行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
□期中進度報告

以大白鼠大腦皮質初級神經細胞培養探討血清素及其接受器對 NMDA 接受器引神經分化及細胞毒性的影響

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 94－2314－B －038 －010－
執行期間：2005 年 08 月 01 日至 2006 年 07 月 31 日

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執行單位：台北醫學大學

中　華　民　國　95 年　12 月　25 日

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以大白鼠大腦皮質初級神經細胞培養探討血清素及其接受器對 NMDA 接受器引神經分化及細胞毒性的影響

The role of serotonin and its receptor on the NMDA receptor-mediated neuronal differentiation and neurotoxicity in the rat primary cortical neuronal culture

中文摘要：

血清素接受器在中樞神經功能發育過程的調控中扮演一個重要的角色。在本研究中，我們探討在大白鼠大腦皮質初級神經細胞培養中，以血清素接受器的拮抗劑長期壓抑其活性，是否會對 NMDA 接受器－一種興奮性胺基酸的受體－的表現造成影響。我們由培養的第三天開始，在大腦皮質初級神經細胞培養中加入 1 μM 非選擇性血清素接受器拮抗劑 methysergide maleate 或 dihydroergocristine mesylate、5-HT_1 接受器之選擇性拮抗劑 pindolol、或 5-HT_2 接受器之選擇性拮抗劑 cyproheptadine hydrochloride，持續培養 9 天。這些拮抗劑的處理並不會對細胞造成明顯的毒害，但降低了 NMDA 接受器在引發和細胞死亡以及細胞內鈣離子蓄積方面的效力，同時也提高了 NR2B 選擇性拮抗劑 ifenprodil 在抑制細胞內鈣離子蓄積方面的效力，並降低了 NMDA 接受器非選擇性拮抗劑 MK-801 在抑制細胞內鈣離子蓄積方面的效力。此外，免疫轉漬法的結果也顯示，NMDA 接受器次單元 NR1A 和 NR2A 的表現量降低，但 NR2B 則否。以上結果顯示，長期壓抑血清素接受器的活性，可能會降低大腦皮質神經細胞中 NMDA 接受器特定次單元的表現，並進而影響 NMDA 接受器所調節的神經傳導。

關鍵字：血清素接受器、NMDA 接受器、大腦皮質初級神經細胞培養、血清素接受器拮抗劑。

Abstract

Serotonin (5-HT) receptor is an important receptor system regulating the development of neuronal function in the CNS. In the present study we determined whether long-term suppression of the activity of serotonin receptor by serotonin receptor antagonist would affect the expression of N-methyl-D-aspartate (NMDA) receptor, one subtype receptor of excitatory amino acid, in rat primary cortical cell culture. The cultured cells were incubated with 1 μM of non-selective 5-HT receptor antagonist, methysergide maleate or dihydroergocristine mesylate, or 5-HT_1 selective antagonist, pindolol, or 5-HT_2 selective antagonist, cyproheptadine hydrochloride, for 9 consecutive days since the third days after cells were plated. Treatment of these antagonists did

not produce significant cell toxicity but decreased the potency of NMDA in inducing cell death and intracellular $^{45}Ca^{2+}$ accumulation. Associating with these change are an increase in the potency of NR2B-selective antagonist, ifenprodil, and a decrease in the potency of MK-801, a non-selective NMDA receptor antagonist, in inhibiting NMDA-induced intracellular accumulation of $^{45}Ca^{2+}$. Furthermore, immunoblotting assay showed a decrease in the expression of NMDA receptor subunit protein NR1A and NR2A, but not the NR2B. These results indicated that long-term suppression of the activity of 5-HT receptor, probably the 5-HT_1 and 5-HT_2 receptor, could result into a subunit-specific down-regulation of NMDA receptor in cortical neurons, which further alters the NMDA receptor-mediated neurotransmission.

Key Words: Serotonin receptor, NMDA receptor, primary cortical culture, 5-HT receptor antagonist, etc.

Introduction

The role of serotonin (5-hydrosytrypamine, 5-HT)-mediated neurotransmission in the development of central nervous system brain is well recognized for decades (Ahmad and Zamenhof, 1978; LoTurco et al., 1995; Mattson, 1988; Sodhi and Sanders-Bush, 2004). The serotonergic neurons develop early in the brain during embryogenesis (Okado et al., 1992; Verney, 2003), and the activation of 5-HT receptors participates in many developmental processes, including morphogenesis, glial cell proliferation, neuronal differentiation, synaptogenesis, and in the formation of connections of serotonergic neurons with its target areas (Ahmad and Zamenhof, 1978; Bennett-Clarke et al., 1994; Lauder, 1990; Lauder and Krebs, 1978; LoTurco et al., 1995; Mattson, 1988). In brain, serotonin interacts with at least 14 different receptors, divided into 7 families, from 5-HT_1 to 5-HT_7 receptor (Baez et al., 1995; Gothert, 1992; Hen, 1992; , 1993; Hoyer et al., 2002; Peroutka, 1990a; 1990b). Each subtype of receptor has its distinct expression profile, affinities for serotonin, amino acid homology, signaling pathway and functions (Backstrom et al., 1999; Baez et al., 1995; Bottenstein and Sato, 1979; Gothert, 1992; Hen, 1993; Hoyer et al., 2002; Lanfumey and Hamon, 2004; Martin and Humphrey, 1994; Nelson, 2004; Saudou and Hen, 1994; Waeber et al., 1990; Woolley et al., 2004). For example, 5-HT_{1A} receptor develops early in the CNS and is associated with secretion of S-100β from astrocytes (Whitaker-Azmitia et al., 1990) and reduction of c-AMP levels in neurons (Dumuis et al., 1988). These actions provide intracellular stability for the

