Increase of β_1-adrenergic receptor gene expression induced by nicotine in hippocampal slice of rat[1]

WANG Yong, ZHU Xiao-Nan, YAN Jie[2], YU Jian-Ping, HUANG Xiao-Hui, CHEN Ru-Zhu[3]

Department of Pharmacology, Sun Yat-sen University of Medical Science, Guangzhou 510089, China

KEY WORDS nicotine; β-adrenergic receptors; hippocampus; reverse transcriptase polymerase chain reaction; Western blotting; radioligand assay

ABSTRACT

AIM: To investigate the effect of nicotine on β_1-adrenergic receptor (β_1-AR) in the hippocampal slice of rat. **METHODS:** Hippocampal slices (400 μm thick) were incubated in artificial cerebrospinal fluid (ACSF) previously saturated with 95 % O_2 and 5 % CO_2 at 28 ºC for 120 min, and then incubated with nicotine 10 μmol/L for 30, 60, 90, and 120 min. mRNA of the β_1-adrenergic receptor was examined with semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), and the protein level was measured by Western blot and RIA. **RESULTS:** The mRNA gene expression and the protein level of β_1-adrenergic receptor in hippocampal slices were increased after nicotine treatment. The peak of protein occurred later but higher than that of mRNA level. **CONCLUSION:** Both expression of β_1- adrenergic receptor gene transcription and post-transcriptional protein level in rat hippocampus were altered by nicotine.

INTRODUCTION

The effect of nicotine on learning and memory function has been focused recently, some observation from animal and human being behavior experiments suggested that nicotine could improve animal and human being learning and memory[1-3]. Long-term potentiation (LTP) as the cellular model for learning and memory has been always used to explore the molecular and cellular mechanisms of the cognitive function. LTP involves a number of different neurotransmitter systems, and adrenergic system plays important role in learning and memory[4,5]. Norepinephrine (NE) is one of these major neuromodulators involved in the induction of LTP; activation of β-adrenergic receptor (AR) on synapse enhances LTP strongly[6], and β-AR blocker could block the LTP elicited by the titanic stimulation in the hippocampal slice of rat or induce distribution of learning and memory function in mice[7], which indicated that norepinephrine (NE) and β-adrenergic receptors in the CNS were involved in the learning and memory. It has been confirmed that nicotine can stimulate release of NE[8,9], facilitate or induce LTP in the hippocampal slice of rat[10,11]. LTP induced by nicotine with different parameters of electric stimulation could be blocked with proporanolol, a nonselective β-AR antagonist[7], so adrenergic receptor signaling is important in LTP induced by nicotine[12]. However, the molecular mechanisms responsible for the effects of that nicotine facilitate LTP induction via β_1 adrenergic receptors are unknown, the present ex-

[1] Supported by Ministry of Public Health Foundation. No 98-1-086.
[2] Now in *Department of Statistics, the 3ed Military Medical University.*
[3] Correspondence to Prof CHEN Ru-Zhu.
Phn 86-20-8733-0561. Fax 86-20-8733-0561.
E-mail 196673@163.com
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periment aimed to investigate whether nicotine influences the gene expression of β_1 adrenergic receptors.

MATERIALS AND METHODS

Reagents and animals Nicotine was obtained from Sigma. Antibody for β_1-AR was a product of Santa Cruz Biotechnology Inc. Tripure isolation reagent, PVDF membrane and Lumi-Light^{plus} Western blotting Substrate Kit were purchased from Rohe. [^{3}H]DHA was from Amersham Pharmacia Biotech Ltd. Sprague-Dawley rats were from the Experimental Animal Center of Sun Yat-sen University (100-120 g, Grade II, Certificate No 26-001 conferred by Medical Animal Management Committee, Guangdong Province). Other chemicals from Sigma were of analytic grade.

