The molecular events occur during MK-801-induced cytochrome oxidase subunit II down-regulation in GT1-7 cells

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Abstract

Previously, we showed that predominant expression of the *N*-methyl-D-aspartate (NMDA) receptor in the neurons of the sexually dimorphic nucleus of the preoptic area of male rats plays an important role in preventing neurons from apoptosis during sexual development. Blocking of the NMDA receptor by dizocilpine ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-iminemaleate (MK-801) causes down-regulation of some survival-related genes including cytochrome oxidase subunit II (*COII*), a mitochondria-encoded complex IV subunit, which in turn induces ATP depletion and the occurrence of apoptosis. The aim of this study is to investigate the molecular events during down-regulation of the *COII* gene expression induced by blocking of the NMDA receptor. Treatment of the GnRH cell line (GT1-7) with MK-801 caused 1) a decrease of intracellular calcium concentration ($[Ca^{2+}]_i$) after 20 h; 2) significant decreases of the levels of peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) mRNA and protein after 24 h; 3) down-regulation of *COII* mRNA after 36 h; and 4) the occurrence of neuronal apoptosis after 48 h. Accordingly, we hypothesize that blocking of the NMDA receptor may cause a decrease of the $[Ca^{2+}]_i$, which in turn inhibits the expressions of PGC-1 and *COII* and then leads to subsequent neuronal apoptosis.

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Introduction

Activation of the *N*-methyl-D-aspartate (NMDA) receptor may be critical for neuronal survival during a certain period of the brain development (Hwang *et al.* 1999). Blocking of the NMDA receptor by MK-801 (dizocilpine), a noncompetitive NMDA receptor antagonist, has been shown to promote cell death in the developing brain (Ikonomidou *et al.* 1999), suggesting that physiological activation of the NMDA receptor is an important regulator of neuron survival (Fiske & Brunjes 2001). The apoptotic incidence of neurons in the sexually dimorphic nucleus of the preoptic area (SDN-POA) of male rats is significantly increased when the NMDA receptor is blocked by MK-801 (Hsu *et al.* 2001). Recently, we reported that some neurotropic genes (RNA binding motif protein 3 (*RBM3*), cytosolic phosphoprotein (*P19*), cytoskeletal γ -actin, and α -tubulin) as well as survival-related genes (*Bcl-2*, cytochrome oxidase subunits II, III (*COII*), (*COIII*), and cytochrome *b*) in SDN-POA of neonatal male rats were significantly down-regulated when the NMDA receptor was blocked (Hsu *et al.* 2005).

COII, a mitochondria-encoded subunit of complex IV (Wong-Riley 1989, Zhang & Wong-Riley 2000), is an enzyme involved in the mitochondrial electron

transport chain that catalyzes the transfer of electrons to generate ATP via the coupled process of oxidative phosphorylation (Capaldi 1990b). Abnormal mitochondrial respiratory chain function due to genetic defects or chemical disruption results in a deficiency in ATP generation and subsequent cell death (Chandra *et al.* 2002). It has been suggested that ATP depletion caused by *COII* reduction may play an important role in the hypoxia-induced apoptotic cell death (Bae *et al.* 1998). However, the molecular mechanism underlying MK-801-induced *COII* suppression is still unclear.

The transcription of mitochondria DNA-encoded genes such as *COII* could be activated by mitochondrial transcriptional factor A (mtTFA; Tfam; Dong *et al.* 2002, Vijayasathiy *et al.* 2003), which could be induced by raising cytosolic Ca^{2+} concentrations (Ojuka *et al.* 2003). Activation of the NMDA receptor may elevate cytosolic Ca^{2+} concentrations via extracellular calcium influx (Dayanithi *et al.* 1995), subsequently activates calcium/calmodulin-dependent protein kinase IV (CaMKIV; See *et al.* 2001) and induces the expression of proliferator-activated receptor γ coactivator-1 (PGC-1; Wu *et al.* 2002) as well as the nuclear respiratory factor (NRF-2), a transcriptional activator of mtTFA (Wu *et al.* 1999, 2002, Ojuka 2004). On the other hand, blocking of the NMDA and

alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite receptor may produce neuronal apoptosis due to Ca^{2+} deficiency (Yoon *et al.* 2003). Accordingly, we hypothesize that blocking of the NMDA receptor may cause a decrease in intracellular Ca^{2+} concentration, which in turn inhibits the gene expression of PGC-1 and NRF-2, and finally leads to the suppression of COII expression and neuronal apoptosis. The present study was designed to investigate the possible signaling pathway of NMDA receptor-mediated regulation of COII gene expression in mitochondria.

