Gene regulation by NMDA receptor activation in the SDN-POA neurons of male rats during sexual development

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Abstract

The present study was designed to identify possible signaling pathways, which may play a role in prevention of neuronal apoptosis in the sexually dimorphic nucleus of the preoptic area (SDN-POA) after physiological activation of the N-methyl-D-aspartate (NMDA) receptor. Gene response to the blockage of the NMDA receptor by an antagonist (dizocilpine hydrogen maleate; MK-801) was screened after suppression subtractive hybridization (SSH). The results showed that differential screening after SSH detected the presence of some neurotrophic genes (RNA binding motif protein 3 (RBM3), α -tubulin) as well as apoptosis-related genes (Bcl-2, cytochrome oxidase subunit II, cytochrome oxidase subunit III) in the SDN-POA of male rats, which were down-regulated by blocking the NMDA receptor. The RT-PCR products of the aforementioned genes in MK-801-treated males were significantly less than that in untreated males. In particular, the expression of Bcl-2 mRNA, including Bcl-2 protein, in male rats were significantly suppressed by MK-801 treatment. Moreover, the binding activity of nuclear factor κ B (NF κ B) was significantly higher in male rats than in females, but significantly diminished by blocking the NMDA receptor with MK-801 in male rats. No significant difference in cAMP response element-binding protein (CREB) binding activity was observed among untreated male, MK-801-treated male, untreated female and MK-801-treated female groups. These results suggest that genes regulated by NMDA receptor activation might participate in neuronal growth and/or anti-apoptosis, and support an important signaling pathway of NF κ B activation and its target gene, Bcl-2, in preventing neuronal apoptosis in the SDN-POA of male rats during sexual development.

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Introduction

Sexually dimorphic structures in the vertebrate brain are important for controlling sexual differences in behavior and function (Clemens *et al.* 1976, Gorski *et al.* 1978). The sexually dimorphic nucleus of the preoptic area (SDN-POA) contributes to the development and expression of male-specific sexual behavior in adulthood (Jacobson *et al.* 1980, Jacobson & Gorski 1981, Arnold & Gorski 1984). Morphologically, the nuclear volume of the SDN-POA in male rats is about seven times greater than in females (Gorski *et al.* 1978). The lower level of neuronal apoptosis in male rats during the critical period of sexual development contributes to this morphological difference. In the perinatal period, testosterone levels are elevated in male, but not in female fetuses (Lalau *et al.* 1990) and serum levels of estradiol are undetectable in female rats during the postnatal period. Davis *et al.* (1996) indicated that there is a significant neuronal apoptosis in the SDN-POA of female rats between postnatal days 6 and 10. Although, testosterone, after being converted to estrogen, had a profound inhibitory effect on the incidence of apoptosis (Davis *et al.* 1996).

Therefore, the low apoptotic incidence in the SDN-POA in male rats may contribute to its large volume compared to that in female rats. Our recent report provided evidence that estradiol converted from testosterone might prevent apoptosis in the SDN-POA neurons of male rats by enhancing expression of the N-methyl-D-aspartate (NMDA) receptor (Hsu *et al.* 2001). However, the molecular mechanism by which activation of NMDA receptor mediates the fate of neurons remains unclear. Previous reports also indicate that activation of the NMDA receptor results in an increase of intracellular Ca^{+2} , NF κ B nuclear translocation and some gene expression, which reportedly mediates the anti-apoptotic effect of NMDA in cerebellar granular neurons (Baeuerle & Henkel 1994, Mattson *et al.* 2000). Considerable evidence also indicated that NF κ B induces the expression of anti-apoptotic gene products such as MnSOD, IAPs, calbindin and bcl-2 (Yu *et al.* 1999, Bui *et al.* 2001, Mattson & Camandola 2001, Brivanlou & Darnell 2002). In addition, Ca^{+2} signaling may activate nuclear calcium/calmodulin-dependent protein kinase (CaMK) and result in phosphorylation of CREB, which induces

the expression of anti-apoptotic genes such as Bcl-2, BDNF, Mcl-1 (Wilson *et al.* 1996, Walton & Dragunow 2000, Sheriff *et al.* 2002). These results suggest that NMDA receptor activation is involved in transcriptional regulation of gene expression. Therefore, the present study was designed to identify a possible pathway leading to gene regulation and subsequent apoptosis after blocking the physiological function of the NMDA receptor.

