capsaicin alone without NMDA treatment (data not shown).

qPCR 14-3-3 Gene Expression Analysis

14-3-3 η and 14-3-3 ζ , which are widely expressed in various tissues (Watanabe et al., 1994) were chosen for measurement by quantitative real time RT-PCR. Expression of both the 14-3-3 η and 14-3-3 ζ transcripts was decreased 16 hr after exposure to 7.5 mM NMDA, as compared to the untreated control condition. After exposure to NMDA plus THC, the expression of 14-3-3 η and 14-3-3 ζ were both increased as compared to either NMDA alone, or to untreated control cells. The effect of THC in reversing the decreased 14-3-3 η or 14-3-3 ζ induced by NMDA was considerably greater than the effect of WIN 55,212-2 or capsaicin (Fig. 2A,B). There were no changes in 14-3-3 η or 14-3-3 ζ when cells were treated with cannabinoids or capsaicin alone, without NMDA (data not shown). These data are therefore consistent with the microarray results.

14-3-3 ζ mRNA was quantified by qPCR at different time points after NMDA stimulation. Figure 2C shows the time course of 14-3-3 ζ mRNA expression under different conditions. When cells were treated with THC plus NMDA, 14-3-3 ζ mRNA expression was up-regulated at early time points. A significant increase in 14-3-3 ζ expression was seen by 30 min after NMDA treatment. In contrast, down-regulation after treatment with NMDA alone was not observed until 16 hr after stimulation.

14-3-3 Protein

14-3-3 protein was detectable in AF5 cells by Western blotting, with the antibody detecting a single band at approximately 30kDa. Figure 3 shows that 14-3-3 protein levels were increased after exposure to THC plus NMDA. There were no statistically significant changes after treatment with WIN 55,212-2 or capsaicin plus NMDA. Although 14-3-3 protein expression was not significantly down-regulated after exposure to NMDA alone, there is a possibility that changes in 14-3-3 η or 14-3-3 ζ were not detected, because the 14-3-3 β antibody is broadly reactive among 14-3-3 protein family members. In cells exposed to NMDA there was, however, a trend in the direction of a decrease, consistent with the changes which were seen in mRNA expression.

Transient Transfection of 14-3-3 Suppresses NMDA-Induced Cell Death

To directly test whether 14-3-3 expression can regulate AF5 cell survival, we monitored NMDA-induced cell death in the presence of transiently expressed 14-3-3 η or 14-3-3 ζ or the control vectors pcDNA3.1 or pDEST26. In addition, the 14-3-3 ζ K49E plasmid, a ligand binding-defective mutant, was also used as another control.

To examine transfection efficiency, Pc3/hrGFP was transiently transfected into AF5 cells using the same conditions. Transfection efficiency for Pc3/hrGFP was $47.5\% \pm 1.9$ (Fig. 4A), counting 220 GFP-positive cells in a total of 468 cells.

Exposure of AF5 cells to 5 to 10 mM NMDA reduced cell viability in a dose-dependent manner, as measured by the LDH activity assay (Fig. 4B). LDH activity was then measured when AF5 cells were transiently transfected with 14-3-3 η or 14-3-3 ζ plasmids, or the control vectors, followed by treatment with 7.5 mM of NMDA. Overexpression of either 14-3-3 η or 14-3-3 ζ suppressed NMDA-induced cell death, as compared to cells transfected with the control vectors. The 14-3-3 ζ K49E mutant did not have a protective effect (Fig. 4C). When cells were treated with the higher 10 mM NMDA dose, however, transient transfection with either the 14-3-3 η or 14-3-3 ζ plasmids did not induce a protective effect (data not shown).

Expression of 14-3-3 Proteins is Increased in the Transfected Cells Treated with NMDA

Under the condition of NMDA exposure, 14-3-3 protein levels were significantly increased in cells transfected with 14-3-3 η or 14-3-3 ζ . There was no increase in 14-3-3 protein in cells transfected with the control vectors pCDNA3.1 or pDEST26 (Fig. 5).

DISCUSSION

The present study demonstrates that decreased expression of two forms of 14-3-3, 14-3-3 η , (Ywhah) and 14-3-3 ζ (Ywhaz) was caused by toxic concentrations of NMDA in the AF5 cell line, which is sensitive to excitotoxic cell death by NMDA in high concentrations. THC, which has a neuroprotective effect in this model, was able to reverse these decreases induced by NMDA under a neuroprotective condition. Notably, under conditions of NMDA-induced cellular stress, THC treatment increased 14-3-3 η or 14-3-3 ζ expression to well above control levels. Transfection of AF5 cells with plasmids expressing 14-3-3 η or 14-3-3 ζ partially blocked NMDA toxicity, while control plasmids were ineffective. 14-3-3 protein levels were increased both in cells treated with THC before NMDA exposure, and in cells transiently transfected with 14-3-3 η or 14-3-3 ζ plasmids before NMDA exposure. Therefore, changes in the level of expression of 14-3-3 η or 14-3-3 ζ may play an important role in the neuroprotection produced by THC under conditions of NMDA-induced cellular stress.

In previous studies, we found that THC produces a more potent neuroprotective effect in AF5 cells as compared to WIN 55,212-2 or capsaicin. The effect may be related to the antioxidant properties of this compound, and is independent of the cannabinoid receptor CB1 (Chen et al., 2005b). The present study was undertaken to further investigate the protective mechanisms of cannabinoids and capsaicin.

Of the five isoforms of 14-3-3 represented in the mouse microarray, only 14-3-3 η was significantly down-regulated after exposure to NMDA and reversed by pre-treatment with THC. A relationship between 14-3-3 and cannabinoid receptor activation has previously been identified by cDNA microarray studies. 14-3-3 γ subunit expression was up-regulated in rat hippocampus after 24 hr THC treatment (Kittler et al., 2000). Another large scale array study also found that cannabinoid exposure (WIN 55,212-2) *in vivo* or *in vitro* up-regulated 14-3-3 expression (Grigorenko et al., 2002). In the present model microarray analysis, qPCR, and Western blotting demonstrated that 14-3-3 η or 14-3-3 ζ down-regulation can be reversed by pretreatment with THC before exposure to NMDA, but not by WIN 55,212-2. Although, to some degree, the neuroprotective effect of THC is shared by capsaicin (Chen et al., 2005b), the microarray and qPCR results did not show a similar change in 14-3-3 expression after capsaicin treatment. Therefore, the present results indicate that changes in 14-3-3 η or 14-3-3 ζ expression might be specific, and of functional importance related to the neuroprotective effect of THC.