cytoskeleton and result in cell differentiation and cessation of proliferation (Azmitia, 2001). The 5-HT_{2A} receptor, with binding affinity for serotonin about 1000 fold lower than that of the 5-HT_{1A} receptor, develops later and is associated with glycogenolysis in astrocytes (Poblete and Azmitia, 1995) and increased Ca^{++} availability in neurons (Azmitia, 2001). These actions destabilize the internal cytoskeleton and result in cell proliferation, synaptogenesis, and apoptosis (Azmitia, 2001).

In the developing rat brain, specific 5-HT receptors have been localized in the developing cortex, and axons of serotonergic neuron reach the edge of the cortex around embryonic day 16 and invade all areas shortly then (Lidov and Molliver, 1982a; 1982b; Wallace and Lauder, 1983). Some subtypes have been shown to be ‘‘functional’’ before birth (Whitaker-Azmitia et al., 1987). The serotonergic innervation of the rat cerebral cortex originates in the mesencephalic dorsal and median raphe nuclei (Kosofsky and Molliver, 1987; Moore et al., 1978). The early development of the serotonergic system in the brain has prompted the speculation that 5-HT receptor plays a role in a number of developmental processes in its target areas including the cortex.

Activation of the N-methyl-D-aspartate receptor (NMDA) receptor has been found to take a role in variety activities during the development of brain (Balazs et al., 1988; Burgoyne et al., 1993; Contestabile, 2000; Kleinschmidt et al., 1987; McDonald and Johnston, 1990; Moran and Patel, 1989; Nelson et al., 1986). These include neuronal migration (Komuro and Rakic, 1993), neurite outgrowth (Constantine-Paton, 1990; Heresco-Levy and Javitt, 1998; Mattson and Kater, 1988), synaptogenesis (Constantine-Paton, 1990; Heresco-Levy and Javitt, 1998; Mattson and Kater, 1988), and the activity-dependent synaptic plasticity (Collingridge and Singer, 1990). Such propensity of the NMDA receptor-mediated activity underscores the importance of normality of the development expression of this receptor in the developmental brain. Thus, aberration of the ontogenesis of the NMDA receptor in either quantitative or qualitative manner may potentially disrupt critical neuronal developmental processes, thereby underling neuro-psychological disorders in a long run.

Previous investigation had shown that acute application of 5-HT enhances the effects of NMDA effect on the neocortical cells (Nedergaard et al., 1986; , 1987; Rahman and Neuman, 1993; Reynolds et al., 1988), including the development of rhythmic bursting and TTX-resistant ‘‘depolarization shifts’’ (Nedergaard et al., 1986; , 1987). On the contrary, serotonin could inhibit the NMDA receptor-mediated production of nitric oxide and cyclic GMP in human neocortex slices through activation of 5-HT_{2C} and

5-HT_{1A} receptors (Maura et al., 2000). Agonists of 5-HT_{1A} (e.g., Bay × 3702) are known to protect the brain from anoxia or ischemia- induced excessive release of glutamate (Alessandri et al., 1999; Mauler et al., 2001), which is believed mediated by the activation of NMDA receptor, and to protect cultured neurons from apoptosis caused by serum deprivation, staurosporin treatment and excitotoxic damage (Ahlemeyer et al., 1999; Semkova et al., 1998; Suchanek et al., 1998). In contrary, activation of 5-HT_{2A} receptor by 3,4-methylenedioxy- methamphetaine (MDMA) induced Ca^{++} dependant toxicity in cultured serotonergic neurons (Azmitia et al., 1990). More interestingly, it has been shown that long-term incubation with serotonin promotes the survival of cultured cortical glutamatergic neuronsin rat primary cortical cell culture (Dooley et al., 1997), an effect through the action on the 5-HT_{1A} receptor and 5-HT_{2C} receptor. Thus, the neurotrophic action of serotonin might include a regulation on the NMDA receptor. Particularly, the development of serotonin neurotransmitter in developing cortex has been thought to be earlier than that of NMDA receptor. Given all these observations, it is possible that the developmental expression of the NMDA receptor in the brain might require normal activity of the 5-HT receptors. To test this possibility, we examined the effect of 5-HT receptor antagonists, which could suppress the activity of 5-HT receptors, on the expression of the NMDA receptor in primary rat cortical cell culture. The antagonists used were methysergide maleate and dihydroergocristine mesylate, both are non-selective 5-HT receptor antagonist, pindolol, a 5-HT_1 selective antagonist, and cyproheptadine hydrochloride, a 5-HT_2 selective antagonist. The function of NMDA receptor was examined by NMDA-induced cytotoxicity and intracellular accumulation of $^{45}Ca^{2+}$ in the cell cultures. The density of the NMDA receptor in the cultured cells were quantified by immunoblotting assay of three essential subunit proteins of NMDA receptor expressed in the cortex, namely, the NR1, NR2A and NR2B.