Materials and methods

Cell cultures

The immortalized GT1-7 cells, which express the NMDA receptor (NMDAR) and mitochondrial COII genes (Lawson *et al.* 1995), were derived from hypothalamic gonadotrophin-releasing hormone (GnRH) neurons and generously provided by Dr Ke-Wen Dong (The Jone Institute for Reproductive Medicine, East Virginia Medical School). The cells were cultured in Dulbecco's Modified Eagle's Medium (4.5 mg/ml glucose, 0.6 mg/ml L-glutamine, 3.7 mg/ml NaHCO_3), supplemented with 15% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco), at 37 °C in a humidified air containing 5% CO_2 (Urbanski *et al.* 1994, Yang *et al.* 2003). Media were changed every other day. The cells were grown until 60% confluence and then treated with MK-801 at a concentration of 10^{-2} or 10^{-3} µM for the indicated time intervals.

Intracellular calcium measurement by flow cytometry

The levels of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) were analyzed using a calcium-sensitive fluorescence dye Fluo-3 (Molecular Probe Inc., Carlsbad, CA, USA; June & Rabinovitch 1994, Yoon *et al.* 2003). Cells were plated at a density of 1×10^5 cells/cm² in a 25T flask, maintained for 1 day, and then treated with MK-801 (10^{-2} or 10^{-3} µM) for the indicated time intervals. Cells were then trypsinized (0.25% trypsin, 0.02% EDTA in calcium-magnesium free Hank's A solution) for 1 min and centrifuged at 1500 r.p.m for 5 min at 4 °C. The cell pellets were resuspended in 1 ml Hank's A solution, 5 µM Fluo-3 AM dye added, and then incubated at 37 °C in a CO_2 incubator for 30 min with gentle mixing every 10 min. After being centrifuged at 1500 r.p.m for 5 min at 4 °C, the cell pellets were washed twice with 1 ml Hank's A solution, and resuspended in 1 ml Hank's A solution. The green fluorescence of Fluo-3 was excited at 488 nm and the emission (525 nm) was detected using Beckman Coulter flow cytometry (Burchiel *et al.* 2000) and then

analyzed by software (WinMDI 2.8; Becton Dickinson Immunocytometry System, San Jose, CA, USA).

Isolation of total RNA

The GT1-7 cells were homogenized in TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). After homogenization, 200 µl chloroform per 1 ml TRI-Reagent was added and mixed well. The mixture was then incubated at room temperature for 10 min. The aqueous phase was separated after centrifugation at 12 000 r.p.m for 15 min at 4 °C and the total RNA was precipitated with 500 µl isopropanol. The total RNA was pelleted by centrifugation at 12 000 r.p.m for 8 min at 4 °C, washed with 1 ml of 75% ethanol, and then resuspended in DEPC-water (Mahesh *et al.* 1999).

Semi-quantitative reverse transcription-PCR (RT-PCR) analysis

RNA was transcribed into cDNA (cDNA) with reverse transcriptase (Promega Corporation) using random primers (Promega Corporation) in 50 mmol/l Tris-HCl; 50 mmol/l KCl; 10 mmol/l MgCl_2 ; 10 mmol/l dithiothreitol (DTT); 0.5 mmol/l spermidine and 1 mmol/l dNTPs at 42 °C for 60 min (Hsu *et al.* 2001).