Additional transcripts of potential interest which were identified by the microarray analysis include *Csnk1a1*, *Hsp60*, *Pdcd4* and *Pdcd7*. *Csnk1a1*, casein Kinase-1 $\alpha 1$ (CK1) was down regulated by 4.2-fold after NMDA toxicity and increased 4.9-fold by THC. CK1 is a ubiquitous eukaryotic protein kinase. Interaction of CK1 with protein turnover machinery may contribute to neurodegenerative diseases. In AD, CK1 co-localizes to ubiquitinated cytoplasmic lesions, including both granulovacuolar degeneration bodies and the tau filaments of neurofibrillary tangles (Kannanayakal et al., 2006), suggesting that it may play a role in disease pathogenesis. Therefore the neurotoxicity of NMDA and neuroprotection of THC may also involve CK1. Examples of transcripts decreased by THC after NMDA exposure which are related to cell death include heat shock protein 60 kDa (*Hsp60*) and programmed cell death proteins 4 (*Pdcd4*) and 7 (*Pdcd7*). There is an extensive literature related to the function of these transcripts in normal and injured cells and tissues and the molecular mechanisms of their expression in response to stress (Didelot et al., 2006). *Pdcd4* and 7 were increased by 4.6 and 2.6-fold after NMDA, and *Hsp60* was increased by 2.6-fold. Pre-treatment with THC decreased expression of these genes by 2.0, 2.2 and 2.0-fold, to levels below that of the control condition.

There are few prior studies which report changes in 14-3-3 η or 14-3-3 ζ expression in cellular models under physiological conditions. Nevertheless, increases in 14-3-3 η have been reported after methamphetamine (Muratake et al., 1995), and regulation of 14-3-3 ζ has been reported to participate in radiation-induced adaptive response (Guo et al., 2003). Studies of schizophrenia have identified changes in 14-3-3 η expression (Vawter et al., 2001) as well as possible genetic associations with 14-3-3 η polymorphisms (Toyooka et al., 1999; Wong et al., 2003). Changes in expression of 14-3-3 η have also been found in cocaine abuse (Lehrmann et al., 2003). Recently, Satoh and coworkers (2006) reported that human astrocytes in culture showed increased expression of 14-3-3 σ in response to oxidative and DNA-damaging stresses. It therefore appears that changes in 14-3-3 expression occur under a number of conditions involving cellular or physiological stress.

The present data are more consistent with a possible role of 14-3-3 expression in neuroprotection, rather than in the initiation of cell death or apoptosis. THC treatment increased 14-3-3 η and 14-3-3 ζ expression to well above control levels, but only under conditions of NMDA treatment. 14-3-3 protein expression showed a similar pattern. Moreover, THC increased 14-3-3 ζ expression within 30 min, whereas the decreases in expression of 14-3-3 ζ expression after NMDA were not seen until 16 hr after treatment. The pattern of changes in 14-3-3 η and 14-3-3 ζ expression is therefore more consistent with a primary role as a mediator of THC-induced neuroprotection, rather than being involved in the initiation of cell death. Although decreases in 14-3-3 ζ expression may contribute somewhat to NMDA-induced cell death, it does not seem that the changes occur rapidly enough to be a primary initiator of NMDA-induced cell death in the current model.

The up-regulation of 14-3-3 ζ was seen at an earlier time point, 30 min after treatment, as compared to the decreases produced by NMDA. This time course is consistent with our previous report that THC protects against NMDA-induced apoptosis in AF5 cells by blocking ROS generation and inhibiting the activation of p38-MAPK (Chen et al., 2005a). Many studies suggest that 14-3-3 can modulate the function of cellular signaling pathways, including p38-MAPK, and can modulate apoptosis induced by p38-MAPK pathway induction (Xing et al., 2000).

Transfection experiments were performed in order to test the possibility that the increases in expression of 14-3-3 proteins could be responsible for the protective effect of THC. Using plasmids which have previously been employed to study 14-3-3 functions (Subramanian et al., 2004), we found that over expression of 14-3-3 η or 14-3-3 ζ by transient transfection before NMDA exposure increased overall 14-3-3 protein levels and partially blocked NMDA-induced

cell death. Transfection efficiency was approximately 40%; therefore, the incomplete protection could be related to the levels of expression of 14-3-3. Also, changes in 14-3-3 expression could influence only one of several pathways by which NMDA initiates toxicity in AF5 cells, so that cell death-inducing pathways initiated by NMDA which are not modulated by 14-3-3 η and 14-3-3 ζ may proceed unchecked in cells transfected by 14-3-3 η or 14-3-3 ζ .

Taken together, the present results indicate that the 14-3-3 η and 14-3-3 ζ isoforms perform a survival-promoting function in AF5 cells treated with THC under conditions of NMDA toxicity. The 14-3-3 η and 14-3-3 ζ proteins, as upstream signal mediators, may therefore play an important role in the neuroprotective effect of THC during NMDA-induced apoptosis.

ACKNOWLEDGEMENTS

We are grateful to Dr. Haiyan Fu (Emory University, Atlanta, GA) for providing us with 14-3-3 plasmids and to Dr. Stephen Dewhurst (University of Rochester School of Medicine and Dentistry, Rochester, NY) for providing us with Pc3/hrGFP plasmid. We also thank Diane Teichberg, William H. Wood III (DNA Array Unit, NIA IRP), for their expert assistance. Research supported by the IRP of NIDA, NIDA, DHHS.