Materials and methods:

1. Cell culture:

Primary cortical neuronal cell cultures were prepared by enzymatic dissociation of fetal rat cortical tissues. In brief, Sprague-Dawley rat embryos of gestational days 15 to 17 were removed under ether anesthesia and the cortex was dissected and freed from the meninges. The tissues were then incubated with 0.25% trypsin in Ca^{2+}/Mg^{2+} free minimum Eagle's medium

(MEM) at 37 °C for 60 minutes. The enzyme solution was removed by aspiration and tissues were washed with MEM containing 0.02 % DNase I. The cells were mechanically dissociated by repetitive pipetting with a fire-polished glass pipette. After low-speed centrifugation, the resulting cell pellet was resuspended in MEM containing 5 % fetal calf serum (FBS) and 5 % horse serum and plated in D-poly-lysine coated 10-cm Petri dish, 6- well cultured plates, or 6-well plates containing coated coverslide at a final cell density of 0.5- 1 x 106 cells/ml. These cells were cultured under 37 °C with 5 % CO2 in a humidified incubator.

2. 5-HT receptor antagonist treatment:

The antagonists we used in this study are purchase from Tocirs™. Methysergide maleate is a 5-HT_1/5-HT_2 receptor antagonist, and dihydroergocristine mesylate is a 5-HT antagonist which also partially agonist at adrenergic and dopaminergic receptors. On DIV 4 and every 2 days thereafter, 1, 10 or 100 μM of either antagonists were added to the culture. Aliquots of culture media were taken out on various DIV for LDH activity assays, and on DIV 14, the cells were harvested for immunoblotting assay.

3. Immunoblotting:

Crude cell lysates will be prepared by solubilizing the cells cultured in 10-cm petridishes in lysis buffer (0.1 M Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, 0.005% bromophenol blue, 0.005% pyronine Y and 1% β-mercaptoethanol). After determining the protein content using the Bio-Rad D_c protein assay, aliquots of cell lysates containing 50 μg of protein will be separated on a polyacrylamide- SDS gel and transferred onto polyvinylidenedifluoride (PVDF) membranes. Membranes will be blocked with 5 % nonfat milk and detected with anti- NMDA receptor 1, 2A, 2B subunit- or anti NSE- antibodies, secondary antibodies then developed with enhanced chemiluminescence (ECL) assay. The intensities of resulting bands of proteins on the x-ray film were quantified using computerized image analyzer.

4. Cytotoxicity assay:

For cytotoxicity assay, the media from cells seeded in 6- well plates or 10-cm patri dishes, culture in the presence or absence of 5-HT antagonist, was taken out and assayed for LDH activity using LDH kit (Roche). The increase in the absorbency at 490 nm per minute was calculated and used as the indicator for LDH activity

5. Intracellular $^{45}Ca^{2+}$ Accumulation Assay:

Primary cortical cell cultures at 12 days in vitro were incubated with 0.1 Ci/ml $^{45}Ca^{2+}$, 100

μM NMDA, and 10 μM glycine in the absence or presence tested compounds for 2 min. The cells were washed with fresh MEM buffer three times and disrupted by 0.5 ml of 1% SDS. Radioactivities of cell solutions were counted by a liquid scintillating counter. The nonspecific $^{45}Ca^{2+}$ accumulation was the $^{45}Ca^{2+}$ accumulation in the cell culture incubated with 100 μM NMDA, 10 μM glycine, and 10 μM MK-801.

6. HPLC measurement of serotonin in culture medium

Culture medium samples were randomized taken from each batch of cultures. The sample. The samples were frozen at −70°C immediately before further analyzed by HPLC. HPLC was conducted by using a reverse-phase column with 3-μm particle size (C18, Beckman, CA) thermostated at 32°C. The mobile phase was 0.2 M NaH_2PO_4, 1.5 mM octyl sodium sulphate, 0.5 mM EDTA (pH 2.8) and 21% methanol. 5-HT quantification was carried out using an amperometric electrochemical detection (model 464; Waters). The mobile phase was delivered at a flow rate of 0.8 ml/min. The minimal detectable amount of 5-HT was 10 fmol .

Result

The expression of 5-HT receptor and NMDA receptor subunit in rat primary cortical cell culture

The immunoblotting assay demonstrated the expression of 5-HT receptors, including 5-HT_{1A}, 5-HT_{1B} 5-HT_{2A} and 5-HT_{2B} subtypes, as well as the NMDA receptor subunits, including NR1, NR2A and NR2B in this cortical cell culture since DIV5. (Fig 1).

Determination of serotnin level in the fresh culture medium by HPLC assay.

The HPLC assay showed that the concentration of serotonin in the culture medium used in the present study is 3 ± 0.47 μM.