Preparation of hippocampal slices Hippocampal slices(400 μm thick) used in the experiment were obtained from Sprague-Dawley rats. Animals were anesthetized with diethyl ether and then the brains were removed rapidly and placed in cold artificial cerebrospinal fluid (ACSF) consisting of (in mmol/L: NaCl 124, KCl 3.4, $MgSO_4 \cdot 7H_2O$ 1.7, $NaHCO_3$ 25, $CaCl_2$ 1.8, glucose 10; pH 7.4) and previously saturated with 95 % O_2 and 5 % CO_2. The hippocampal slices were transversed in ACSF and incubated at 28 ºC for 120 min, and then nicotine 10 μmol/L was added. After incubation for 30, 60, 90, and 120 min respectively, the samples were determined.

RNA preparation According to the protocol of Tripure^{TM} isolation reagent, hippocampal slice with Tripure isolation reagent was homogenized, and then centrifuged at 12000×*g* for 10 min at 4 ºC. The supernatant was transferred and incubated for 5 min followed by incubation with chloroform for 2-15 min at room temperature. After isolation of the colorless upper aqueous phase isopropanol was added, and the sample was centrifuged again. The pellet was washed with 75 % ethanol.

Determination of the level of β_1-AR mRNA by RT-PCR The level of β_1-AR mRNA was detected by RT-PCR based on the method of Miyahara *et al*[13]. The cDNA was synthesized with MluV reverse transcripase. Reaction mixture 20 μL containing 0.2-3.2 μg sample RNA, RNasin ribonuclease, oligo d (T)16 primer, dNTP 1 mmol/L was incubated in RT buffer at 37 ºC for 5 min, then with 200 U MluV reverse transcripase, at 42 ºC for 60 min. The reaction was stopped by heating at 72 ºC for 10 min and immediately chilled on ice. The primers were designed according to software and synthesized by Sangon. The sense primer was 5'-CAT CAC GCT GCC CTT TCG CTA-3', and the antisense primer 5'-CGG TTG GTG ACG AAA TCG C -3'. RT reaction mixture 3 μL was then diluted with PCR buffer containing 0.5 μg of sense primer and antisense primer, dNTP 0.2 mmol/L, and 1 U *Tag* DNA polymerase. Amplified reaction was performed with a thermocycler for a single 5-min heating step at 95 ºC followed 28 cycles under the conditions: 94 ºC (60 s), 55 ºC (60 s), 72 ºC (60 s); and final extension at 72 ºC for 10 min. The amplification resulted in an expected product of 188 bp. Initially, the number of cycles was titrated for sufficient but still exponential amplification. Aliquot of 8 μL was removed from PCR mixture after different cycles of amplification and were electrophoresed on a 1.5 % agarose gel containing ethidium bromide in TAE 40 mmol/L. Amplified DNA band was scanned and the relative density was quantified. Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) as internal standard was simultaneously amplified using 5'-TCA CCA TCT TCC AGG AGC GAG A-3' as a sense primer and 5'-ATG AGC CCT TCC ACG ATG C-3' as an antisense primer. The amplified fragment of GAPDH was 303 bp.

Determination of β_1-AR protein level by Western blot analysis Western blot analysis was performed according to the method of Podlowski *et al*[14] with minor modification. The slices were transferred to a microcentrifuge tube and subsequently homogenized by sonication in 120 μL of a cold PBS buffer consisting of phenathroline 1 mmol/L, iodacetamide 1 mmol/L, PMSF 0.4 mmol/L, pepstatin A 1 μmol/L. Protein concentrations were determined by nucleic acid and protein analyzer (BECKMAN DU 640) based on the Bradford method. Samples containing amounts of total protein (20 μg) were boiled for 5 min, separated on 12 % SDS-PAGE gel, transferred on PVDF membranes and blocked for 3 h at room temperature. Then the blots were incubated overnight with primary antibody. A rabbit polyclonal antibody (1:1000 Santa Cruz) was used as a primary antibody. An horseradish peroxidase-conjugated (1:1000) anti-rabbit Ig was used as a secondary antibody. Protein signals were visualized with enhanced chemiluminescence (Donpoint) and quantified with Gel Doc 1000 system (BIO-RAD).