Materials and methods

Animals

Long-Evans rats were mated. The presence of a plug under the cage the following day was marked as embryonic day 1 (ED1). Pregnant rats were housed individually under a controlled temperature ($22 \pm 0.5^\circ\text{C}$) on a 14 h:10 h light/dark cycle. Food and water were available *ad libitum*. All experiments were carried out in accordance with the guidelines of National Institutes of Health in the US and approved by the Committee for the Use of Experimental Animals of Kaohsiung Medical University. The brain tissues were sampled under CO_2 anesthesia to minimize pain or discomfort.

Dizocilpine hydrogen maleate (MK-801) treatment

Neonatal rats pups were administered with an NMDA receptor antagonist, MK-801 (0.4 mg/kg body weight, s.c.) (RBI; Natick, Massachusetts, USA; Speiser *et al.* 1991) at 0800 h of postnatal day 1 (PND1). Then, the brain tissues containing the SDN-POA were sampled at 1800 h of PND1 for RNA extraction and electrophoretic mobility shift assay (EMSA), and 0800 h of PND2 and PND3 for RT-PCR and Western blot analysis, respectively.

RNA preparation

To dissect the brain tissue containing the SDN-POA, perpendicular cuts to the ventral floor of the brain were made at the center of the optic chiasm and the rostral margin of the third ventricle. Bilateral cuts were placed at about 0.5 mm from the ventricle. The tissue was severed from the rest of the brain by a horizontal cut at a depth of 1 mm (Takagi & Kawashima 1993). Total RNA was isolated from the brain tissue containing the SDN-POA. Poly(A)⁺RNA was extracted with an oligo-dT cellulose column from total RNA pooled from tissue block containing the SDN-POA of ten rat brains.

Suppression subtractive hybridization (SSH)

SSH was carried out using a Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Three

micrograms poly(A)⁺RNA from MK-801-treated male SDN-POA was used for a tester and that from intact males as a driver for cDNA synthesis in the forward subtraction hybridization. In the reverse subtractive hybridization, intact male brain sample and MK-801-treated male SDN-POA were used as tester and driver respectively. Driver ds cDNA was synthesized from 3 µg of poly(A)-RNA and 1 ng of oligonucleotide Pr16 as a primer. Synthesis of first and second strand cDNA and blunt-ending of DNA ends were performed by using T4 DNA polymerase according to the manufacturer's protocol. After first and second rounds of hybridization between the tester and driver cDNA, the hybridization mixture was PCR amplified in a volume of 25 µl, containing 1 µl of the diluted subtraction mixture, 1 µl PCR primer 1 (10 µM), 10 xPCR reaction buffer, 0.5 µl dNTPs mix (10 mM) and 50x Advantage cDNA polymerase Mix (Clontech) under the following conditions: incubation at 75 °C for 5 min to extend the adaptors followed by 94 °C for 10 s, 66 °C for 30 s and 72 °C for 1.5 min. The primary PCR mixture was diluted 10-fold and 1 µl was used in a second PCR with nested primers for the primer 1 and primer 2 for ten cycles at 94 °C for 10 s, 68 °C for 30 s and 72 °C for 1.5 min. The subtraction efficiency was evaluated by measuring the level of housekeeping gene (GAPDH) in the subtracted cDNA pool using PCR according to the manufacturer's specification. Thereafter, products of the selective secondary PCR were cloned into pT-Adv vector using T/A cloning kit (Invitrogen, Carlsbad, CA, USA) and transformed into an *E. coli*, TOP10F⁺ (Invitrogen).

Screening of the subtracted cDNA

Screening of the subtracted cDNA sample was carried out using the PCR-Select Differential Screening Kit (Clontech) against randomly selected bacterial colonies cultured overnight in a 96-well plate and blotted onto a nylon membrane. The membrane was treated with 0.5 M NaOH, 1.5 M NaCl for 4 min, 0.5 M Tris-HCl also for 4 min, and then washed for 30 min in $0.2 \times \text{SSC}$ and 0.2% SDS at 63 °C. Pre-hybridization was carried out at 42 °C in a buffer containing 5 xSSPE, 50% formamide, 5x Denhart's solution, 2% SDS, 200 µg/ml salmon sperm DNA, 100 µg/ml Poly A, 10 µl/ml of 0.3 mg/ml of oligonucleotides corresponding to the nested primers and complementary sequences. The probes were generated using random priming method in the presence of [α -³²P] (Perkin Elmer, Shelton, CT, USA) dATP using either the subtracted MK-801-treated male template or untreated male template. The adaptor in the subtracted templates was removed by QIAquick PCR purification kit (QIAGEN, Hilden, Germany) after *Rsa*I, *Eag*I and *Sma*I restriction enzyme (New England Biolabs, Inc. Beverly, MA, USA) (the sites present in the