PCR was performed with specific primers for COII, PGC-1, mtTFA, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 18S. Primers for PGC-1 (5'-TCCTCTGACCC CAGACTCAC-3') and (5'-TAGAGTCTTGGAGCTCCT-3') amplify a 442 bp fragment (Portilla *et al.* 2002) primer for mtTFA (5'-GGGTCTTGTCTGTATTCCGAA-3') and (5'-TGAAACGATCCGGAGACATCT-3') amplify a 633 bp fragment primers for COII (5'-CACCAATGATACTGAAGC-TACG-3') and (5'-GTTACTGTTGCTTGATTTAGTCG-3') amplify a 250 bp fragment primers for GAPDH (5'-ACCACAGTCCATGCCATCAC-3') and (5'-TCCAC-CACCCTGTTGCTGTA-3') amplify a 450 bp fragment; primers for 18S (5'-CCGCAGCTAGGAATAATGGAATAG-GAC-3') and (5'-ACGACGGTATCTGATCGTCTTCG-3') amplify a 220 bp fragment. The cDNA amplifications were carried out in a 25 µl mixture containing 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1 mmol/l MgCl_2 , 0.1 mmol/l primer, and 1 U Taq DNA polymerase. The reaction profile was as follows. COII: denaturation for 1 min at 94 °C, annealing for 2 min at 59 °C, and extension for 2 min at 72 °C running for 28 cycles; PGC-1: denaturation for 1 min at 94 °C, annealing for 2 min at 63 °C, and extension for 2 min at 72 °C running for 35 cycles; mtTFA: denaturation for 1 min at 94 °C, annealing for 2 min at 58 °C, and extension for 2 min at 72 °C running for 28 cycles; GAPDH: denaturation for 1 min at 94 °C, annealing for 2 min at 60 °C, and extension for 2 min at 72 °C running for 25 cycles; 18S: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and

Figure 1 The effects of MK-801 on the expression of cytochrome oxidase subunit II (COII) mRNA in the GT1-7 cells. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} or 10^{-2} μ M) for 16, 20, 24, 36 or 48 h. Expression of mRNA was quantified by real-time PCR. The GAPDH product was used as an internal control. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$.

extension for 1 min at 72 °C running for 25 cycles. PCR-amplified DNA fragments were visualized by ethidium bromide staining after agarose gel electrophoresis (Chandrasekaran *et al.* 1998).

Quantitative real-time PCR

RNA was transcribed into cDNA (cDNA) with reverse transcriptase (Promega Corporation) using random primers (Promega Corporation) in 50 mmol/l Tris-HCl; 50 mmol/l KCl; 10 mmol/l $MgCl_2$; 10 mmol/l DTT; 0.5 mmol/l spermidine and 1 mmol/l dNTPs at 42 °C for 60 min. Real-time PCR was performed using the SYBR Green method on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems,

Foster City, CA, USA). The sequences of primers used in this study were designed using the ABI Prism Primer Express Program (Applied Biosystems). PCR was performed using specific primers for COII, PGC-1, mtTFA, and GAPDH. Primers for PGC-1 (5'-ACAGTCTCCCCGTGGATGAA-3') and (5'-TCAGTGGTCACGGCTCCAT-3') primers for mtTFA (5'-CCAAAAGACCTCGTTCAGCAT-3') and (5'-TTTCCCTGAGCCGAATCATC-3') primers for COII (5'-GAGCAGTCCCCTCCCTAGGA-3') and (5'-CCTGGTCGGTTTGATGTTACTGT-3') primers for GAPDH (5'-ATGTGTCCGTCGTGGATCTGA-3') and (5'-ATGCCTGCTCACCACCTTCT-3'). The cDNA amplifications were carried out in a 25 μ l mixture containing 12.5 μ l of 2 $\times$ SYBR Green PCR Master Mix

(Applied Biosystems), 300 nM primers and 50 ng cDNA. All samples were performed with triplicate and nontemplate control (NTC). GAPDH mRNA was used as the internal control. Analysis was performed using SDS version 2.1 software (Applied Biosystems). The comparative C_T method was used for quantification of gene expression.