Radioligand binding assay Rat hippocampal crude membrane was prepared according to the method based on Lai *et al* with modification[15]. Hippocampal slice was homogenized with 20 fold volume buffer con-

taining Tris-HCl 50 mmol/L, $MgCl_2$ 10 mmol/L, and then centrifuged. The concentration of protein was measured by nucleic acid and protein analyzer with Bradford method. β_1 Adrenergic receptor binding assay was carried out according to Stutz *et al*[16]. Sample 50 μL was incubated with [^{3}H]DHA at different concentration in a total volume of 250 μL for 30 min at 25 ºC. The incubation buffer consisted of Tris-HCl 50 mmol/L and $MgCl_2$ 10 mmol/L, pH 7.4. Non-labelled competitor propranolol was added to measure non-specific binding. After incubation, samples were filtered under vacuum over Whatman GF/G filter, and washed with ice-cold buffer. The amount of [^{3}H]DHA was determined by LKB-1214-Rackbeta Counter at 60 % efficiency. B_{max} and K_d values were calculated by nonlinear regression analysis and normalized to the control.

Statistical assay The data were presented as mean±SD. Semiquantitative data of RT-PCR and Western blot were expressed as the percentage relative to controls in the same experiment. Statistical assay was performed using 2-Way ANOVA of SPSS software. $P<0.05$ was considered statistical significant.

RESULTS

RT-PCR RNA extracted from sample was scanned on the 1.5 % agarose gel with ethidium bromide staining, no degradation of RNA and contamination of DNA were found. The reverse transcription and amplification of total RNA without digestion isolated from hippocampcal slice resulted in a single band of the expected size of 188 bp using β_1-AR specific primer; No amplified products could be revealed in RNA preparation digested with RNase A, suggesting no contamination of DNA in the RNA samples. The amplified products were increased proportionally to the increase in the amount of template RNA and the number of cycles, indicating that the RT-PCR used in this study is sufficiently sensitive and accurate to detect changes in β_1-AR mRNA level. On the basis of similar titration, 28 cycles were adopted for measurements of β_1-AR mRNA and GAPDH mRNA (Fig 1).

Determination of β_1-AR mRNA in the hippocampal slice of rat No change in β_1-AR mRNA was found in the hippocampal slice without nicotine treatment (data not shown), but significant increase in β_1-AR mRNA level was demonstrated after nicotine 10 μmol/L treatment. The mRNA level of β_1-AR was increased significantly to 153 %±20 % of control after nicotine treatment for 60 min, but after 90-min and 120-min treatment, the mRNA level of β_1-AR was 136.8 %± 15.6 % and 128.2 %±17.7 % of control, respectively. Prolonged application of nicotine did not have further effects on β_1-AR mRNA expression (Fig 2).