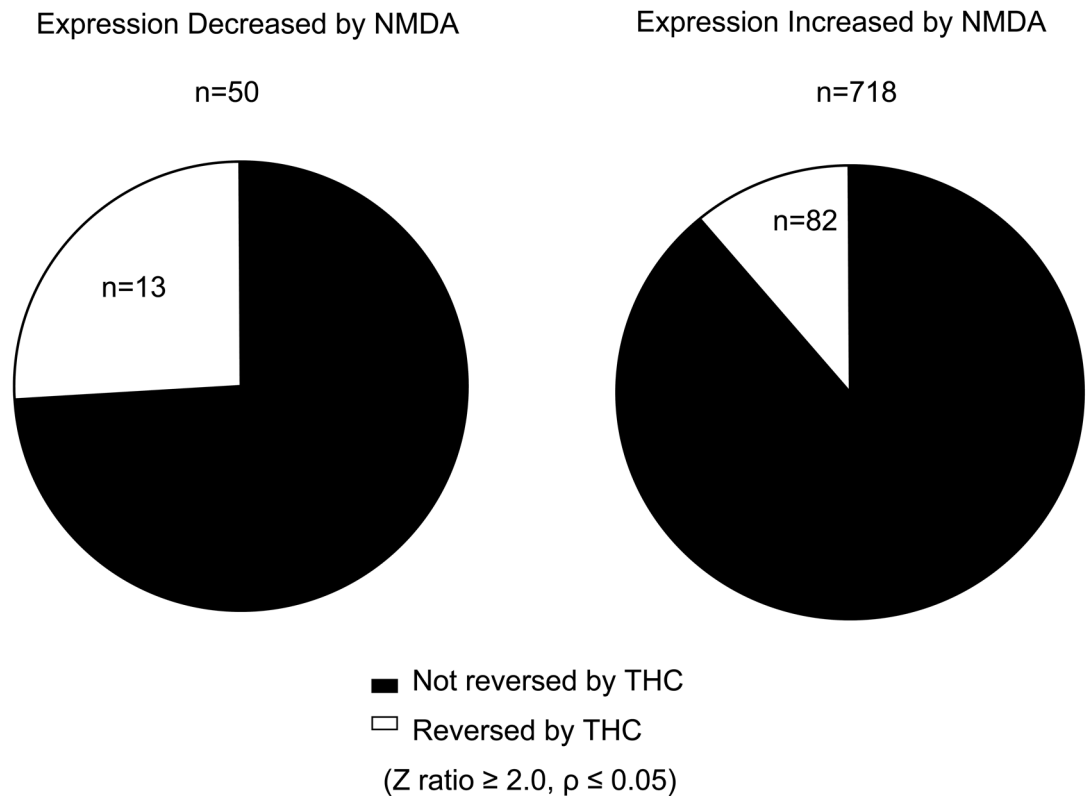
REFERENCES

- Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999;96:857–868. [PubMed: 10102273]
- Cheadle C, Vawter MP, Freed WJ, Becker KG. Analysis of microarray data using Z score transformation. *J Mol Diagn* 2003;5:73–81. [PubMed: 12707371]
- Chen J, Errico SL, Freed WJ. Reactive oxygen species and p38 phosphorylation regulate the protective effect of Delta (9)-tetrahydrocannabinol in the apoptotic response to NMDA. *Neurosci Lett* 2005a; 389:99–103. [PubMed: 16098661]
- Chen J, Lee CT, Errico SL, Deng X, Cadet JL, Freed WJ. Protective effects of Delta (9)-tetrahydrocannabinol against N-methyl-d-aspartate-induced AF5 cell death. *Brain Res Mol Brain Res* 2005b;134:215–225. [PubMed: 15836919]
- Chen XQ, Fung YW, Yu AC. Association of 14-3-3 γ and phosphorylated bad attenuates injury in ischemic astrocytes. *J Cereb Blood Flow Metab* 2005;25:338–347. [PubMed: 15660102]
- Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell* 2000;6:41–51. [PubMed: 10949026]
- Didelot C, Schmitt E, Brunet M, Maingret L, Parcellier A, Garrido C. Heat shock proteins: Endogenous modulators of apoptotic cell death. *Handb Exp Pharmacol* 2006;172:171–198. [PubMed: 16610360]
- Grigorenko E, Kittler J, Clayton C, Wallace D, Zhuang SY, Bridges D, Bunday S, Boon A, Paget C, Hayashizaki S, Lowe G, Hampson R, Deadwyler S. Assessment of cannabinoid induced gene changes: tolerance and neuroprotection. *Chem Phys Lipids* 2002;121:257–266. [PubMed: 12505705]
- Guo G, Yan-Sanders Y, Lyn-Cook BD, Wang T, Tamae D, Ogi J, Khaletskiy A, Li Z, Weydert C, Longmate JA, Huang TT, Spitz DR, Oberley LW, Li JJ. Manganese superoxide dismutase-mediated gene expression in radiation-induced adaptive responses. *Mol Cell Biol* 2003;23:2362–2378. [PubMed: 12640121]
- Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 1997;275:90–94. [PubMed: 8974401]
- Kannanayakal TJ, Tao H, Vandredy DD, Kuret J. Casein kinase-1 isoforms differentially associate with neurofibrillary and granulovacuolar degeneration lesions. *Acta neuropathol* 2006;111:413–421. [PubMed: 16557393]
- Kargul GJ, Dudekula DB, Qian Y, Lim MK, Jaradat SA, Tanaka TS, Carter MG, Ko MS. Verification and initial annotation of the NIA mouse 15K cDNA clone set. *Nat Genet* 2001;28:17–18. [PubMed: 11326268]