Figure 1 Genes down-regulated by blocking NMDA receptor with MK-801 in SDN-POA of male rats. cDNA clones were generated by SSH, transformed into *E. coli* and cultured in 96-well dish as described in detail in Materials and methods. The bacterial cultures were transferred onto a nylon membrane and then analyzed by Southern hybridization. Two identical membranes containing cDNA clones amplified after forward (A) and reverse (B) SSH were hybridized with a probe generated from the forward subtracted cDNA library (I and III) and reverse subtracted cDNA library (II and IV). Circled clones were selected for identification of DNA sequence.

adaptor sequence) digestions. Hybridization was performed overnight with $1-2 \times 10^6$ c.p.m./ml of labeled probe. The membranes were washed at room temperature for 15 min with $2 \times$ SSC, followed by 66°C with $2 \times$ SSC and 0.5% SDS for 3×15 min, and $0.2 \times$ SSC

and 0.1% SDS for 10–15 min. The hybridization results were analyzed using autoradiography. After differential screening, the selected clones were sequenced and nucleic acid homology searches were performed using the BLAST program.

Table 1 Expression of gene clones screened after suppression subtractive hybridization. Homology search was performed by using BLAST program of National Center for Biotechnology Information

	Homology gene	Species (similarity %)		Change (%)	
Clone					
2E	RNA binding motif protein 3 (RBM3)	Mouse	(97)	D	(56±25.28**)
2F	Cytochrome oxidase subunit II (COII)	Rat	(98)	D	(46±6.56**)
3D	Cytochrome oxidase subunit III (COIII)	Rat	(100)	D	(62±11.46**)
7C	Cytochrome b gene	Rat	(99)	N	
7E	Cytosolic phosphoprotein (p19); SCG10	Rat	(99)	N	
8A	Cytochrome oxidase subunit III (COIII)	Rat	(100)	D	(62±11.46**)
9B	Cytochrome oxidase subunit III (COIII)	Rat	(100)	D	(62±11.46**)
9F	Bcl-2	Rat	(99)	D	(42±12.28**)
10D	Cytosolic phosphoprotein (p19); SCG10	Rat	(99)	N	
10F	α -tubulin	Rat	(100)	D	(40±11.79**)
11D	Cytoskeletal gamma-actin	Rat	(97)	N	
11E	Cytochrome oxidase subunit II (COII)	Rat	(98)	D	(46±6.56**)

D: decreased; N: non-significantly changed. Percent of change was evaluated by measuring the RT-PCR product and comparing that of MK-801-treated with un-treated male group.

Reverse transcription (RT)-PCR for quantitation of the mRNA expressions of RNA binding motif protein 3 (RBM3), α -tubulin, cytochrome oxidase subunit II (COII) cytochrome oxidase subunit III (COIII) and Bcl-2

RNA was isolated and transcribed into complementary DNA (cDNA) with reverse transcriptase (AMV reverse

transcriptase; Roch, Basel, Switzerland) using random primers in 50 mmol/l Tris-HCl; 50 mmol/l KCl; 10 mmol/l MgCl₂; 10 mmol/l DTT; 0.5 mmol/l spermidine and 1 mmol/l dNTPs at 42 °C for 60 min. PCR was performed with specific primers for RBM3 (forward: 5'-AAGAAGGGAAACTCTTCGTAGG-3'and reverse:

Figure 2 Effect of MK-801 on the expression of RNA binding motif protein 3 (RBM3) mRNA in the SDN-POA of male and female rats. MK-801 (0.5 mg/kg body weight, s.c.) was administered at 0800 h of postnatal day 1, and brain tissue was sampled at 0800 h of postnatal day 2. Expression of mRNA was quantified by RT-PCR. The RT-PCR product of RBM₃ was 242 bp. β -actin (450 bp) was used as an internal control. The data shown indicates mean±s.d. of five samples in each group. **P*<0.05, ***P*<0.01.