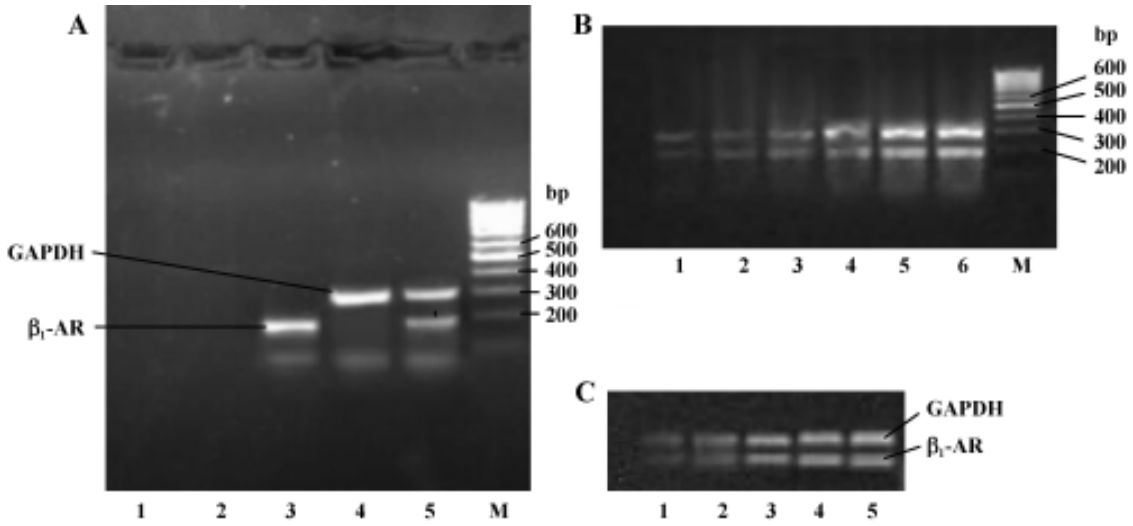

Fig 1. (A) Representative picture of ethidium bromide staining of agarose gel for assessment of DNA-free RNA preparation from the hippocampal slice of rats. Lane 1: RNA samples were digested with RNase A; Lane 2: without reverse transcription before PCR; Lane 3-5: Normal RT-PCR with β_1-AR specific primer, GAPDH specific primer, and bis β_1-AR and GAPDH primers, respectively. Lane M: marker. (B) RT-PCR was performed using 3.0 μg total RNA with 18, 21, 24, 27, 30, and 35 cycles, respectively (Lane 1-6). (C) Lane1-5 with 28 cycles using 0.2, 0.4, 0.8, 1.6, and 3.2 μg total RNA respectively with 28 cycles.

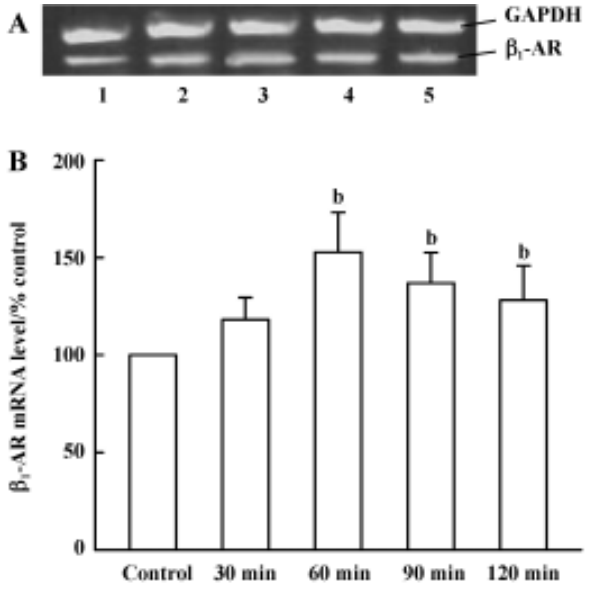

Fig 2. (A) Expression of β_1-AR mRNA by RT-PCR in the hippocampal slice exposed to nicotine 10 μmol/L. Lane 1-5: Control and treatment of nicotine for 30, 60, 90, and 120 min. (B) β_1-AR mRNA level was normalized to GAPDH signal. *n*=6. Mean±SD. $^bP< 0.05$ *vs* control (2-Way ANOVA of SPSS).

Effect of nicotine on β_1-AR protein level in hippocampal slice No signal was found in lane 1 because of no β_1-AR protein in loading sample. However, one band weight of 63 kDa appeared at the other lanes loaded with sample extracted from hippocampus, consistent to the other investigation[9]. This findings indicated that the antibody was specific for β_1-AR. β_1-AR protein level in hipoocampal slice began to increase at 30 min. It was elevated by 54.3 % at 60 min (P<0.05) and reached peak level (by 81.5 %, P<0.01) at 90 min. It dropped at 120 min (67.4 % *vs* 81.5 %) (Fig 3).