Western blot analysis of PGC-1

The cells were homogenized in lysis buffer (50 mmol/l Tris-base, 150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l NaF, 0.1 mmol/l Na_3VO_4 , 1% Triton X-100, 1 mmol/l AEBSF and 10 $\mu\text{g}/\text{ml}$ leupeptin, pH 7.4) and incubated at 4 °C for 10 min. Supernatant containing the total protein was collected by centrifuging for 15 min at 22 000 *g*. The protein concentrations were estimated using the Bio-Rad protein microassay procedure (Bradford 1976). Samples were heated for 5 min in boiling water before loading. Equal amounts of protein (20 μg) were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane by electroblotting for 1 h (100 V), and then blocked with the Tween-Tris buffer saline solution (t-TBS; 20 mmol/l Tris-base, 0.44 mmol/l NaCl, 0.1% Tween 20, pH 7.6) containing 5% nonfat dry milk and 0.1% Tween 20 at 4 °C for overnight. The blot was then incubated with polyclonal PGC-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:200 dilution in t-TBS containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h (Baar *et al.* 2002). Subsequently, membranes were incubated for 1 h with the secondary antibody (1:5000) at room temperature, and then washed for 1 h with the t-TBS. The membrane was also probed with β -actin antibody to correct for difference in protein loading. Immunoreactive protein was visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's specifications (Siegel *et al.* 1994, Yang *et al.* 2003).

Extraction of nuclear protein and electrophoretic mobility shift assay (EMSA)

Cells were plated at a density of 6×10^5 cells/ cm^2 in a 10 cm dish. The culture medium was removed when the cells reached 90% confluence and 1 ml PBS was added to cover the cells. The cells from eight dishes were scraped into conical centrifuge tube and centrifuged for 5 min at

1500 r.p.m. The packed cells were resuspended in five volume of hypotonic buffer (10 nmol/l HEPES, pH7.9, 10 mmol/l KCl, 1.5 mmol/l MgCl_2 , 0.5 mmol/l DTT, 1 mmol/l AEBSF and 10 $\mu\text{g}/\text{ml}$ leupeptin), centrifuged for 5 min at 1500 r.p.m., and the supernatant was discarded. The pellet was resuspended in a hypotonic buffer and allowed swelling for 10 min on the ice. The nuclei were collected by centrifuging for 15 min at 4000 r.p.m. A volume of high-salt buffer (20 mmol/l HEPES, pH 7.9, 0.42 mol/l NaCl, 20% glycerol, 1 mmol/l EDTA, 1 mmol/l DTT, 1 mmol/l 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) and 10 $\mu\text{g}/\text{ml}$ leupeptin) equal to nuclear packed volume was added and stirred at 4 °C for 30 min. The nuclear extracts were centrifuged for 15 min at 14 000 r.p.m and the supernatant was dialyzed using a 100 $\times$ volume of dialysis buffer for 1 h. For determination of protein–DNA interactions, the double-stranded oligonucleotide containing a functional NRF-2 consensus binding site in the mtTFA promoter (the recognition sequence for NRF-2 is oligo 5'-CTGCAGACCGAAGTCTGGG-3' (Choi *et al.* 2002) and mutant NRF-2 consensus binding sequence is 5'-CCCA-GACTGAAATGATGCAG-3') were purchased from Promega and end-labeled with $\gamma^{32}\text{P}$ -ATP according to the manufacturer's recommendation. The binding reactions of NRF-2 or mutant NRF-2 to mtTFA promoter were performed in a final volume of 20 μl mixtures containing buffer (10 mmol/l HEPES, pH7.5, 1 μg poly(dI-dC), 0.1 mol/l NaCl, 0.8 mmol/l EDTA, 1 mmol/l DTT, 0.05% NP-40, and 4% glycerol), 10 μg nuclear proteins, and 100 000 c.p.m of the radiolabeled probe. The mixtures were incubated at room temperature for 20 min. For competition assay, a 50-fold excess amount of cold NRF-2 or mutant NRF-2 oligonucleotide was preincubated with nuclear proteins for 45 min before the addition of labeled probe. The DNA–protein complexes were resolved on 5% nondenaturing polyacrylamide gel in 0.5 $\times$ TGE (Tris base, glycine, EDTA) buffer. The gel was dried and exposed to X-ray film overnight at -70 °C. (Kanke *et al.* 1998, Kain *et al.* 2000, Choi *et al.* 2002, Dong *et al.* 2002).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL stain) for apoptosis detection