- Kittler JT, Grigorenko EV, Clayton C, Zhuang SY, Bunday SC, Trower MM, Wallace D, Hampson R, Deadwyler S. Large-scale analysis of gene expression changes during acute and chronic exposure to [Δ] 9-THC in rats. *Physiol Genomics* 2000;3:175–185. [PubMed: 11015613]
- Lehrmann E, Hyde TM, Vawter MP, Becker KG, Kleinman JE, Freed WJ. The use of microarrays to characterize neuropsychiatric disorders: postmortem studies of substance abuse and schizophrenia. *Curr Mol Med* 2003;3:437–446. [PubMed: 12942997]
- Martin H, Patel Y, Jones D, Howell S, Robinson K, Aitken A. Antibodies against the major brain isoforms of 14-3-3 protein. An antibody specific for the N-acetylated aminoterminal of a protein. *FEBS Lett* 1993;331:296–303. [PubMed: 8375512]
- Masters SC, Fu H. 14-3-3 proteins mediate an essential anti-apoptotic signal. *J Biol Chem* 2001;276:45193–45200. [PubMed: 11577088]
- Masters SC, Subramanian RR, Truong A, Yang H, Fujii K, Zhang H, Fu H. Survival-promoting functions of 14-3-3 proteins. *Biochem Soc Trans* 2002;30:360–365. [PubMed: 12196095]
- McGowan MH, Russell P, Carper DA, Lichtstein D. Na⁺, K⁺-ATPase inhibitors down-regulate gene expression of the intracellular signaling protein 14-3-3 in rat lens. *J Pharmacol Exp Ther* 1999;289:1559–1563. [PubMed: 10336553]
- Moore, BW.; Perez, VJ. Specific Acidic Proteins of the Nervous System. In: Carlson, FD., editor. *Physiological and Biochemical Aspects of Nervous Integration*. Prentice Hall: Englewood Cliffs, New Jersey; 1967. p. 343-359.
- Muratake T, Hayashi S, Ichikawa T, Kumanishi T, Ichimura Y, Kuwano R, Isobe T, Wang Y, Minoshima S, Shimizu N, Takahashi Y. Structural organization and chromosomal assignment of the human 14-3-3 eta chain gene (YWHAH). *Genomics* 1996;36:63–69. [PubMed: 8812417]
- Muratake T, Hayashi S, Ichimura Y, Morii K, Kuwano R, Ichikawa T, Kumanishi T, Isobe T, Watanabe M, Kondo H. The effect of methamphetamine on the mRNA level for 14.3.3 eta chain in the human cultured cells. *Mol Neurobiol* 1995;11:223–230. [PubMed: 8561965]
- Noailles PA, Becker KG, Wood WH III, Teichberg D, Cadet JL. Methamphetamine-induced gene expression profiles in the striatum of male rat pups exposed to the drug in utero. *Brain Res Dev Brain Res* 2003;147:153–162.
- Sato J, Tabunoki H, Nanri Y, Arima K, Yamamura T. Human astrocytes express 14-3-3 sigma in response to oxidative and DNA-damaging stresses. *Neurosci Res* 2006;56:61–72. [PubMed: 16797759]
- Subramanian RR, Zhang H, Wang H, Ichijo H, Miyashita T, Fu H. Interaction of apoptosis signal-regulating kinase 1 with isoforms of 14-3-3 proteins. *Exp Cell Res* 2004;294:581–591. [PubMed: 15023544]
- Tanaka TS, Jaradat SA, Lim MK, Kargul GJ, Wang X, Grahovac MJ, Pantano S, Sano Y, Piao Y, Nagaraja R, Doi H, Wood WH III, Becker KG, Ko MS. Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proc Natl Acad Sci USA* 2000;97:9127–9132. [PubMed: 10922068]
- Toyooka K, Muratake T, Tanaka T, Igarashi S, Watanabe H, Takeuchi H, Hayashi S, Maeda M, Takahashi M, Tsuji S, Kumanishi T, Takahashi Y. 14-3-3 protein eta chain gene (YWHAH) polymorphism and its genetic association with schizophrenia. *Am J Med Genet* 1999;88:164–167. [PubMed: 10206237]
- Truckenmiller ME, Tornatore C, Wright RD, Dillon-Carter O, Meiners S, Geller HM, Freed WJ. A truncated SV40 large T antigen lacking the p53 binding domain overcomes p53-induced growth arrest and immortalizes primary mesencephalic cells. *Cell Tissue Res* 1998;291:175–189. [PubMed: 9426306]
- Truckenmiller ME, Vawter MP, Zhang CP, Conejero-Goldberg C, Dillon-Carter O, Morales N, Cheadle C, Becker KG, Freed WJ. AF5, a CNS cell line immortalized with an n-terminal fragment of SV40 large T: Growth, differentiation, genetic stability and gene expression. *Exp Neurol* 2002;175:318–337. [PubMed: 12061863]
- van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays* 2001;23:936–946. [PubMed: 11598960]
- Vawter MP, Barrett T, Cheadle C, Sokolov BP, Wood WH III, Donovan DM, Webster M, Freed WJ, Becker KG. Application of cDNA microarrays to examine gene expression differences in schizophrenia. *Brain Res Bull* 2001;55:641–650. [PubMed: 11576761]

- Watanabe M, Isobe T, Ichimura T, Kuwano R, Takahashi Y, Kondo H, Inoue Y. Molecular cloning of rat cDNAs for the zeta and theta subtypes of 14-3-3 protein and differential distributions of their mRNAs in the brain. *Brain Res Mol Brain Res* 1994;25:113–121. [PubMed: 7984035]
- Wong AH, Macciardi F, Klempan T, Kawczynski W, Barr CL, Lakatoo S, Wong M, Buckle C, Trakalo J, Boffa E, Oak J, Azevedo MH, Dourado A, Coelho I, Macedo A, Vicente A, Valente J, Ferreira CP, Pato MT, Pato CN, Kennedy JL, Van Tol HH. Identification of candidate genes for psychosis in rat models, and possible association between schizophrenia and the 14-3-3zeta gene. *Mol Psychiatry* 2003;8:156–166. [PubMed: 12610648]
- Xing H, Zhang S, Weinheimer C, Kovacs A, Muslin AJ. 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. *EMBO J* 2000;19:349–358. [PubMed: 10654934]
- Zhang L, Chen J, Fu H. Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. *Proc Natl Acad Sci USA* 1999;96:8511–8515. [PubMed: 10411906]
- Zhang L, Wang H, Liu D, Liddington R, Fu H. Raf-1 kinase and exoenzyme S interact with 14-3-3zeta through a common site involving lysine 49. *J Biol Chem* 1997;272:13717–13724. [PubMed: 9153224]

Numbers of transcripts changed by NMDA and by NMDA plus THC treatment

**Fig. 1.**

Microarray analysis of NMDA and THC treated AF5 cells. Subpopulation of genes with significantly changed expression after NMDA or THC plus NMDA treatment. Genes which show both Z ratio \geq 2.0 and $p < 0.05$ ($N = 3$ microarrays per treatment) were considered as being changed significantly. The black area represents transcripts altered by NMDA treatment as compared to control, while the white area represents transcripts for which the change after THC plus NMDA was reversed as compared to NMDA alone.