Figure 3 Effect of MK-801 on the expression of α -tubulin mRNA in the SDN-POA of male and female rats. MK-801 (0.5 mg/kg body weight, s.c.) was administered at 0800 h of postnatal day 1, and brain tissue was sampled at 0800 h of postnatal day 2. Expression of mRNA was quantified by RT-PCR. The RT-PCR product of α -tubulin mRNA was 213 bp. β -actin (450 bp) was used as an internal control. The data shown indicates mean $\pm$ S.D. of five samples in each group. * $P < 0.05$, ** $P < 0.01$.

5'-ATGATCAACTCGGATTTGGCG-3'), α -tubulin (forward: 5'-GAGAGGCTCTCTGTCGACTAC-3' and reverse: 5'-TAGTGTAGGTTGGGCGCTCAA-3'), COII (forward: 5'-CAATCCTCCCAGCTGTCATTC-3' and reverse: 5'-GAAGCTGTGATTTGAGCCGCA-3'), COIII (forward: 5'-CTAGTTCCTACCCACGACCTA-3' and reverse: 5'-TGAAGTGGAAATTTTAGTTGTCG-3'), bcl-2 (forward: 5'-CGTCATAACTAAAGACACCC-3' and reverse: 5'-TTCATCTCCAGTATCCCACTC-3'), β -actin (internal control for RBM3, α -tubulin, COIII and bcl-2; forward: 5'-CTACAATGAGCTGCGTGTGG-3' and reverse: 5'-TAGCTCTTCTCCAGGGAGGA-3'), or 18S (internal control for COII; QuantumRNA 18S internal standards; Promega, Madison, Wisconsin, USA). RT-PCR products for RBM3, α -tubulin, COII, COIII, Bcl-2, β -actin and 18S were 242 bp, 213 bp, 400 bp, 366 bp, 255 bp, 450 bp and 324 bp, respectively. The cDNA amplification was carried out in 25 μ l of 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1 mmol/l MgCl₂, 0.1 mmol/l primer, and 1U of Taq DNA polymerase. Each cycle of RBM3 PCR consisted of precycle for 90 s at 95 °C, denaturation for 60 s at 95 °C, annealing for 60 s at 55 °C, extension for 120 s at 72 °C and 35 cycles were repeated. For α -tubulin, PCR consisted of precycle for 120 s at 95 °C, denaturation for 60 s at 95 °C, annealing for 60 s at

58 °C, and extension for 120 s at 72 °C and 35 cycles were repeated also. For COII, COIII and 18S, PCR consisted of precycle for 120 s at 94 °C, denaturation for 60 s at 94 °C, annealing for 120 s at 55 °C, extension for 180 s at 72 °C and 30 cycles were repeated. Each cycle of Bcl-2 PCR consisted of precycle for 120 s at 95 °C, denaturation for 60 s at 95 °C, annealing for 60 s at 58 °C, and extension for 120 s at 72 °C and 35 cycles were repeated. Finally, the DNA fragments were visualized by ethidium bromide staining after agarose gel electrophoresis.

EMSA

Brain tissues containing the SDN-POA were isolated from untreated male, MK-801-treated male, untreated female and MK-801-treated female rats for quantifying DNA binding activity of the transcriptional factor. Eight samples were pooled in each group and homogenized in a microfuge tube with buffer A (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.5 mmol/l DTT, 1 mmol/l AEBSF and 10 μ g/ml leupeptin; all chemicals for preparing buffer solution were supplied by Sigma). The nuclei fraction was collected by centrifugation at 500 g for 10 min and washed once with buffer A containing 0.2% NP-40. After 2nd centrifugation at

Figure 4 Effect of MK-801 on the expression of cytochrome oxidase subunit II (COXII) mRNA in the SDN-POA of male and female rats. MK-801 (0.5 mg/kg body weight, s.c.) was administered at 0800 h of postnatal day 1, and brain tissue was sampled at 0800 h of postnatal day 2. Expression of mRNA was quantified by RT-PCR. The RT-PCR product of COXII mRNA was 400 bp. 18S (324 bp) was used as an internal control. The data shown indicates mean $\pm$ S.D. of six samples in each group. ** $P < 0.01$.