The effect of long-term exposure to methysergide maleate, dihydroergocristine mesylate, pindolol or cyproheptadine hydrochloride on the NMDA induced cell death and $^{45}Ca^{2+}$ accumulation in the cultured cells

In the preliminary study, we found that a 9-day incubation with these 5-HT receptor antagonists did concentration-dependently produce significant cell death as determined by visual examination under light microscope and LDH assay of the culture medium (data not shown). The minimal concentration of antagonists produced apparent increase in the LDH in the medium after a 9-day incubation is 10 μM for dihydroergocristine mesylate, and 30 μM for methysergide maleate, pindolol and cyproheptadine hydrochloride. However, 1 μM of each of these antagonists

did not produce significant increase in the LDH level in the medium or visualized cell death. Therefore, we added 1 μM of each antagonists in the following studies. A 30-minute incubation of NMDA concentration-dependently increased cell death on DIV12-14 as measured by LDH assay. The EC_{50} of NMDA for this cytotoxic effect is 28 ± 4 μM. The EC_{50} of NMDA in the cultured cells after being treated with methysergide maleate, dihydroergocristine mesylate, pindolol and cyproheptadine hydrochloride were 51 ± 6 μM, 6 1± 6 μM, 84 ± 11 μM and 54 ± 8 μM, respectively (Fig 2). The EC_{50s} of NMDA in cells treated with antagonists are all significantly higher than that in cells without antagonist treatment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keuls test). There is no significant change in the maximal level of LDH induced by NMDA found between the treated and untreated cultured cells (data not shown). Furthermore, a 2-minute incubation of NMDA concentration-dependently increased the intracellular accumulation of $^{45}Ca^{2+}$ accumulation in the cultured cell. The EC_{50} of NMDA for this effect was 14 ± 7 μM, and the EC_{50s} of NMDA in the cultured cell treated with methysergide maleate, dihydroergocristine mesylate, pindolol and cyproheptadine hydrochloride were 39 ± 5 μM, 59 ± 11 μM, 72 ± 17 μM and 48 ± 9 μM, respectively (Fig 3). The EC_{50s} of NMDA in cells with antagonist treatment are all significantly higher than that in the cells without antagonists treatment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keuls test). There is no significant change in the maximal level of $^{45}Ca^{2+}$ accumulation found between the treated and untreated cultured cells (data not shown).

The effect of co-administration of methysergide maleate, dihydroergocristine mesylate, pindolol or cyproheptadine hydrochloride on the NMDA induced cell death and $^{45}Ca^{2+}$ accumulation in the cultured cells

We tested whether co-administration of 1 μM of the 5-HT receptor antagonists with NMDA in the cells without prior long-term treatment with antagonists wound affect the potency of NMDA in inducing calcium accumulation. The EC_{50} of NMDA in the absence of co-administration of antagonist was 17 ± 5 μM, and the EC_{50s} of NMDA in the cultured cells in the presence of methysergide maleate, dihydroergocristine mesylate, pindolol and cyproheptadine hydrochloride was 16 ± 4 μM, 19 ± 3 μM, 22 ± 6 μM and 11 ±7 μM, respectively (data not shown). There is no significant difference between these EC_{50s}. Besides, the maximal level of $^{45}Ca^{2+}$ accumulation induced by NMDA was not significantly changed by the presence of antagonists (data not shown).

The effect of long-term exposure to dihydroergocristine mesylate and cyproheptadine hydrochloride on the potency of MK-801 and ifenprodil in inhibiting the NMDA-induced

intracellular $^{45}Ca^{2+}$ accumulation in the cultured cells

Co-administration MK-801 or ifenprodil with NMDA inhibited NMDA-induced $^{45}Ca^{2+}$ accumulation in a concentration-dependent manner. The inhibitory kinetics curve for MK-801 is better fitted with two-site kinetics, a high potency with IC_{50} of 19±0.3 nM and a low potency with IC50 of 24 ± 9 μM (Fig 4). The IC_{50s} of high potency of MK-801 in the cells treated with dihydroergocristine mesylate and cyproheptadine hydrochloride were 59 ± 8 nM and 78 ± 16 nM respectively, and the IC_{50s} of low potency were 49 ± 15 μM and 19 ± 13 μM, respectively (Fig.5). The IC_{50} of high potency effect of MK-801 in the cells treated with antagonist are all significantly higher than that in cells without antagonists treatmetment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keul t test). However, no significant difference was found in the IC_{50} of low potency effect. Ifenprodil concentration-dependently inhibited NMDA-induced intracellular accumulation of $^{45}Ca^{2+}$ with IC_{50} of 0.84 ± 0.17 μM (Fig 5). In contrast to that of MK801, the inhibitory curve of ifenrpodil is better fitted by one-kinetic phase. The IC_{50s} of ifenprodil in the cells treated with dihydroergocristine mesylate and cyproheptadine hydrochloride were 0.41 ± 0.14 μM and 0.48 ± 0.11 μM, respectively. In contrast to that of MK-801, the IC_{50} of ifenprodil in the cell treated with antagonist are all significantly lower than that in the cells without treatment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keuls test).