For TUNEL assay, a TdT FragEL DNA Fragmentation Detection kit (Oncogene, Cambridge, MA, USA) was

Figure 2 MK-801 dose dependently decreases intracellular Ca^{2+} concentrations in GT1-7 cells. Intracellular calcium concentrations in the GT1-7 cells were measured using Fluo-3 green fluorescence following treatment with MK-801 (0, 10^{-3} , or 10^{-2} μM) for (a) 16, (b) 20, (c) 24, (d) 36 or (e) 48 h. These results demonstrate that viable single cell-suspensions of GT1-7 can be obtained for intracellular Ca^{2+} analysis using flow cytometry. (f) Quantitative results ($n=3$). Values represent means $\pm$ s.e.m. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P<0.05$. * $P<0.05$, ** $P<0.01$, MK-801 (10^{-2} μM) versus control; $^{\#}P<0.05$, MK-801 (10^{-3} μM) versus control; $^{\Phi}P<0.05$, MK-801 (10^{-3} μM) versus MK-801 (10^{-2} μM).

Figure 3 Effects of MK-801 on the expression of PGC-1 mRNA in GT1-7 cells. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} or 10^{-2} μ M) for 16, 20, 24, 36, or 48 h. The mRNA levels were quantified by real-time PCR. The GAPDH mRNA was used as an internal control. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$.

used according to the manufacturer's instructions. The cells were plated at a density of 1×10^5 on a six-well plate and incubated at 37 °C for 1 day. After treating with or without MK-801 (10^{-2} or 10^{-3} μ M) for 16, 20, 24, 36 or 48 h, the cells (including the floating cells) were harvested, fixed in 4% paraformaldehyde, and then centrifuged at 1500 r.p.m for 5 min. Pellets were re-suspended with 80% ethanol. The cells at a concentration of 2×10^5 cells/cm³ were fixed onto glass slides using a Cytospin at 2000 r.p.m for 5 min, pretreated with proteinase K and H₂O₂, and then incubated in equilibration buffer followed by working-strength TdT enzyme (TdT labeled dUTP nucleotides to free 3'-OH DNA termini) at 37 °C for 1.5 h. The cell

slides were incubated in a stop/wash buffer for 15 min, stained with 3,3'-diaminobenzidine (DAB) solution, and then counterstained with methyl green. The incidence of apoptosis was derived from the quotient of apoptotic nucleus number divided by the sum of total neuron numbers in each slides (Hsu *et al.* 2001).

Statistical analysis

All data were expressed as the mean value $\pm$ S.E.M. Comparisons were subjected to ANOVA followed by Scheffe's least significant difference test. $P < 0.05$ was considered as significant difference.

Figure 4 Effect of MK-801 on the protein levels of PGC-1 in GT1-7 cells. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} , or 10^{-2} μ M) for 16, 20, 24, 36, or 48 h. Molecular weight of PGC-1 is 86 kDa. β -Actin (43 kDa) was used as an internal standard. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. * $P < 0.05$.

Results

Effect of MK-801 on the expression of COII mRNA

Initially, we examined whether MK-801 treatment could affect the expression of *COII* gene in GT1-7 cells. The levels of *COII* mRNA were quantified by real-time PCR. As illustrated in Fig. 1, treatment of GT1-7 with MK-801 (10^{-2} or 10^{-3} μ M) for 36 or 48 h resulted in a decrease of *COII* mRNA level ($P < 0.05$). No significant change of the level of *COII* mRNA was observed before 36 h treatment with MK-801.

Effect of MK-801 on intracellular free calcium levels in GT1-7 cells

To examine whether the change of $[Ca^{2+}]_i$ was involved in the MK-801-induced increase of *COII* in GT1-7 cells, we conducted flow cytometric analysis to determine intracellular free calcium concentrations using Fluo-3 AM as the fluoro probe. As illustrated in Fig. 2a–e, a significant decrease of $[Ca^{2+}]_i$ induced by MK-801 (10^{-2} and 10^{-3} μ M) was observed at 24 h after

treatment. This decrease was in a time- and dose-dependent manner (Fig. 2f).