500 μ g for 10 min, the nuclear pellet was then mixed with buffer B (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 AEBSF and 10 μ g/ml leupeptin), stayed for 30 min at 4 °C and centrifuged for 30 min. For determination of protein-DNA interactions, the ds oligonucleotide containing NF κ B consensus binding sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') or cAMP response element-binding protein (CREB) consensus binding sequence (5'-AGAGATTGCCTGAC GTCAGACAGCTAG-3') was purchased from Promega and end-labeled with γ^{32} P-ATP according to manufacturer's recommendation. The NF κ B or CREB binding reactions were performed in final volume of 20 μ l mixtures containing buffer (10 mmol/l HEPES, pH 7.5, 1 μ g poly(dI-dC), 0.1 mol/l NaCl, 0.8 mmol/l EDTA, 1 mmol/l DTT, 0.05% NP-40, 4% glycerol), 10 μ g nuclear proteins, and 100 000 c.p.m. of radiolabeled probe. In order to confirm the specific protein-DNA interaction, the mixture was incubated at room temperature with antibody for 45 min before adding labeled probe. For competition assay, 50-fold excess amount of cold CREB or NF κ B oligonucleotide was pre-incubated with nuclear proteins for 15 min before the addition of labeled oligonucleotide probe. The DNA-protein com-

plexes as well as DNA-protein-Ab complex were resolved on 5% nondenaturing polyacrylamide gel in 0.5x TGE buffer. The gel was dried and exposed to x-ray film overnight at -70 °C (Yang *et al.* 1995).

Western blot analysis of Bcl-2

Brain tissues containing the SDN-POA were isolated and pooled from untreated male, MK-801-treated male, untreated female and MK-801-treated female for quantifying protein content of Bcl-2. Four $\times$ volume of extraction buffer (20 mM Tris-acetate, pH 7.4, 50 mM NaCl, 10% SDS, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 μ g/ml pepstatin, 0.5 μ g/ml leupeptin, 0.2 mM Na₃VO₄, 5 mM mercaptoethanol) was added and homogenized in homogenizer for 12–15 strokes. The suspension was subsequently centrifuged for 80 min at 16000 μ g and the clear lysate was ready for protein quantitation. Equal amounts of protein were heated in boiling water for 5 min and separated by 15% SDS-polyacrylamide gel. The gel was transferred onto polyvinylidene difluoride (PVDF) transfer membrane by electroblotting for 1 h (100 v), and the membrane was blocked overnight at 4 °C by the Tween-Tris buffer saline solution (TBS-T) containing 5% nonfat dry milk

Figure 5 Effect of MK-801 on the expression of cytochrome oxidase subunit III (COXIII) mRNA in the SDN-POA of male and female rats. MK-801 (0.5 mg/kg body weight, s.c.) was administered at 0800 h of postnatal day 1, and brain tissue was sampled at 0800 h of postnatal day 2. Expression of mRNA was quantified by RT-PCR. The RT-PCR product of COXIII mRNA was 366 bp. β -actin (450 bp) was used as an internal control. The data shown indicates mean $\pm$ S.D. of six samples in each group. ** $P < 0.01$.

for Bcl-2 and 0.1% Tween 20. The blot was then incubated with Bcl-2 antibody (1:500, monoclonal; Transduction Lab, Cat no. B46620, Lexington, KY, USA) in TBS-T containing 5% nonfat milk and 0.1% Tween 20 for 1 h. Then washed in TBS-T and incubated with goat anti-mouse IgG (1:2000, HRP conjugated, Transduction Lab. Cat no. M15345) in TBS-T containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h and subsequently washed for 1 h in the TBS-T. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Statistics

The data, with two variables (i.e. sex and MK-801 treatment), was analyzed by using 2x2 factorial analysis of variance followed by Scheffe's test. A 95% confidence was considered statistically significant.

Results

Downregulated gene expression after blocking NMDA receptor with MK-801 in the SDN-POA of male rats

SSH was used to forward subtract the mRNA of MK-801-treated male brain samples containing the

SDN-POA from that of untreated male samples. The selected colonies cloned with subtracted cDNA after SSH were screened by comparative Southern dot-blot hybridization (Fig. 1). Twelve clones (circled; Fig. 1) revealed significant suppression of gene expression in MK-801-treated males. DNA sequencing analysis and homology searching using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information showed that these clones shared a highly homologous sequence with rat RBM3, α -tubulin, COII, COIII, cytochrome b, cytosolic phosphoprotein (p19), cytoskeletal gamma actin and bcl-2. After confirmation of these differentially expressed genes by RT-PCR, expression difference (% of change) between MK-801-treated and untreated samples of these genes were recorded (Table 1). The result showed that two neurotrophic genes (RBM3, α -tubulin) and three apoptosis-related genes (COII, COIII and bcl-2) were down-regulated by blocking NMDA receptor.