T**he effect of long-term exposure to dihydroergocristine mesylate on the expression of NR1, NR2A and MR2B in rat primary cortical cell culture**

Immunoblotiing assay showed that long-term incubation of the 1 μM dihydroergocristine mesylate or methysergide maleate significantly decreased the abundance of NR1A and NR 2A but had no effect on the abundance of NR2B in the cultured cell (Fig 6 and 7).

Discussion

The present study demonstrated that long-term incubation of serotonin antagonist, either non-selective or selective for 5-HT_1 or 5-HT_2 receptor, produced a significant alteration in the NMDA receptor-mediated responses in cortical neuronal cultured cells, including a decrease in the potency of NMDA in stimulating the NMDA receptor-mediated intracellular $^{45}Ca^{2+}$ accumulation and cytotoxicity, and an increase in the potency of ifenprodil and a decrease in the potency of MK-801 in inhibiting NMDA receptor-mediated intracellular $^{45}Ca^{2+}$ accumulation. Associated with these changes is a down-regulation of NMDA receptor subunits only for NR1A and NR2A, but spared the NR2B.

The antagonists used in this study include non-selective antagonists, methysergide maleate (Leigh, 1963) and dihydroergocristine mesylate (D'Agostino et al., 1984), and selective antagonist, pindolol for 5-HT_1 receptor (Tricklebank et al., 1985) and cyproheptadine hydrochloride (Fillion et al., 1980; Krstic and Katusic, 1982) for 5-HT_2 receptor. As demonstrated by our data from the immunoblotting assay, it appears that the targeting 5-HT receptor subtypes for these applied antagonists are indeed expressed on the cultured cells during the incubation period. The concentration of each 5-HT receptor antagonists applied to the culture (1 μM) is at least 10-folds lower than the toxic concentration range defined by our preliminary experiments, which showed concentration over 10 μM of each antagonist produced significant increase in the LDH activity in cell culture medium after long-term incubation. Therefore, the effect of 5-HT receptor antagonists on the NMDA–mediated responses in cultured cell is not attributed to the drug cytotoxicity.

The binding affinities of the applied 5-HT receptor antagonists to their receptors had been reported to be all within nanomolar range (Choudhary et al., 1993; Corradetti et al., 1998; Honrubia et al., 1997; Korneyev and Cincotta, 1996). Therefore, 1 μM of these antagonists should be sufficient to saturate its targeting receptor in the absence of agonist competitor to the same receptor binding site. However, the ambient concentration of serotonin in our culture medium is estimated around 3 μM, a super-saturable concentration for 5-HT receptor on the cultured cell. Given that the binding affinity of the applied antagonists have been estimated about 1-10 fold lower than that of serotonin, which is varying among different subtype of 5-HT receptor, it is reasonable to justify that the antagonism efficacy of each antagonist would be less than 50%. On the other word, the 5-HT receptor activity of the cultured cells is only partially suppressed in the presence of the applied antagonist. The reason that we used such partial suppression condition instead of total suppression is based upon our preliminary observation, which showed that once the applied concentration of 5-HT receptor antagonists is higher then 30 μM, a concentration that theoretically could almost overcome the effect of the ambient serotonin, apparent cytotoxicity

was noted after a 3- or 7- day incubation. Consistently, other reports had also shown that serotonin-deprivation could cause cultured neuronal cell death (Ahlemeyer et al., 1999; Ahlemeyer and Krieglstein, 1997; Menegola et al., 2004). These findings strongly suggest that long-term survival of cortical cell culture requires the 5-HT receptor activity maintaining in a sufficient level.

The principle finding of this study is that long-term incubation of any one of the four 5-HT receptor antagonist produce a similar decreases in the potency of NMDA in inducing intracellular accumulation of $^{45}Ca^{2+}$ in cultured cells, which is paralleled well with the decrease of the potency of NMDA in inducing cell death. This finding indicates that long-term and partial suppression of 5-HT receptor activity induces a decrease in the sensitivity to the applied NMDA in these cultured cells. Since the intracellular calcium accumulation induced by the NMDA is mainly caused by the binding of NMDA to its receptor on the cell surface membrane thereby opening the NMDA receptor-coupled ion channel to allow the influx of extracellular calcium into the cells, such sensitivity changes of NMDA is likely due to an alteration of the NMDA receptor on the cultured cells. This alteration might include a decrease in the number of NMDA receptor expressed on the cells, or a decrease in the binding affinity of NMDA to its site on the NMDA receptor, or both. To discriminate between these two possibilities, radio-ligand binding assay of NMDA receptor in cultured cells is required. Unfortunately, our previous attempt on the binding experiment was not successful. However, the subsequent immunoblotting assay did show a significant decrease in the quantity of the NR1 and NR2A subunit protein, providing evidence in supporting a quantitative decrease in the NMDA receptor. Still, the change in the affinity of NMDA can not be excluded at present.