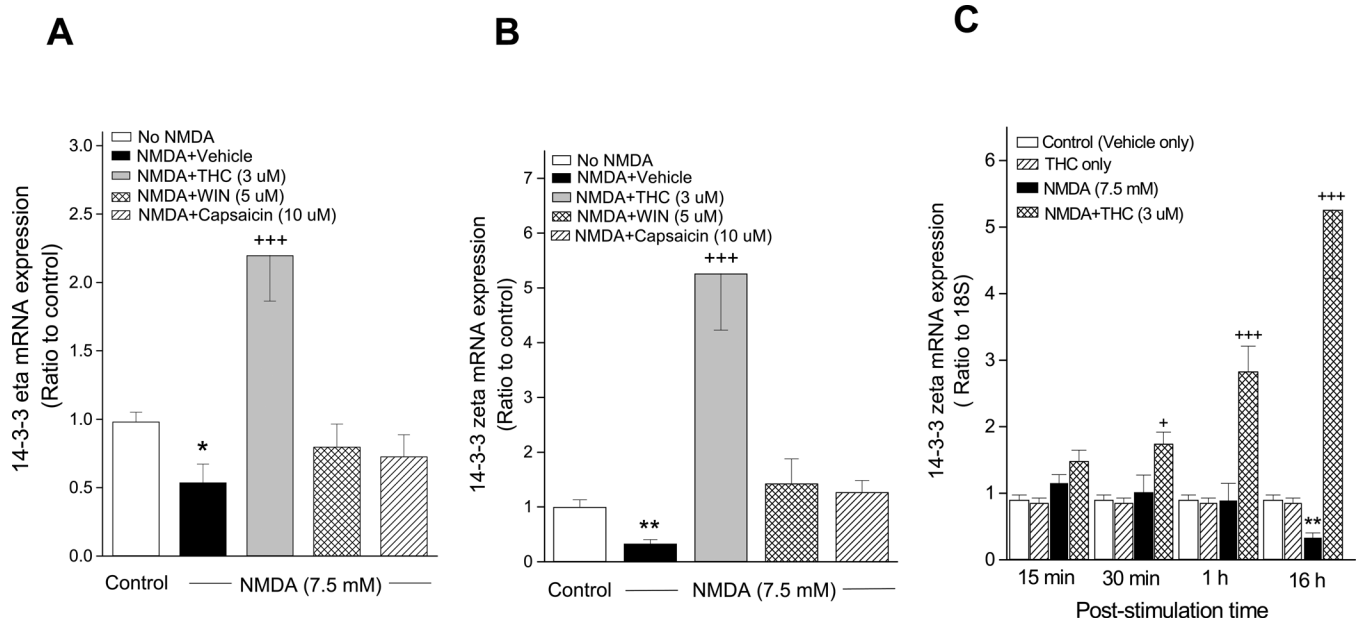


Fig. 2.

Expression of the 14-3-3 eta and 14-3-3 zeta transcripts were quantified by qPCR. 14-3-3 eta (14-3-3 η), (A) and 14-3-3 zeta (14-3-3 ζ), (B) were significantly down-regulated 16 hr after stimulation with 7.5 mM NMDA. THC reversed the down-regulation of 14-3-3 eta and 14-3-3 zeta in AF5 treated cells with NMDA. For statistical analysis, a one-tailed *t* test was used to compare NMDA and control groups; *, $p < 0.05$; **, $p < 0.01$. A one-way ANOVAs with post-hoc Tukey multiple comparisons were used to compare the remaining groups to NMDA alone; +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$. (C) Time course of 14-3-3 zeta mRNA expression. 14-3-3 zeta was up-regulated significantly 30 min and 1 hr after NMDA stimulation in the THC plus NMDA group. **, $p < 0.01$ as compared to the control group; +++, $p < 0.001$ as compared to the NMDA treatment group. Data shown are means \pm S.E.M. from four identical experiments. The amounts of 14-3-3 eta and 14-3-3 zeta transcripts are expressed as ratios to 18S for each individual experiment.

