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ORIGINAL ARTICLE

Construction of the Subtracted cDNA Library of Striatal Neurons Treated with Long-term Morphine[△]

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Key words: neuron; morphine tolerance; suppression subtractive hybridization; subtracted cDNA library; differential gene expression

Objective To construct a morphine tolerance model in primarily cultured striatal neurons, and screen the differentially expressed genes in this model using suppression subtractive hybridization (SSH).

Methods Subtracted cDNA libraries were constructed using SSH from normal primarily cultured striatal neurons and long-term morphine treated striatal neurons (10^{-5} mol/L for 72 hours). To check reliability of the cell culture model, RT-PCR was performed to detect the cAMP-responsive element-binding protein (CREB) mRNA expression. The subtracted clones were prescreened by PCR. The clones containing inserted fragments from forward libraries were sequenced and submitted to GenBank for homology analysis. And the expression levels of genes of interest were confirmed by RT-PCR.

Results CREB mRNA expression showed a significant increase in morphine treated striatal neurons (62.85 ± 1.98) compared with normal striatal neurons (28.43 ± 1.46 , $P < 0.01$). Thirty-six clones containing inserted fragments were randomly chosen for sequence analysis. And the 36 clones showed homology with 19 known genes and 2 novel genes. The expression of 2 novel genes, mitochondrial carrier homolog 1 (Mtch1; 96.81 ± 2.04 vs. 44.20 ± 1.31 , $P < 0.01$) and thymoma viral proto-oncogene 1 (Akt1; 122.10 ± 2.17 vs. 50.11 ± 2.01 , $P < 0.01$), showed a significant increase in morphine-treated striatal neurons compared with normal striatal neurons.

Conclusions A reliable differential cDNA library of striatal neurons treated with long-term morphine is constructed. Mtch1 and Akt1 might be the candidate genes for the development of morphine tolerance.

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MORPHINE tolerance plays a major role in the generation and maintenance of opiate addiction and subsequently, its treatment. However, the mechanism of opiate tolerance still remains unclear. The striatum is believed to be a crucial structure associated with drug dependence. Morphological studies have revealed that a high level of opioid receptor expression in the stratum is associated with morphine tolerance.¹ The successful identification and cloning of these opioid receptors have helped us to further understand the fundamentals of tolerance mechanism. Many studies have suggested that down-regulation of opioid receptors in long-term opiate use may exert an important effect in the development of drug dependence, tolerance and progression of withdrawal symptoms.² Our previous studies also demonstrated that long-term use of morphine may cause down-regulation of opioid receptors in cultured striatal neurons.³ Besides the opioid receptors, other genes like c-fos, junB are also intimately related to morphine tolerance.⁴ In addition, early experiments have shown that apelin has a close association with endogenous opioid peptides in central function.^{5, 6}

Suppression subtractive hybridization (SSH) is a powerful technique that is capable of isolating differentially expressed genes without knowing their identity.⁷⁻¹⁰ In this study, we used SSH to identify differentially expressed genes in long-term morphine-treated cultured striatal neurons, and to construct a reliable high qualitative subtracted cDNA library, so that we may find some differential or unknown genes in these neurons by cloning and DNA sequencing.

MATERIALS AND METHODS

Primary rat striatal neuron culture and identification

Adult Wistar rats, supplied by the Animal Center of Taishan Medical College, were used to generate the offspring studied in this experiment. All applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. Young Wistar rats born within 24 hours were selected for primary striatal neurons culture using trypsin digestion protocol.¹¹ After incubation of primary cells for 24 hours, cytarabine was added to inhibit proliferation of colloid cells. The cells were cultured in DMEM (Hyclone, Utah, USA) supplemented with 10% of fetal bovine serum (Gibco, CA, USA) in a humidified 5% CO₂ atmosphere at 37°C. The complete medium was completely replaced at 48 hours and thereafter at every 2 days.

The primary cultured cells were fixed with 4% para-

formaldehyde in PBS at day 4. The cells were then rinsed with PBS, incubated with anti-neuron specific enolase (NSE) polyclonal antibody (Wuhan Boster Biological Technology, Ltd., Hubei, China) at a concentration of 20 µg/mL, and then detected with SP kit (Zhongshan Biotechnology Co., Beijing, China). Brown yellow colour in cytoplasm indicated presence of NSE. The NSE ratio was calculated as NSE containing cells/total cells.

Administration of morphine

Neurons seeded in 6 well plates were randomly divided into morphine group and control group with 6 wells in each group. After incubated with serum-free DMEM solution for 3 hours, cells in morphine group were cultured with 10⁻⁵ mol/L morphine (Northeast General Pharmaceutical Factory, Shenyang, Liaoning, China), and control cells were cultured with equal volume of serum-free DMEM solution. At 72 hours after administration, cells were collected for SSH.