Interestingly, the decrease in the expression of the NMDA receptor subunit protein is only limited to the NR1A and NR2A, but not on the NR2B. It is well known that the NMDA receptor in the rat cortex is formed mainly by NR1 with NR2A or NR2B (Conti et al., 1996; Hoffmann et al., 1997; Zhong et al., 1995), in a multi-meric conformation. Thus, the NMDA receptors in the cortex could be classified into two major populations, one is NR1/NR2A subset and the other one is NR1/NR2B subset. With this respect, a decrease in the quantity of NR1 will reflect a total reduction in the quantity of both of NR1/NR2A and NR1/NR2B receptors, but a decrease in the quantity of NR2A without similar change in the NR2B would further suggest that the decrease in the number of NR1/NR2A receptor is more significant than that in the NR1/NR2B, or the decrease in the NMDA receptor is mainly restricted to the NR1/NR2A population. If so, then the down-regulation in the NMDA receptor expression is accompanied by a change in the ratio of the NMDA receptor subset population, in a way that the proportion of the NR1/NR2A subset is decreased while the proportion of the NR1/NR2B subset is increased. The down-regulation in the

expression of NMDA receptor could account for the decrease in the sensitivity of cells to NMDA, and the shift in NMDA receptors to predominantly NR2B-containing receptors matches well with the finding that the potency of ifenprodil, an NR2B receptor preferential antagonist, in inhibiting the NMDA-induced response is increased after cells were long-exposed to 5-HT receptor antagonist. However, the finding that the potency of the high-affinity site for MK-801 potency in inhibiting NMDA-elicited response is decreased without a change in the potency of low-affinity site, could not be explained by the present result since MK-801 is not selective between NR2A and NR2B receptors. Given that the change in the NMDA receptor is induced by long-term and partial suppression of the 5-HT receptors, and the ambient concentration of serotonin is able to over-saturate the 5-HT receptors, it appears that in this cultur system, to obtain a normal ontogeneic expression of the NMDA receptor requires that the activity of 5HT receptor keeping in its maximal level.

The answer regarding which type of 5-HT receptor that produced such alteration in the NMDA receptor expression is still obscured. Both non-selective and selective 5-HT receptor antagonist produced similar change, suggesting that there is no subtype selectivity for this particular effect. At the least, suppression of 5-HT_1 and 5-HT_2 receptor is involved. Interestingly, these two types of receptors acts differently on the brain, and some experiment showed that they could induce contrary response in neuronal cells (Azmitia, 2001). For example, 5-HT_{1A} stimulates the differentiation and maturation of neurons, while 5-HT_{2A} promotes proliferation and of astrocytes (Azmitia, 2001); 5-HT_{1A} agonist protects neuronal cells against apoptosis (Ahlemeyer et al., 1999; Ahlemeyer and Krieglstein, 1997; Suchanek et al., 1998), while 5-HT_{2A} enhances apoptosis through intracellular Ca^{2+} build up and the IP3 signaling (Neuman and Rahman, 1996; Poblete and Azmitia, 1995). In the 1A neuronal cell line, 5-HT_{2A} can increase the cAMP level by PKC- dependent calcium/calmodulin- dependent mechanisms (Berg et al., 1994), in contrast to inhibitory effect of 5-HT_1 on adenyl cyclase (De Vivo and Maayani, 1990; Dunlop et al., 1998). Such complexity in the function of 5-HT receptor, combining with the fact that the selectivity of 5-HT antagonists is not complete, will make it more difficult to resolve this issue by conventional pharmacological studies. Perhaps, using siRNA method to selectively inhibit the expression of each subtype receptor in the culture will be helpful.

One interesting question raised by the present study is that what impact such alteration of the expression of the NMDA receptor will bring in terms of NMDA receptor-related developmental synaptic plasticity on the cortical neuron. In particular, such long-term suppression of 5-HT receptor not only reduces the NMDA receptor expression, but also increases the proportion of NR2B- containing receptor. It has been found that NR2A and NR2B receptor has their distinct role in the neuropathophysiology (Bartlett et al., 2006; Kew et al., 1998; Mallon et al., 2005;

Yoshimura et al., 2003). For example, in the hippocampus, NR2A receptor is predominant in the generation of long-term depression (LTD), while NR2B receptor is predominant in the generation of long-term potentiation (LTP). Both neurophysiological responses are critical for the developmental plasticity of hippocampal neuron (Bartlett et al., 2006). Thus, restriction in the expression of both subtype receptors to a certain level becomes critical for ensuring the normal developmental plasticity. In addition, during the development of the rat, especially during postnatal days (PND) 7–14, the central nervous system (CNS) exhibits enhanced susceptibility to the toxic effects of modulation of the NMDA receptor system (Miyamoto et al., 2001). This enhanced susceptibility has been suggested to be derived from the increased expression of NR2B receptor (Miyamoto et al., 2001). Whether similar implications could be applied to the present result is worthy to be determined both in vitro and in vivo.

Taking together, the present study demonstrated that long-term but partial suppression of 5-HT receptor altered the expression of NMDA receptor in cortical neuron. This particular phenomenon further emphasizes the important role of 5-HT receptor function in the expression of NMDA receptor in developing brain.