Effects of MK-801 on the mRNA expressions of RBM3, α -tubulin, COII, COIII and bcl-2 in the SDN-POA of male and female rats

In order to confirm the differentially expressed genes between rats with and without MK-801 treatment and

Figure 6 Effect of MK-801 on the expression of Bcl-2 mRNA in the SDN-POA of male and female rats. MK-801 (0.5 mg/kg body weight, s.c.) was administered at 0800 h of postnatal day 1, and brain tissue was sampled at 0800 h of postnatal day 2. Expression of mRNA was quantified by RT-PCR. The RT-PCR product of Bcl-2 mRNA was 255 bp. β -actin (450 bp) was used as an internal control. The data shown indicates mean $\pm$ s.d. of four samples in each group. * $P < 0.05$; ** $P < 0.01$.

to clarify the possible role of these genes on sexual development, the differentially expressed genes screened after SSH were compared between untreated male, MK-801-treated male, untreated female and MK-801-treated female groups. The results showed that five of the differentially expressed genes (RBM3, α -tubulin, COII, COIII and bcl-2) showed similar trends among the above four groups. As shown in Figs 2–6, the RT-PCR product was significantly higher in male rats than in females, and the prominent expression of mRNA in male rats was significantly decreased by MK-801 treatment. No significant difference in RT-PCR product between intact females and MK-801-treated females was observed.

Effect of MK-801 on the DNA binding activities of NF κ B and CREB in the SDN-POA of male and female rats

The binding activity of NF κ B was significantly higher in male rats than in females, and the binding activity of NF κ B in male rats was significantly diminished after blocking the NMDA receptor with MK-801. No significant difference was observed between MK-801-treated and untreated female rats. Moreover, the binding of NF κ B was significantly blocked by 50-fold unlabeled NF κ B consensus sequence but not by

non-specific CREB consensus sequence (Fig. 7). On the other hand, no significant difference in CREB binding activity was observed between untreated male, MK-801-treated male, untreated female and MK-801-treated female groups. The binding of CREB was significantly competed by 50-fold unlabeled CREB oligonucleotide but not by excess NF κ B oligonucleotide as shown in Fig. 8.

Effect of MK-801 on the protein content of Bcl-2 in the SDN-POA of male and female rats

As shown in Fig. 9, the protein content of Bcl-2 was significantly higher in male rats than in females. In addition, the high level of Bcl-2 in male rats was significantly decreased after MK-801 treatment. However, no significant difference in Bcl-2 protein content was observed between untreated female and MK-801-treated female groups.

Discussion

The present results show that expression of neurotrophic and apoptosis-related genes in the SDN-POA during the neonatal period was suppressed by blocking the NMDA receptor with MK-801. Furthermore, the binding

Figure 7 Effect of MK-801 on the DNA binding activity of NFκB in SDN-POA of male and female rats at postnatal day 1. Left panel: representative data of electrophoretic mobility shift assay (EMSA). The brain tissues of 5–6 rats containing SDN-POA were homogenized in a microfuge tube with hypotonic buffer and high-salt buffer. Lane 1: intact male; lane 2, MK-801-treated male; lane 3, intact female; lane 4, MK-801-treated female; lane 5, intact male, nuclear protein was incubated with p65 antibody at room temperature for 45 min before adding labeled probe, left side arrow indicates the supershift of p65Ab-NFκB-DNA complex; lane 6, specific competition, 50x specific NFκB consensus sequence (5'-AGTTGAGGGGACTTCCCAGGC-3') was pre-incubated with nuclear proteins for 20 min before the addition of labeled probe; lane 7, non-specific competition, 50x non-specific CREB consensus sequence was used as non-specific competition probe. Right panel: quantitative results of EMSA. The data shown indicates mean ± S.D. of nine samples in each group. *P < 0.05.

activity of NFκB and expression of Bcl-2 were significantly diminished by MK-801. These results suggest that NMDA receptor activation may regulate neuronal survival by modulating gene expressions and provide a signaling pathway for regulating neuronal apoptosis through NFκB in the SDN-POA during sexual development.