MK-801 decreases the expression of PGC-1 in GT1-7 cells

The expression of PGC-1, which regulates the expression of NRF-2 and subsequent mtTFA and *COII*, could be affected by intracellular free calcium concentrations. To examine the effect of MK-801 on the *PGC-1* gene expression in GT1-7 cells, we conducted real-time PCR and western blot analyses to measure the levels of PGC-1 mRNA and protein respectively. The results showed that both the RT-PCR product of PGC-1 (Fig. 3) and protein level of PGC-1 (Fig. 4) in GT1-7 cells were significantly decreased after 24 h treatment with MK-801. These inhibitions were in a dose- and time-dependent manner.

Effect of MK-801 on the DNA-binding activity of NRF-2 and the levels of mtTFA mRNA

It has been shown that PGC-1 could induce the expression of NRF-2, which regulates multiple target

Figure 5 Effect of MK-801 on the DNA-binding activity of NRF-2 in GT1-7 cells. 1. Left panel was treated with MK-801 for 36 h, a representative data of EMSA. Cultured cells were homogenized with hypotonic buffer and high-salt buffer. Lane 1: control, NRF-2 probe; lane 2: 10^{-3} μ M MK-801, NRF-2 probe; lane 3: 10^{-2} μ M MK-801, NRF-2 probe; lane 4: control, mutated NRF-2 probe; lane 5: control, competition with 50 $\times$ specific mutant NRF-2 consensus sequence, mutant NRF-2 oligonucleotide (5'-CTG CAT CAT TTC AGT CTG GG -3') was preincubated with nuclear proteins for 45 min before the addition of labeled probe; lane 6: control, mutated NRF-2 consensus sequence, nonspecific competition with 5 pmol/ μ l p53 consensus sequence; lane 7: mutated NRF-2 consensus sequence probe only; lane 8: control, competition with 50 $\times$ specific NRF-2 consensus sequence, NRF-2 oligonucleotide (5'-CTG CAG ACC GGA AGT CTG GG-3') was preincubated with nuclear proteins for 45 min before the addition of labeled probe; lane 9: control, NRF-2 consensus sequence, nonspecific competition with 5 pmol/ μ l p53 consensus sequence; lane 10: NRF-2 consensus sequence probe only. Right panel: quantitative results of EMSA. Eight samples were analyzed in each group, and values represent the means $\pm$ S.E.M.

genes including *mtTFA*, which could activate COII expression. Accordingly, we examined the effect of MK-801 on binding activity of NRF-2 on *mtTFA* in GT1-7 cells by conducting the EMSA. Surprisingly, no significant difference of *mtTFA*/NRF-2 binding was observed between MK-801 treated and control cells as shown in Fig. 5. In specific competition analysis, binding of NRF-2 was significantly competed by 50-fold unlabeled NRF-2 oligonucleotide 5'-CTG CAG ACC GGA AGT CTG GG-3'. In nonspecific competition analysis, unlabeled p53 did not compete with labeled NRF-2 oligonucleotide. Since the binding between *mtTFA* DNA and NRF-2 was not affected by MK-801 treatment, we further examined whether the levels of *mtTFA* and *mtTFB1M* and *mtTFB2M* were affected by MK-801 treatment or not. The *mtTFA* (Fig. 6), *TFB1M* (Fig. 7), and *TFB2M* (Fig. 8) mRNA levels were significantly decreased at 24, 48, and 48 h after MK-801 treatment respectively.

Neuronal apoptosis induced by blocking of the NMDA receptor

To study whether blocking of the NMDA receptor could induce apoptosis in GT1-7 cells, the TUNEL staining assay was performed in the cultured GT1-7 cells treated with or without MK-801. As shown in Fig. 9, treatment of

GT1-7 cells with MK-801 (10^{-2} or 10^{-3} μ M) for 36 or 48 h significantly increased the population of apoptotic cells when compared with the cells without MK-801 treatment ($P < 0.01$).