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FIRURES:

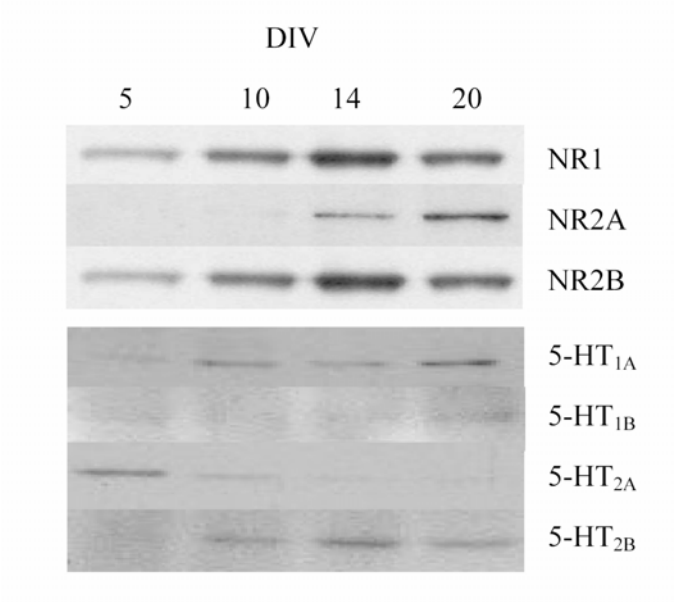

Figure 1: The expression of NMDA receptor and 5-HT receptor subunit proteins in primary cortical cell culture on different day *in vitro* (DIV).

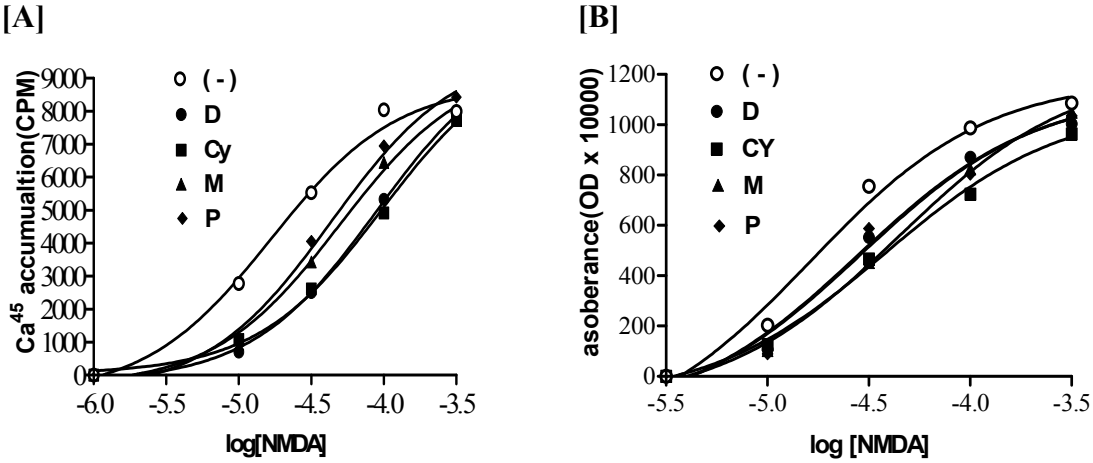

Figure 2: The effect of long-term exposure to 1 μM of methysergide maleate (M), dihydroergocristine mesylate (D), pindolol (P) or cyproheptadine hydrochloride (Cy) on the NMDA induced $^{45}Ca^{2+}$ accumulation and LDH release in the cultured cells. Data are one representative of four similar experiments. **[A] NMDA induced $^{45}Ca^{2+}$ accumulation:** The EC_{50} of NMDA in the cultured cell without antagonist treatment was 14 ± 7 μM, and the EC_{50} of NMDA in the cultured cells after treatment of methysergide maleate,

dihydroergocristine mesylate, pindolol and cyproheptadine hydrochloride were 39 ± 5 μM, 59 ± 11 μM, 72 ± 17 μM and 48 ± 9 μM, respectively. The EC_{50s} of NMDA in cells with antagonist treatment are all significantly higher than that in the cells without antagonists treatment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keuls test). **[B] LDH release:** The EC_{50} of NMDA in the cultured cells without antagonist treatment is 28 ± 4 μM, and the EC_{50} of NMDA in the cultured cells with treatment of methysergide maleate, dihydroergocristine mesylate, pindolol and cyproheptadine hydrochloride were 51 ± 6μM, 61 ± 6μM, 84 ± 11μM and 54 ± 8 μM, respectively. The EC_{50s} of NMDA in cells with antagonist treatment were all significantly higher than that in the cells without antagonist treatment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keuls test).