Total RNA isolation

Total RNA was extracted from cultured neuron samples using TRIzol reagent (Invitrogen, CA, USA), and electrophoresed on 1% agarose/EtBr gel. Total RNA concentration was determined with a Biophotometer (Bio-Rad, CA, USA) by measuring absorbance at 260 nm (A₂₆₀) and 280 nm (A₂₈₀).

Isolation of polyA+RNA

PolyA+RNA was isolated from purified total RNA using a PolyATtract[®] mRNA isolation system II kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The concentration of mRNA was determined spectrophotometrically by measuring absorbance at 260 nm. mRNA was stored at -80°C until it was used.

SSH

SSH was performed using Clontech PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA, USA). Forward subtraction was performed to compare the differentially expressed genes between morphine group and control group. We constructed forward subtraction using cDNA of morphine group as tester and cDNA of control group as driver. cDNA was synthesized with 2 µg pooled mRNA according to the manufacturer's instructions, then was digested by *Rsa*I. The digested product was hybridized with adaptors for 2 cycles, then suppression and a nested PCR amplification were performed. The nested PCR conditions consisted of 27 cycles at 94°C for 30 seconds, 66°C for 30 seconds, and 72°C for 90 seconds, and 12 cycles at 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 90 seconds.

At least 25% of cDNA should have adaptors on both ends to meet experimental requirements. The fragments spanning adaptor/cDNA junctions were amplified by primer adaptor 1 or adaptor 2 and G3PDH 3'- or 5'- primers. After 25 cycles, the products were identified by 2.0% agarose gel electrophoresis.

Subtraction efficiency was estimated by PCR comparison of G3PDH cDNA abundance in the subtracted or unsubtracted tester cDNA pools. After 18, 23, 28 and 33 cycles, 5 μ L amplified products were removed from each reaction and electrophoretically examined on a 2.0% agarose/ethidium bromide gel.

Transformation

The nested PCR products were cloned into pMD18-T vector (TaKaRa, Shiga, Japan), transformed into competent cell *Escherichia coli* DH5 α (TaKaRa, Shiga, Japan), and screened on Luria broth plates containing ampicillin/X-gal/iso-propyl β -D-1-thiogalactopyranoside (IPTG). Approximately, there were 200 clones on each culture plate, and over 100 clones were isolated and incubated overnight in Luria broth-ampicillin broth at 37°C. Clones were then randomly picked up for PCR amplification using nested PCR primers.

Sequencing and BLAST homology search

The clones containing inserted fragments from forward libraries were sequenced using ABI-PRISM3730 DNA sequencer (ABI, CA, USA). Nucleic acid homology searches were performed using the BLAST program at NCBI.

RT-PCR

To check reliability of the cell culture model, RT-PCR was performed to detect the cAMP-responsive element-binding protein (CREB) mRNA expression. To further confirm the differential expression between control and morphine groups, several genes of interest were selected to confirm their differential expression by RT-PCR.

The extracted RNA was quantified by a Biophotometer (BIO-RAD, CA, USA). cDNA was synthesized using 2 μ g total RNA in 25 μ L reaction volume with 1 μ L reverse transcriptase (M-MLV, 200 U/ μ L; Promega, Wis., USA), 4 μ L 5 $\times$ first-strand buffer, 1.25 μ L dNTPs (10 mmol/L), RNAase inhibitor fermentas (40 U/ μ L) 1 μ L, and 500 ng random primer (Sangon, Shanghai, China) according to manufacturer's instructions. PCR primers were synthesized by Invitrogen Company. The sequences of primers and product length were as follows: CREB: forward 5'-GTTCAAGCTG-CCTCTGGTGAT-3', reverse 5'-TCTTCAGCAGGCTGTGTAG-GA-3' (119 bp); mitochondrial carrier homolog 1 (Mtch1): forward 5'-AACGCCTTGTCCTGTGACC-3', reverse 5'-TT-