In the present result show the expression of RBM3 in SDN-POA of male rats was significantly higher than that in females, and the prominent expression of RBM3 in male rats was significantly suppressed by MK-801. Previous reports have indicated that RBM3 is a highly homologous member of the glycine-rich RNA-binding protein family. It has been described as a heterogeneous nuclear ribonucleoprotein (hnRNP) closely related to hnRNP-G, which enhances survivability of motor neurons (Hofmann & Wirth 2002). However, whether RBM3 is involved in neuronal survival during development and how NMDA receptor activation regulates expression of the RBM3 gene remains unclear. Another MK-801-down-regulated gene, α -tubulin, is a neuron-specific gene, and its expression is highly dependent on the stage of neuronal growth and differentiation (Wu W *et al.* 1997). According to a previous report, the expression of α -tubulin genes is

highly elevated during developmental growth and is subsequently down-regulated around the time of target contact and neuronal maturation in the embryonic rat nervous system. Transcription of α -tubulin genes is induced at the time of neuronal birth, immediately prior to migration away from the ventricular zones (Gloster *et al.* 1999), while the mRNA levels of α -tubulin decrease as maturational processes progress (Lee *et al.* 1990). Previous reports have indicated that DNA synthesis ceases on day 16 post-fertilization (pf) for those neurons destined for the medial preoptic area (MPOA) but still proliferates as late as day 18 pf until birth, for neurons destined for the SDN-POA. In addition, the neuroblast divisions resulting in neurons destined for the SDN-POA begin earlier and terminate sooner in females than in males (Jacobson & Gorski 1981). The present result showed that the expression of α -tubulin mRNA of female rats was significantly less than that of males at PND1, and the predominant expression of α -tubulin mRNA in male rats was significantly diminished by MK-801 treatment. Although the physiological role of α -tubulin requires further investigation, the expression of α -tubulin appears to be involved in neuronal growth or maturation in the SDN-POA during sexual development. Except for the aforementioned neurotrophic

Figure 8 Effect of MK-801 on the DNA binding activity of CREB in SDN-POA of male and female rats at postnatal day 1. Left panel: representative data of electrophoretic mobility shift assay (EMSA). The brain tissues of 5–6 rats containing SDN-POA were homogenized in a microfuge tube with hypotonic buffer and high-salt buffer. Lane 1, intact male; lane 2, MK-801-treated male; lane 3, intact female; lane 4, MK-801-treated female; lane 5, intact male, nuclear protein was incubated with CREB antibody at room temperature for 45 min before adding labeled probe, left side arrow indicates the supershift of Ab-CREB-DNA complex; lane 6, intact male, specific competition, 50x CREB consensus sequence (5'-AGAGATTGCCTGACGTCAGACAGCTAG-3') was pre-incubated with nuclear proteins for 20 min before the addition of labeled probe; lane 7, non-specific competition, 50x NFκB consensus sequence was used as a non-specific probe. Right panel: quantitative results of EMSA. The data shown indicates mean±S.D. of six samples in each group.

genes, apoptosis-related genes in the SDN-POA were down-regulated by NMDA receptor blockage. The RT-PCR products of mRNA of cytochrome oxidase (CO), COII and COIII were significantly decreased in male rats after MK-801 treatment. COII and COIII are components of complex IV, which catalyzes the oxidation of cytochrome c and reduced oxygen to yield ATP and water in the electron transport chain. As one of the electron-driven proton pumps of oxidative phosphorylation, CO plays an important role in determining the rate of energy production in eukaryotes. Therefore, the activity of CO and its subunit mRNA levels are highly regulated and correlated with neuronal survival (Wong *et al.* 1989, Capaldi 1990, Zhang *et al.* 2000). For example, in a patient with Alzheimer's disease (AD), hippocampal oxidative metabolism is directly associated with down-regulation of mRNA for the COII (Chandrasekaran *et al.* 1998). In addition, a previous report indicated that ATP depletion caused by reduced levels of COII mRNA under hypoxia might be a significant factor in the induction of apoptotic cell death (Bae *et al.* 1998). Therefore, it seems reasonable to suggest that down-regulation of COII and COIII may be involved in neuronal apoptosis caused by NMDA

receptor blockage via un-coupling of the respiratory chain, which usually causes mitochondrial permeability transition (MPT), ATP depletion and subsequent cell death.