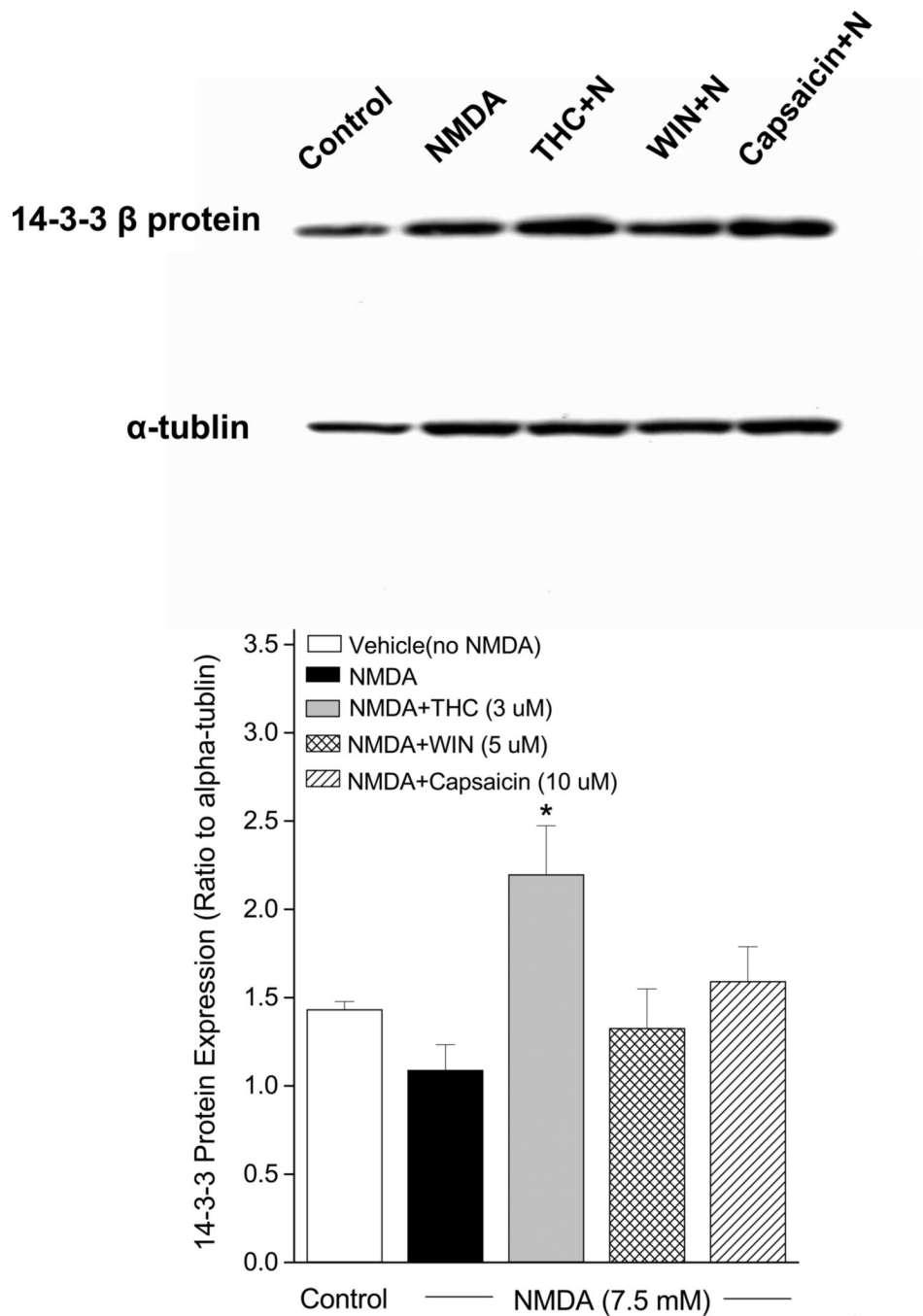
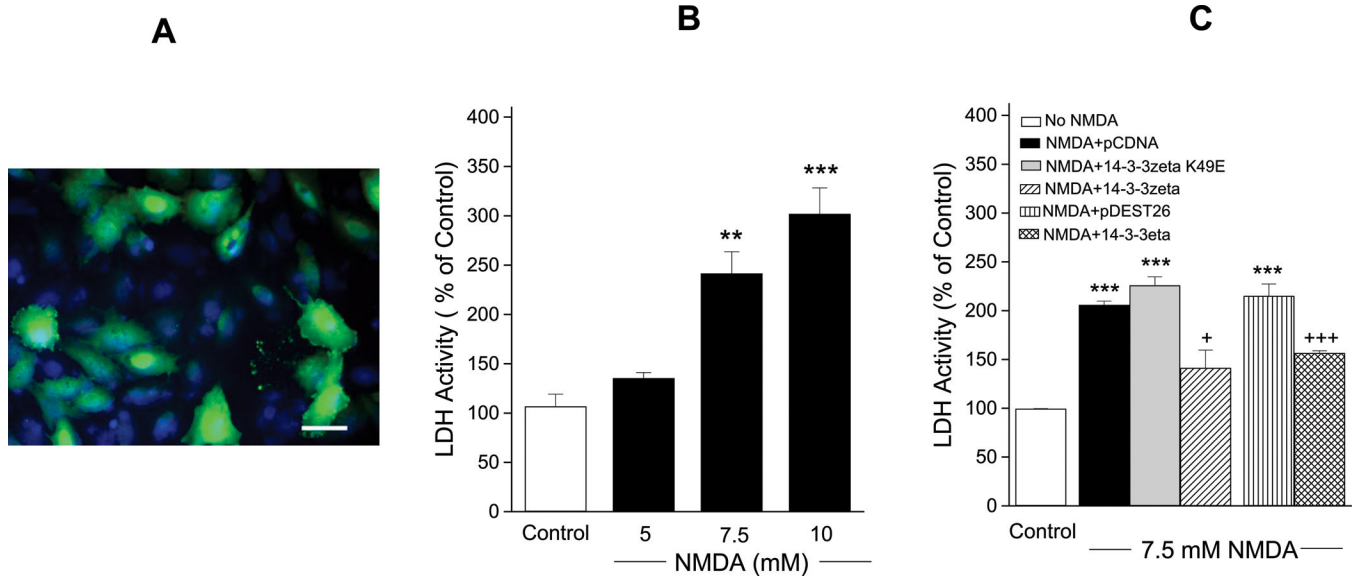


Fig. 3.

Expression of 14-3-3 protein as measured by Western blotting. Cultured cells were harvested 16 hr after stimulation with NMDA (7.5 mM). The 14-3-3 antibody recognized a single band in AF5 cells at 30 KDa. Total 14-3-3 proteins were significantly up-regulated after exposure to THC plus NMDA, as compared to NMDA alone (*, $p < 0.05$). Statistical analysis was based on three independent experiments. Data shown are means \pm S.E.M.