Effect of nicotine on maximum binding capacity of β_1-AR The maximal binding capacity (B_{max}) for [^{3}H]DHA binding was greatly increased to 171.4 %± 17.3 % of control after treatment with nicotine 10 μmol/L for 60 min and reached to the peak (193.1 %±20.2 % of control) after nicotine treatment for 90 min. But when the period was prolonged to 120 min, B_{max} was 155.4 %±11.3 % of control (P<0.01 *vs* 90 min). The results reinforced the data that β_1-AR protein expression was up-regulated in the hippocampal slice after nicotine 10 μmol/L treatment. In contrast, no effect of nicotine on the affinity of β_1-AR was investigated (data not shown).

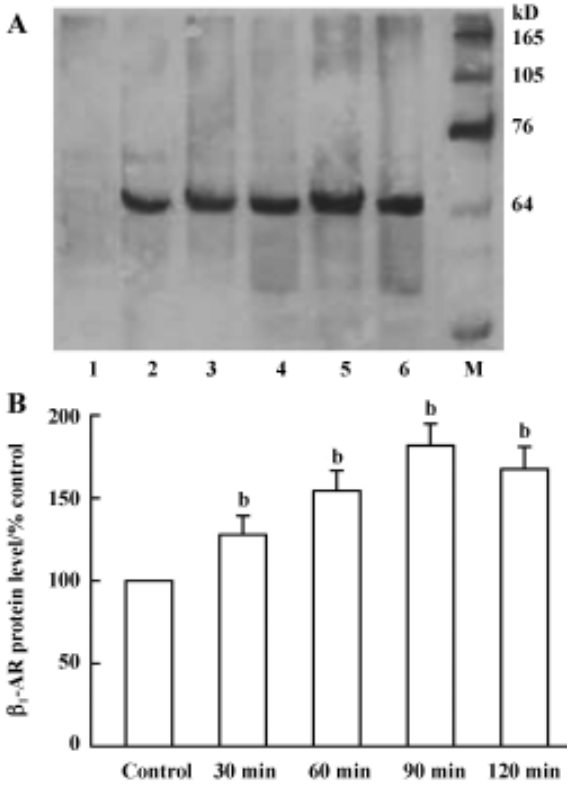

Fig 3. Increase of β_1-AR protein level after treatment with nicotine 10 μmol/L. (A) Autograph of Western blot. Lane 1, negative control without β_1-AR protein, no band was revealed; Lane 2-6, 30 μg total protein extracted from hippocampal slice after nicotine 10 μmol/L treatment for 0, 30, 60, 90, and 120 min, respectively, only one band was revealed in each lane, the molecular weight is 63 kDa. Lane M: standard protein marker. (B) Western blot analysis of β_1-AR protein level was quantified with Doc Gel 1000 system. *n*=6. Mean±SD. $^bP<0.05$ *vs* control (2-Way ANOVA of SPSS).

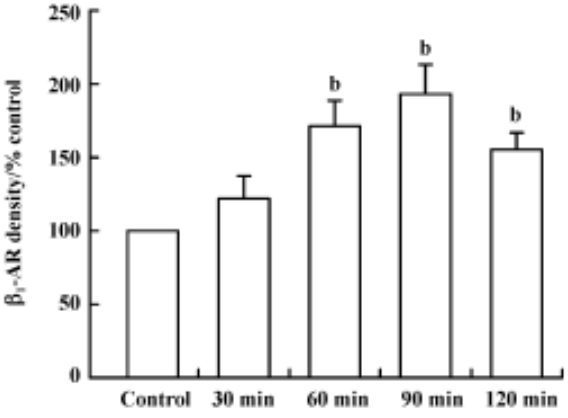

Fig 4. Effect of nicotine on the maximal binding capacity of β_1-AR in hippocampal slice using [^{3}H]DHA. *n*=5. Mean±SD. $^bP< 0.05$ *vs* control (2-Way ANOVA of SPSS). The density of control group is (67±16) pmol·g^{-1}protein.