Discussion

Previously, we have demonstrated that the apoptotic incidence of neurons in SDN-POA of male rats is significantly increased when NMDA receptor is blocked by MK-801 (Hsu *et al.* 2001) and the physiological activation of NMDA receptor may protect against neuronal death by transcriptional increase of some survival-related genes, including COII, in SDN-POA during sexual development (Hsu *et al.* 2005). In this study, we attempted to map the signaling pathway of NMDA receptor-mediated neuronal survival in the GT1-7 cell lines, which were derived from hypothalamic GnRH neurons and expressed NMDA receptor as well as mitochondrial COII genes, by comparing the time course of intracellular events, which have been suggested to be involved in the neuronal apoptosis, induced by blocking the activation of NMDA receptor. The results from the present study suggest that blocking of the NMDA receptor causes a decrease of $[Ca^{2+}]_i$, which in turn may inhibit the expressions of PGC-1,

Figure 6 Effect of MK-801 on the expression of mitochondrial transcription factor A (mtTFA) mRNA in GT1-7 cells. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} , or 10^{-2} μ M) for 16, 20, 24, 36, or 48 h. Expression of mRNA was quantified by real-time PCR. The GAPDH mRNA was used as an internal control. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$.

NRF-2 and *COII* gene, and finally leads to the occurrence of apoptosis in the GT1-7 cell line.

It is well accepted that activation of the neuronal NMDA receptor results in elevation of intracellular calcium and phosphorylation of CaMKIV (Yano *et al.* 1998). Activation of CaMK could induce expression of PGC-1 (Wu *et al.* 2002). In the present study, we observed a decrease in the $[Ca^{2+}]_i$ and the occurrence of neuronal apoptosis in the MK-801 treated GT1-7 cell line. These findings are consistent with previous reports suggesting that intracellular Ca^{2+} deficiency plays a causative role in MK-801/6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-induced activation of caspase-3 and neuronal apoptosis (Yoon *et al.* 2003). By comparing

the time course of intracellular events induced by MK-801 treatment, we found that a decrease of $[Ca^{2+}]_i$ began at 20 h after MK-801 (Fig. 1), whereas the levels of PGC-1 mRNA and protein began to decline at 24 h (Figs 3 and 4), suggesting that declination of $[Ca^{2+}]_i$ may be a cause of the MK-801-induced suppression of PGC-1 expression.

It has been shown that PGC-1 could induce the expression of NRF-2, which regulates multiple target genes including *mtTFA* and *TFBMs* (Gleyzer *et al.* 2005). The mtTFs usually translocate into mitochondria and directly activate the transcription and replication of mtDNA (mitochondrial DNA) such as *COII*. Since it has been shown that the transcription of *COII* is

Figure 7 Effect of MK-801 on the expression of mitochondrial transcription factor B1M (TFB1M) mRNA in GT1-7 cells. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} , or 10^{-2} μ M) for 16, 20, 24, 36, or 48 h. Expression of mRNA was quantified by real-time PCR. The GAPDH mRNA was used as an internal control. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. ** $P < 0.01$.

activated by mtTFA, Tfam (Dong *et al.* 2002, Vijayasathy *et al.* 2003), it is likely that a decrease in PGC-1 expression would lead to a decrease of COII expression in GT1-7 cells. In the present study, a decrease of COII mRNA level was not observed until 36 h after MK-801 treatment (Fig. 7), suggesting that COII might be in the downstream of PGC-1, which started to decline at 24 h after MK-801 treatment.

Previously, it has been indicated that the COII activity and mRNA levels are highly regulated and correlated closely with neuronal activity (Wong-Riley 1989, Capaldi 1990a, Zhang & Wong-Riley 2000). It was also reported that reduction of hippocampal oxidative metabolism in Alzheimer disease is directly associated with a down-regulation of COII mRNA (Chandrasekaran *et al.* 1998). Moreover, it has been suggested that ATP depletion

caused by decreased COII expression might play a significant role in the hypoxia-induced apoptotic cell death (Bae *et al.* 1998). Administration of ionotropic glutamate receptor antagonists (MK-801/CNQX) induces neuronal apoptosis *in vivo* and *in vitro* through activations of caspase-9 and caspase-3, that is preceded by release of mitochondrial cytochrome *c* and translocation of Bax into mitochondria (Yoon *et al.* 2003). In the present study, we demonstrated that treatment with MK-801 decreased the COII levels and increased the number of TUNEL positive cells in GT1-7 cells at both 36 and 48 h after MK-801 treatment. Particularly, a prominent increase of apoptotic cells occurred at 48 h after MK-801 treatment, suggesting that the decreased COII expression might account for, at least in part, the MK-801-induced apoptosis in GT1-7 cells.