**Fig. 4.**

Overexpression of 14-3-3 eta or 14-3-3 zeta suppressed NMDA-induced cell death. AF5 cells were transiently transfected with expression vectors, encoding 14-3-3 eta (14-3-3 η), 14-3-3 zeta (14-3-3 ζ), or one of the three control vectors (14-3-3 ζ K49E, pCDNA3.1, pDEST26) for 6 hr, then cells were exposed to 7.5 mM NMDA for 30 min. Cells were harvested after an additional 16 hr. Increased LDH in the medium reflects cell lysis and loss of viability. (A) Transfection efficiency with Pc3/hrGRP in AF5 cells; as indicated by GFP expression (green cells) after 16 hr, Bar = 20 μ M. (B) Dose-dependency of the loss of cell viability by NMDA. **, $p < 0.01$; ***, $p < 0.001$, respectively, as compared to untreated controls. (C) Effects of the 14-3-3 expression vectors transfection followed by 7.5 mM NMDA treatment. Each condition was assessed by three independent experiments and data shown are means \pm S.E.M. A one-way ANOVA with post-hoc Tukey multiple comparison tests was used for statistical analysis. ***, $p < 0.001$, compared to control; +, $p < 0.05$ compared to pCDNA3.1 vector plus 7.5 mM NMDA; +++, $p < 0.001$ compared to pDEST26 vector plus 7.5 mM NMDA.

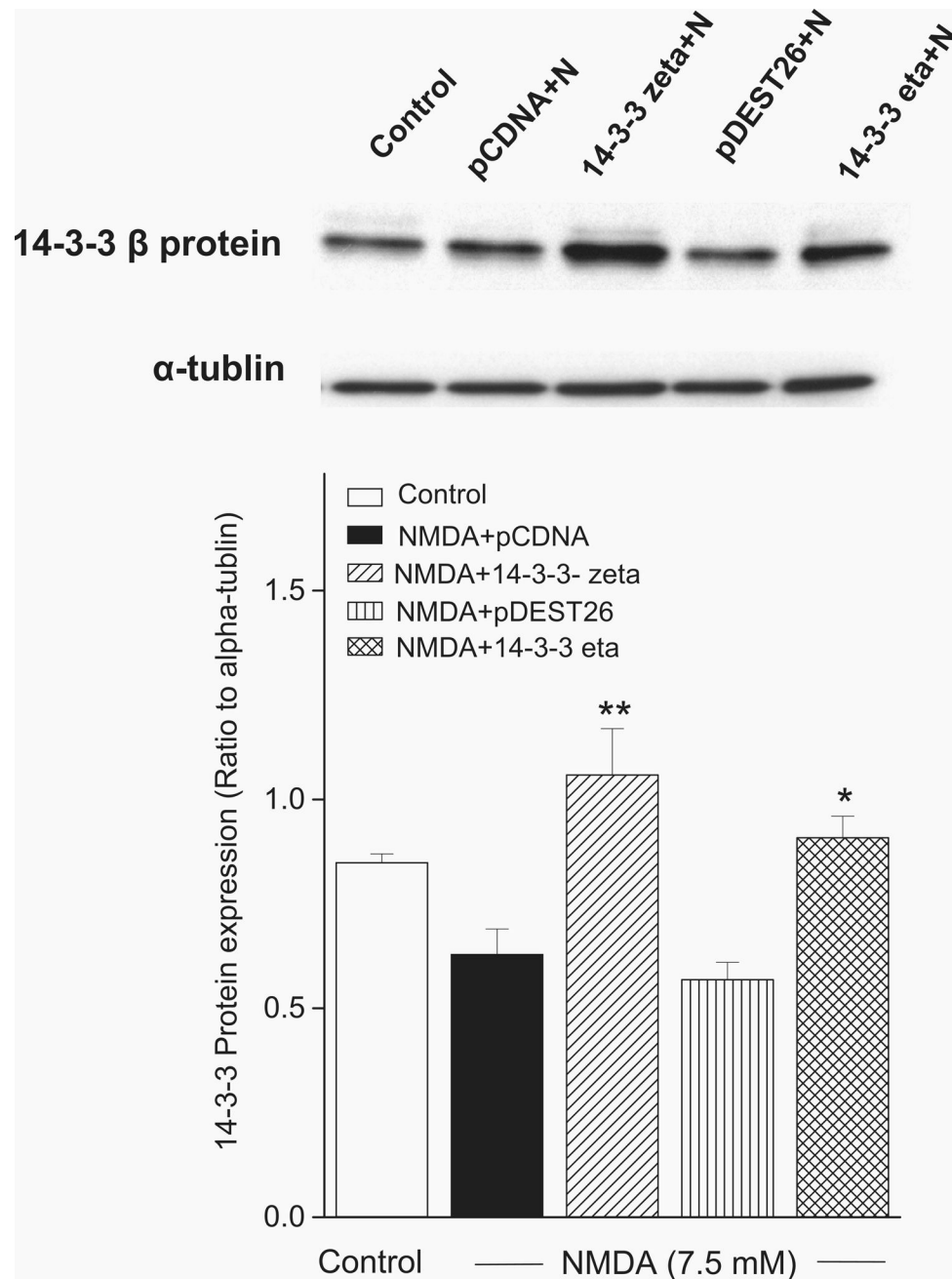


Fig. 5.

Expression of 14-3-3 protein in transfected cells exposed to NMDA as measured by Western blotting. AF5 cells were transiently transfected with expression vectors encoding 14-3-3 eta (14-3-3 η), 14-3-3 zeta (14-3-3 ζ), or one of the two control vectors (pCDNA3.1, pDEST26). Cells were harvested 16 hr after NMDA exposure. Total 14-3-3 proteins were significantly up-regulated when cells were transfected with the 14-3-3 eta or 14-3-3 zeta encoding plasmids plus NMDA treatment, as compared to the control vectors plus NMDA (*, $p < 0.05$; **, $p < 0.01$). Statistical analysis was based on three independent experiments. Data shown are means \pm S.E.M.