Bcl-2 is highly expressed during neurogenesis and overexpression of Bcl-2 protects neurons from naturally occurring cell death in the CNS (Abe-Dohmae *et al.* 1993, Martinou *et al.* 1994). Our previous results showed that the protein content of Bcl-2 in the POA of male rats was higher than that of females at PND 8, and MK-801 pre-treatment diminished the expression of Bcl-2 in male rats (Hsu *et al.* 2000). It suggests that the prominent expression of Bcl-2 in male rats might inhibit naturally occurring neuronal apoptosis in SDN-POA during normal sexual development. Additionally, activation of NMDA receptors may protect against neuronal death in the SDN-POA by modulating the expression of Bcl-2 (Hsu *et al.* 2000), which is transactivated by NFκB (Mattson & Camandola 2001) and inhibits the release of cytochrome c and/or mitochondrial apoptogenic proteins (Cai & Jones 1998, Cai *et al.* 1998). In the present study, the expressions of Bcl-2 mRNA and protein in the SDN-POA of male rat were significantly suppressed by MK-801 treatment. In addition, the binding activity of

Figure 9 Effect of MK-801 on the protein content of Bcl-2 in the SDN-POA of male and female rats. MK-801 (0.5 mg/kg B.W, s.c) was administered at 0800 h of postnatal day 1, and brain tissue was sampled at 0800 h of postnatal day 3. Molecular weight of Bcl-2 is 26 kDa. β -tubulin (59 KD) was used as an internal standard. The data shown indicates mean $\pm$ S.D. of four samples in each group. ** $P < 0.01$.

NF κ B was also significantly suppressed by MK-801 treatment. This indicates that the signaling pathway of NF κ B-regulated bcl-2 expression might participate in NMDA receptor-mediated neuronal survival in the SDN-POA during sexual development.

Previous reports indicated that the NMDA receptor is an important route to NF κ B activation during ischemic brain injury (Shen *et al.* 2002). In addition, activation of the NMDA receptor results in Ca^{+2} influx associated with the phosphorylation of CaMK IV (Bito *et al.* 1996), which controls gene expression through the phosphorylation of key regulatory sites on nuclear transcription factors. CaMK IV serves to decode the Ca^{+2} signal to the nucleus and modulates a multitude of cellular responses including cell cycle, apoptosis and synaptic efficacy (Heist & Schulman 1998). Activated CaMK IV shows specificity for phosphorylation of Ser133, the site necessary for transactivation by CREB (Enslen *et al.* 1994, 1995). The interaction of the p65 (Rel A) subunit of NF κ B with CREB binding protein (CBP) involves the KIX region of CBP, which is the same region responsible for binding the transcriptionally active Ser133-phosphorylated form of CREB (Zhong *et al.* 1998, Sheppard *et al.* 1999). Since transcriptional activation of both NF κ B and CREB requires association with the co-activator CBP, competition between NF κ B and CREB for binding to CBP has been postulated to be

important in regulating the transcriptional activity of these factors (Shenkar R 2001). The present result showed that the binding activity of NF κ B but not CREB was significantly suppressed by MK-801, suggesting that, during sexual development, the expression of bcl-2 may be regulated primarily by NF κ B rather than CREB after activation of NMDA receptor.

In addition to NF κ B and CREB, recent reports indicated that CaMK IV activates the nuclear respiratory factor (NRF; Wu *et al.* 2002), which regulates the mitochondrial transcriptional factor A (mtTFA; Tfam)(Dong *et al.* 2002) and subsequently activates mitochondrial DNA-encoded bio-energetic genes such as COII and COIII. This suggests the possibility of a mitochondria-dependent pathway of NMDA receptor mediated neuronal survival during sexual development. Additionally, the present results showed an interesting phenomenon that both the neurotrophic genes (RBM3, α -tubulin) and apoptosis-related genes (COII, COIII, Bcl-2) were all significantly down-regulated by blocking the NMDA receptor. However, no gene expression was up-regulated after NMDA receptor blockage. Although false negative results are possible due to method of suppression subtractive hybridization, the neuronal survival signals were significantly diminished after blocking the NMDA receptor in the present study. The fact that MK-801 turns off neuronal survival genes

suggests that physiological NMDA receptor activation might be functionally essential for neuronal survival.

In conclusion, these NMDA responsive genes, such as RBM3, α -tubulin, COII, COIII and Bcl-2 might contribute to neuronal survival and/or anti-apoptosis during sexual development. Furthermore, physiological activation of the NMDA receptor may prevent SDN-POA neurons from apoptosis through NF κ B activation and subsequent expression of bcl-2. These facts further elucidate the critical role of the NMDA receptor in the sexual development of SDN-POA. In addition, the suppression of mitochondrial genes, i.e. COII and COIII, by blocking the NMDA receptor opens a new window for further understanding of how activation of NMDA receptor regulates mitochondrial gene expression.

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