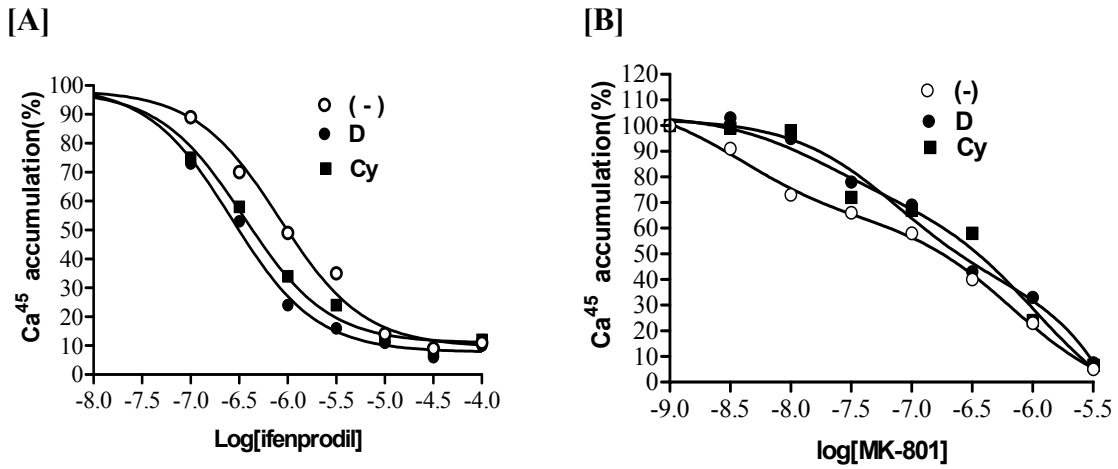

Figure 3: The effect of long-term exposure to dihydroergocristine mesylate or cyproheptadine hydrochloride on the potency of MK-801 and ifenprodil in inhibiting the NMDA-induced intracellular $^{45}Ca^{2+}$ accumulation in the cultured cells. Data are one representative of four similar experiments. **[A]** The inhibitory kinetics curve for MK-801 is better fitted with two-site kinetics, a high potency with IC_{50} of 19 ± 0.3 nM and a low potency with IC50 of 24 ± 9 μM (Fig 4). The IC_{50} of high potency of MK-801 in the cells after treated with dihydroergocristine mesylate and cyproheptadine hydrochloride were 59 ± 8 nM and 78 ± 16 nM respectively, and the IC_{50s} of low potency were 49 ± 15 μM and 19 ± 13 μM, respectively. The IC_{50} of high potency effect of MK-801 in the cells treated with antagonists are all significantly higher than that in the cells without antagonists ($p < 0.05$, one-way ANOVA with post hoc Newman-Keul t test). **[B]** Ifenprodil concentration -dependently inhibit NMDA-induced intracellular accumulation of $^{45}Ca^{2+}$ with IC_{50} of 0.84 ± 0.17 μM (Fig 5). The IC_{50} of ifenprodil in the cells treated with dihydroergocristine mesylate and cyproheptadine hydrochloride were 0.41 ± 0.14 μM and 0.48 ± 0.11 μM, respectively. In contrast to that of MK-801, the IC_{50s} of ifenprodil in the cells treated with antagonist were all

significantly lower than that in the cells without treatment ($p < 0.05$, one-way ANOVA with post hoc Newman-Keuls test).

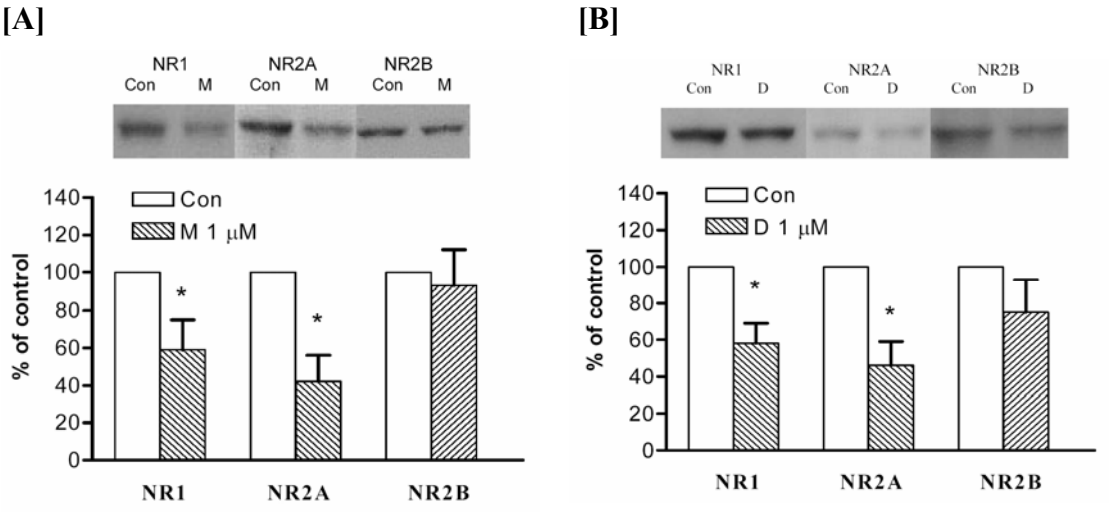

Figure 4: The effect of long-term exposure to methysergide maleate (M) or dihydroergocristine mesylate (D) on the expression of NR1, NR2A and NR2B in rat primary cortical cell culture. Immunoblotting assay showed that long-term incubation of the 1 μM methysergide maleate **[A]** or dihydroergocristine mesylate **[B]** significantly decreased the abundance of NR1A and NR 2A but had not effect on the abundance of NR2B in the cultured cell. Con: control. * means significant difference to that of control (paired Student t test, $p< 0.05$)