DISCUSSION

Adrenergic system regulates forms of synaptic plasticity is involved in memory formation. LTP induced by nicotine is blocked by propranolol, a nonselective antagonist[7,12], which indicated that β-AR activation was involved in cognitive function improved by nicotine and other nicotinic receptor agonists. In present study, we directly observed that nicotine up-regulated β_1-AR mRNA and protein level.

Both β_1 and β_2 receptors are expressed in hippocampus, but β_1-AR is predominant for cognitive function because it was expressed on neurons, while β_2-AR was expressed in blood vessels, thus we selected β_1-AR in present study. β_1-AR mRNA level and protein level were increased in hippocampcal slice after nicotine treatment. The fact that the peak of protein level appeared later than that of mRNA indicated the protein increase was referred to gene transcription. However, mRNA level was unchanged after treatment with nicotine for 30 min while the protein expression was markedly increased, which suggested there was at least a path way independent to gene transcription. So the change of protein level of β_1-AR induced by nicotine was due to not only the altered β_1-AR gene transcription but also regulation of post-transcription, such as post-transcriptional modification, translation, even degradation of protein. The effect of nicotine on β_1-AR gene post-transcription may be more significant. In this study, it should be noticed that the magnitude of changes in the β_1-AR density measured by radioligand assay is not corresponded with that determined by Western blot analysis, which may be due to the difference in the sensitivity of the two methods. Western blot is specific for β_1-AR but radioligand binding assay is not.

Induction of LTP is mediated by G protein signaling pathway. Activation of β_1-AR is one of the important components to G protein signaling pathway. β_1-AR agonist elicited increase in cAMP level and ionic concentration, which caused phosporylation of ERK[17-19]. So the increase in β_1-AR mRNA and protein expression will improve cognitive function through G protein signaling pathway.

Interestingly, prolonged treatment with nicotine did not further up-regulated both mRNA and protein expression of β_1-AR. One of the possibility is that acute release of neurotransmitter induced by nicotine will up-regulate β_1-AR mRNA expression and protein synthesis, but lasting neurotransmitter release will inhibit up-regulation of β_1-AR mRNA transcription and protein synthesis[20]. Another possibility is that nicotine receptor will desensitize after long-lasting stimulation by nicotine. Fu *et al* reported that nicotine receptor desensitized at 40 min after consecutive stimulation by high concentration of nicotine[21].

In conclusion, nicotine induced LTP by up-regulating mRNA and protein expression of β_1-AR in hippocampcal slice.

REFERENCES

1 Levin ED, Bettegowda C, Blosser J, Gordon J. AR-R17779 and alpha7 nicotinic agonist, improves learning and memory in rats. Behav Pharmacol 1999;10: 675-80.

2 Bernal MC, Vicens P, Carrasco MC, Redolat R. Effects of nicotine on spatial learning in C57BL mice. Behav Pharmacol 1999; 10: 333-6.

3 Kelton MC, Kahn HJ, Conrath CL, Newhouse PA. The effects of nicotine on Parkinson's disease. Brain Cogn 2000; 43: 274-82.

4 McGaugh JL. Involvement of hormonal and neuromodulatory systems in the regulation of memory storage. Annu Rev Neurosci 1989; 12: 255-87.

5 Zhang HT, Frith SA, Wilkins J, O'Donnell JM. Comparison of the effects of isoproterenol administered into the hippocampus, frontal cortex, or amygdala on behavior of rats maintained by differential reinforcement of low response rate. Psychopharmacology (Berl) 2001; 159: 89-97.