Figure 8 Effect of MK-801 on the expression of transcription factor B2M (TFB2M) mRNA in GT1-7 cells. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} , or 10^{-2} μ M) for 16, 20, 24, 36, or 48 hr. Expression of mRNA was quantified by real-time PCR. The GAPDH mRNA was used as an internal control. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. * $P < 0.05$.

Mitochondrial DNA (mtDNA) transcription is initiated bidirectionally from closely spaced promoters, heavy strand promoters and light strand promoters, within the D-loop regulatory and requires mitochondrial RNA polymerase and mtTFA (Gleyzer *et al.* 2005). The mtDNA transcription can also be enhanced by mitochondrial transcription-specific factors (TFB1M and TFB2M; Gleyzer *et al.* 2005). Besides, the high-mobility group (HMG-box) domains of mtTFA proteins are capable of inducing a conformational change in promoter-containing DNA, suggesting that promoter conformation may play an important role in transcription initiation (Tracy & Stern 1995). Our data suggest that changes of both nuclear and mitochondrial gene expression

might be involved in the occurrence of apoptosis induced by blocking of the NMDA receptor. Previous studies indicated that the transcription of COII, a mitochondrial DNA-encoded gene, could be activated by mtTFA, Tfam (Dong *et al.* 2002, Vijayasathya *et al.* 2003). Accordingly, we examined whether mtTFA is involved in the COII expression induced by blocking of the NMDA receptor. Our data indicated that the mtTFA mRNA levels began to decrease at 24 h after MK-801 treatment (Fig. 6). The mRNA levels of TFB1M and TFB2M, on the other hand, were not decreased until 48 h after MK-801 treatment (Figs 7 and 8). Surprisingly, the EMSA assay showed that the NRF-2/mtTFA DNA-binding activity between MK-801 treated and control cells is not significantly different

Figure 9 Induction of neuronal apoptosis in GT1-7 cells by MK-801. Cultured cells were maintained for 1 day before treating with MK-801 (0, 10^{-3} , or 10^{-2} μ M) for 16, 20, 24, 36, or 48 h. (a–c) a representative TUNEL staining after the cells treated for 48 h with (a) control, (b) 10^{-3} μ M MK-801 or (c) 10^{-2} μ M MK-801 respectively. MK-801 dose dependently increased the TUNEL positive cells. (d–f) the insets of (a–c) with a larger magnification. Red arrows indicated representative apoptotic cells. (g) Quantitative results of neuronal apoptosis identified by TUNEL staining. Each slide containing 2×10^5 cells was counted five views. Four samples were analyzed in each group and values represent the means $\pm$ S.E.M. ** $P < 0.01$.

(Fig. 5). These data suggest that the decreases of mtTFA, TFB1, and TFB2 might be involved in the occurrence of apoptosis induced by MK-801.

Recent studies have shown that brain mitochondria undergo swelling and release proteins located in the matrix or the inter-membrane space when exposed to calcium at a concentration of 25 μ M (Kristal & Dubinsky 1997). NMDA receptor agonist causes

cytochrome *c* release and procaspase-3 expression and results in harmful Ca^{2+} overloading of transient global ischemia (Zhang *et al.* 2002). Therefore, the NMDA antagonist, MK-801, has been suggested to act as a therapeutic agent to protect neuron against excitotoxicity and Ca^{2+} overload *in vivo* (Schulz *et al.* 1996). Although the proposed signaling pathway involved in the MK-801-induced neuronal apoptosis

needs to be confirmed by showing some direct evidence, our results from the present study suggest that therapeutic potential of MK-801 might be compromised by its proapoptotic effects on central neurons.

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