GGCCTCACGTCCTACGAAC-3' (220 bp); thymoma viral proto-oncogene 1 (Akt1): forward 5'-GCTTCTACGGTGC-GGAGATTG-3', reverse 5'-CACGGCCATAGTCGTTGTCCT-3' (234 bp); and 18sRNA: forward 5'-GTAACCCGTTGAAC-CCCATT-3', reverse 5'-CCATCCAATCGGTAGTAGCG-3' (151 bp). For PCR reactions, each 1 μ L cDNA was used as template in a 50 μ L reaction volume containing 200 μ mol/L dNTPs, 1.5 mmol/L MgCl₂, 0.25 μ mol/L each primer, and 1 U Taq DNA polymerase. PCR amplification reaction parameters of CREB, Mtch1, Akt1, and 18sRNA consisted of pre-degeneration at 94°C for 4 minutes; 20-34 cycles of degeneration at 94°C for 30 seconds, annealing at 57°C-60°C for 30 seconds, elongation at 72°C for 30 seconds; and elongation at 72°C for 5 minutes. The 10 μ L PCR products were separated on 1% agarose gels, and the signal intensities of bands were analyzed using Molecular Image Gel DocTM XR System (BIO-RAD, CA, USA). The intensities of each band were normalized to the corresponding 18sRNA band. The whole RT-PCR process was repeated at least 3 times.

Statistical analysis

All quantitative data were presented as mean $\pm$ SEM. The differences between control group and morphine group were evaluated using Student's *t*-test. *P*<0.05 was considered statistically significant.

RESULTS

Identification of cultured neuron and total RNA

The cultured striatal neurons with NSE positive rate of 90% ($\pm$) were used in this experiment (Fig. 1).

The electrophoresis result of extracted total RNA is shown in Figure 2. The extracted total RNA of cultured striatal neurons had an A₂₆₀:A₂₈₀ $\geq$ 2.0.

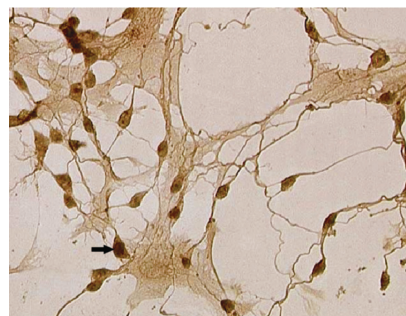


Figure 1. The primary cultured striatal neurons show neuron specific enolase (NSE) expression. SP $\times$ 400 Brown yellow colour in cytoplasm indicates presence of NSE (arrow).

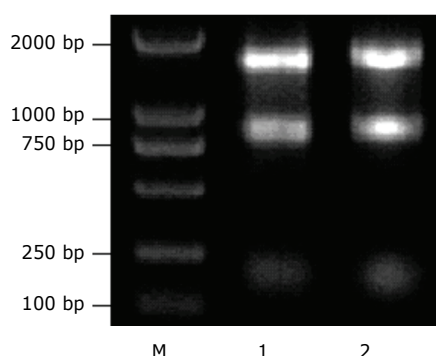


Figure 2. Electrophoresis result of the total RNA extracted from striatal neurons.

M: marker; Lane 1: control group; Lane 2: morphine group.

Analysis results of ligation and subtraction efficiency

The results showed that the adaptors were efficiently ligated with double-stranded cDNA.

G3PDH product was observed after 18 cycles of amplification in unsubtracted cDNA libraries and could be detected after 28 cycles in subtracted cDNA libraries (Fig. 3), so the subtraction efficiency was satisfactory.

Differential screening of subtracted cDNA libraries

PCR amplification showed the fragment length of PCR

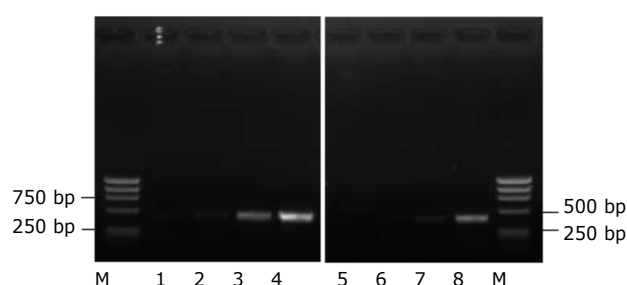


Figure 3. Analysis results of subtraction efficiency for G3PDH cDNA before and after subtraction.

Lanes 1-4: unsubtracted primitive cDNA after 18, 23, 28, and 33 cycles, respectively; Lanes 5-8: subtracted PCR production after 18, 23, 28, and 33 cycles, respectively.

products ranged from 200 to 500 bp, which possibly represented differentially expressed genes. Thirty-six clones from forward library were randomly chosen for sequence analysis and submitted to GenBank for homology analysis. Bioinformatic analysis showed that the 36 clones showed significant homologies with 19 known genes and 2 novel genes (Table 1). Most genes played an important role in cell or extracellular matrix structure and movement, cell defense, material transportation, and the regulation of expression and metabolism.