6 Katsuki H, Izumi Y, Zorumski CF. Noradrenergic regulation of synaptic plasticity in the hippocampal CA1 region. J Neurophysiol 1997; 77: 3013-20.

7 Hamid S, Gavin SD, Jeffrey AG, John DS. Nicotine induces long-lasting potentiation in the dentate gyrus of nicotine-primed rats. Neuroscience Res 1997; 29: 81-8.

8 Clarke PBS, Reuben M. Release of [^{3}H]-noradrenaline from rat hippocampal synaptosomes by nicotine: mediation by different nicotinic receptor subtypes from striatal [^{3}H]-dopamine release. Br J Pharmacol 1996; 117: 595-606.

9 Yitong Fu, Shannon GM, Tressa JJ, Burt M. Nicotine-induced norepinephrine release in the rat amygdala and hippocampus is mediated through brainstem nicotinic cholinergic receptors. Pharmacology 1998; 284: 1188-96.

10 Fujii S, Ji ZX, Morita N, Sumikawa K. Acute and chronic nicotine exposure differentially facilitate the induction of LTP. Brain Res 1999; 846: 137-43.

11 HE Jin, DENG CY, CHEN RZ. Long-term potentiation induced by nicotine in CA1 region of hippocampal slice is Ca^{2+}-dependent. Acta Pharmacol Sin 2000; 21: 429-32.

12 Watabe AM, Zaki PA, O'Dell TJ. Coactivation of beta-adrenergic and cholinergic receptors enhances the induction of long-term potentiation and synergistically activates mitogen-activated protein kinase in the hippocampal CA1 region. J Neurosci 2000; 20: 5924-31.

13 Miyahara S, Komori T, Fujiwara R, Shizuya K, Yamamoto M, Ohmori M, *et al*. Effects of restraint stress on alpha(1)

adrenoceptor mRNA expression in the hypothalamus and midbrain of the rat. Brain Res 1999; 843: 130-5.

14 Podlowski S, Hans PL, Rosemarie M. Agonistic anti-β_1-adrenergic receptor autoantibodies from cardiomyopathy patients reduce the β_1-adrenergic receptor expression in neonatal rat cardiomyocytes. Circulation 1998; 98: 2740.

15 Lai H, Carno MA, Wen YF. Naltrexone pretreatment blocks microwave-induced changes in central cholinergic receptors. Bioelectromagnetics 1991; 12: 27-33.

16 Stutz R, Sanchez M, Salvatierra NA, Scimonelli T. The effect of melanotropic peptides on binding of [^{3}H]dihydroalprenolol-hydrochloride to hypothalamic membranes. Peptides 2001; 22: 135-8.

17 Winder DJ, Martin KC, Muzzo IA. ERK plays a regulatory role in induction of LTP by theta frequency stimulation and its modulation by β-adrenergic receptors. Neuron 1999; 24: 715-26.

18 Giovannini MG, Blitzer RD, Wong T, Asoma K, Tsokas P, Morrison JH, *et al*. Mitogen-activated protein kinase regulates early phosphorylation and delayed expression of Ca^{2+}/calmodulin-dependent protein kinase II in long-term potentiation. J Neurosci 2001; 21: 7053-62.

19 Wang J, Chen YB, Zhu XN, Chen RZ. Activation of p42/44 mitogen-activated protein kinase pathway in long-term potentiation induced by nicotine in hippocampal CA1 region in rats. Acta Pharmacol Sin 2001; 22: 685-90.

20 Hosoda K, Duman RS. Regulation of beta 1-adrenergic receptor mRNA and ligand binding by antidepressant treatments and norepinephrine depletion in rat frontal cortex. J Neurochem 1993: 60: 1335.

21 Fu Y, Matta SG, Valentine JD, Sharp BM. Desensitization and resensitization of norepinephrine release in the rat hippocampus with repeated nicotine administration. Neurosci Lett 1998; 241: 147-50.