TABLE 1

Transcripts Increased by THC after NMDA Exposure

| Gene Name | Gene Symbol | Genebank | Z ratio (NMDA/Control) | Z ratio |
|---|---------------|----------|------------------------|---------|
| expressed sequence AI317223 | AI317223 | BQ552213 | -2.1 | |
| proteasome (prosome, macropain) 26S subunit, non-ATPase, 12 | Psmc12 | BQ552401 | -2.1 | |
| tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide | Ywhah | BG065012 | -2.1 | |
| RIKEN cDNA 1810074P20 gene | 1810074P20Rik | BQ551388 | -2.1 | |
| alveolar soft part sarcoma chromosome region, candidate 1 | Aspsc1 | BG065426 | -2.2 | |
| glycine cleavage system protein H (aminomethyl carrier) | Gcsh | BG078677 | -2.1 | |
| ankyrin repeat domain 27 (VPS9 domain) | Ankrd27 | BG064159 | -2.4 | |
| signal recognition particle receptor ('docking protein') | Srpr | BG078637 | -3.6 | |
| casein kinase 1, alpha 1 | Csnk1a1 | BG072410 | -4.2 | |
| RIKEN cDNA 1810015A11 gene | 1810015A11Ri | | | |
| SERTA domain containing 1 | k | BQ551071 | -2.7 | |
| cystatin C | Sertad1 | BQ551069 | -5.1 | |
| RIO kinase 2 (yeast) | Cst3 | BQ552407 | -3.5 | |
| | Riok2 | BG078437 | -4.6 | |

TABLE 2

Selected Examples (32/82) of Transcripts Decreased by THC after NMDA Exposure

| Gene Name | Gene Symbol | Genebank | Z ratio (NMDA/ Control) | Z ratio (NMDA +THC/NMDA) |
|---|----------------|----------|----------------------------|-----------------------------|
| necdin | Ndn | BG079634 | 5.4 | -2.8 |
| cullin 4B | Cul4b | AW536452 | 5.2 | -2.3 |
| large tumor suppressor | Lats1 | BG081236 | 5.2 | -3.1 |
| interleukin 1 receptor-associated kinase | Il1rak | BG069778 | 4.7 | -2.6 |
| programmed cell death 4 | Pcd4 | BG063238 | 4.6 | -2.0 |
| ubiquitin A-52 residue ribosomal protein fusion product 1 | Uba52 | BG065688 | 4.0 | -3.5 |
| hemoglobin Y, beta-like embryonic chain | Hbb-y | BG079368 | 3.6 | -2.5 |
| ATP-binding cassette, sub-family E (OABP), member 1 | Abce1 | BG068195 | 3.4 | -2.0 |
| ninein | Nin | BG071156 | 3.3 | -2.1 |
| GTP binding protein 3 | Gtpbp3-pending | BG083560 | 3.2 | -2.8 |
| B-cell translocation gene 4 | Btg4 | BG082967 | 3.2 | -3.1 |
| tousled-like kinase 2 (Arabidopsis) | Tlk2 | BQ550791 | 3.2 | -2.2 |
| growth differentiation factor 3 | Gdf3 | BG079333 | 3.1 | -2.2 |
| toll interacting protein | Tollip-pending | C79165 | 3.0 | -2.0 |
| nuclear receptor subfamily 5, group A, member 2 | Nr5a2 | BG079994 | 2.9 | -2.3 |
| ubiquitin-conjugating enzyme E2, J2 homolog (yeast) | Ubc6p-pending | BQ552740 | 2.9 | -2.1 |
| coagulation factor C homolog (Limulus polyphemus) | Coch | BG072342 | 2.8 | -2.3 |
| heat shock protein, 60 kDa | Hsp60 | BG064728 | 2.7 | -2.0 |
| TYRO3 protein tyrosine kinase 3 | Tyro3 | BG066134 | 2.6 | -2.0 |
| ribosomal protein L3 | Rpl3 | BG079511 | 2.6 | -2.4 |
| signal transducing adaptor molecule (SH3 domain and ITAM) | Stam2 | BG063497 | 2.6 | -2.0 |
| programmed cell death protein 7 | Pcd7 | BG066710 | 2.6 | -2.2 |
| Rho GTPase activating protein 5 | Arhgap5 | BQ551551 | 2.5 | -2.6 |
| large tumor suppressor | Lats1 | BG081357 | 2.4 | -2.0 |
| ferrochelatase | Fech | BG066502 | 2.3 | -2.3 |
| meiosis-specific nuclear structural protein 1 | Mns1 | BG078615 | 2.3 | -2.3 |
| histocompatibility 2, D region locus 1 | H2-D1 | BG087558 | 2.3 | -2.9 |
| lurcher transcript 1 | Lt1 | BG065913 | 2.2 | -2.4 |
| hyaluronan and proteoglycan link protein 1 | Hapln1 | BQ551248 | 2.2 | -2.1 |
| lactotransferrin | Ltf | BG070413 | 2.2 | -4.3 |
| DNA segment, Chr 2, ERATO Doi 120, expressed | D2Erd120e | BG065802 | 2.1 | -2.2 |
| LPS-responsive beige-like anchor | Lrba | BG082946 | 2.0 | -2.4 |

TABLE 3
Regulation of 14-3-3 Gene Expression for NMDA Exposure and NMDA Plus Cannabinoids or Capsaicin

| Conditions | Ywhaq 14-3-30 | Ywhag 14-3-3γ | Ywhae 14-3-3ε | Ywhab 14-3-3β | Ywhah 14-3-3η |
|---------------------|---------------|---------------|---------------|---------------|-----------------------|
| NMDA/Normal | -0.46 | -0.75 | -0.83 | -0.56 | -2.142 ^{**} |
| THC+NMDA/NMDA | +1.07 | +0.56 | +0.64 | +0.63 | +3.080 ⁺⁺⁺ |
| WIN+NMDA/NMDA | +0.05 | +0.66 | +0.88 | +0.68 | +1.120 |
| Capsaicin+NMDA/NMDA | +0.78 | +0.54 | +0.68 | +0.63 | +1.900 |
| NMDA/Normal | -0.46 | -0.75 | -0.83 | -0.56 | -2.142 ^{**} |

Statistical analysis was based on an increase or decrease of at least 2.0-fold, $p < 0.05$.

Alterations in expression of five 14-3-3 isoforms after NMDA exposure, or after NMDA plus cannabinoid or capsaicin treatment. 14-3-3η (eta) (Ywhah) was significantly down-regulated after NMDA exposure and up-regulated by THC treatment before exposure to NMDA. The other four isoforms in the microarray, were not significantly changed. Data shown are representative of three independent experiments.

^{**} $p < 0.01$ as compared with the control group

⁺⁺⁺ $p < 0.001$ as compared to the NMDA alone group.