Table 1. Differentially expressed genes in long-term morphine treated striatal neurons

Clone no.	Gene/gene product	GenBank accession no.
A07	Bromodomain containing 2 (Brd2)	NM_212495
A12	TBC1 domain family, member 1	XM_341215
B01	Anaphase promoting complex subunit 7	XM_222171
B03	Thioredoxin domain containing 13 (Txndc13)	XM_001081259
B04	Thymoma viral proto-oncogene 1 (Akt1)	NM_033230
B06	Protein kinase, interferon inducible double stranded RNA dependent activator (Prkra)	NM_001024780
B07	Fibronectin 1 (Fn1)	NM_019143
B09	HGF-regulated tyrosine kinase substrate (Hgs)	NM_019387
B10	Alanyl-tRNA synthetase domain containing 1 (Aarsd1)	NM_001034109
B11	Nestin (Nes)	NM_012987
C01	Protein phosphatase 1 (formerly 2C)-like (Ppm1l)	NM_001107681
C04	Mitochondrial tumor suppressor 1 (Mtus1), nuclear gene encoding mitochondrial protein	NM_178093
C05	Actinin alpha 3 (Actn3)	NM_133424
C06	Dextrin (Dstn)	NM_001033666
C09	Mitochondrial carrier homolog 1 (Mtch1)	XM_215358
C10	Glycogen synthase kinase 3 beta (Gsk3b)	NM_032080
D07	GRB2-associated binding protein 1 (Gab1)	NM_0011084440
E05	Malic enzyme 1, NADP(+)-dependent, cytosolic (Me1)	NM_0126
E11	Signal-regulatory protein alpha (Sirpa)	NM_013016
G05	TRM2 tRNA methyltransferase 2 homolog A (S. cerevisiae) (Trmt2a)	NM_001011895
G06	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae) (Lsm4)	NM_001106073

Quantification of gene expression

CREB mRNA expression showed a significant increase in morphine group (62.85 ± 1.98) compared with control group (28.43 ± 1.46 , $P < 0.01$) (Fig. 4). Among the differentially expressed transcripts identified by nested PCR, two novel genes (Mtch1 and Akt1) were quantitated by RT-PCR. The results showed that the expression of Mtch1 (96.81 ± 2.04 vs. 44.20 ± 1.31 , $P < 0.01$) as well as Akt1 (122.10 ± 2.17 vs. 50.11 ± 2.01 , $P < 0.01$) in morphine group were significantly higher than those in control group (Fig. 4).

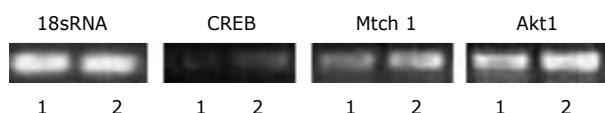


Figure 4. RT-PCR analysis result of mRNA expression levels of positive clones between control group and morphine group.

Lane 1. control group; Lane 2. morphine group.

CREB: cAMP-responsive element-binding protein; Mtch 1: mitochondrial carrier homolog 1; Akt1: thymoma viral proto-oncogene 1.

DISCUSSION

In this study, we constructed a primary cultured neuron morphine tolerance model. Forward subtraction of SSH was performed to compare gene expression difference between normal cultured neurons and morphine treated neurons. We identified differentially expressed genes from subtracted cDNA library.

The signaling mechanism underlying morphine tolerance has not been elucidated. One identified mechanism is μ -opioid receptor signaling that is coupled to several downstream effectors, including adenylyl cyclase, K^+ channels, Ca^{2+} channels, and p42/44 mitogen-activated protein kinases. The basal and forskolin stimulated activities of adenylyl cyclase are significantly increased in morphine tolerant mice.¹² The super-activation of adenylyl cyclase has some regional selectivity. The role for the striatum in the development of morphine tolerance has been previously reported.^{13, 14} So we used striatal neurons.

The main second messenger system linked to the opioid receptor is the cAMP pathway. The level of cAMP significantly increases after morphine withdrawal.¹⁵ Cellular changes occurring during cAMP superactivation include increased expression of certain adenylyl cyclases, protein kinase A, and CREB.^{16, 17} And up-regulation of CREB has been recognized to be an adaptive response to long-term use of opiates.¹⁸ In this study, CREB mRNA expression showed a significant increase in morphine group. Morphine

incubation produced a similar reaction in neurons *in vivo* when morphine tolerance took place, indicating the validity of long-term use of morphine to cultured striatal neurons.

One of the most commonly target genes to prevent opioid tolerance is opioid receptor gene. In this study, 2 novel genes, Mtch1 and Akt1, showed a significantly increased expression in morphine-treated striatal neurons compared with normal striatal neurons. As the new candidate genes that play a role in the development of morphine tolerance are identified, we could overcome morphine tolerance not only at the opiate receptor level, but even through further downstream target genes.

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