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Serotonin Synthesis and Metabolism in Dissociated Cultures of Fetal Rat Brainstem

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ABSTRACT

We established an *in vitro* system of central serotonergic neurons by culturing dissociated rat embryonic (E14) brainstem cells to 14 days *in vitro* and monitored the serotonergic neuronal growth by measuring 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) contents in the cells with high performance liquid chromatography with electrochemical detection (HPLC-EC). We studied also the effects of various drugs on the contents of 5-HT and 5-HIAA, confirming *in vivo* reports.

The 5-HT content (13 ng/mg protein) and 5-HT turnover rate (17 pmol/mg protein/h) at 14 days *in vitro* were in good agreement with those reported in the adult rat brain. The 5-HT content was more easily depleted with *p*-chlorophenylalanine, a tryptophan hydroxylase inhibitor than with NSD 1015 (3-hydroxybenzylhydrazine), an aromatic L-amino acid decarboxylase (AADC) inhibitor. Incubation of the cultures with tryptophan or 5-hydroxytryptophan increased the rate of serotonin formation implying that neither tryptophan hydroxylase nor AADC is saturated with its amino acid substrate in this *in vitro* system. The 5-HT content was depleted by reserpine. The 5-HT and 5-HIAA contents were increased and decreased, respectively, by monoamine oxidase inhibitors.

All the above results indicate that the biochemical properties of the central seotonergic neurons in this culture system reflect reliably those of central serotonergic neurons *in vivo*. We suggest that measuring 5-HT and 5-HIAA contents in the primary cultured dissociated brainstem-cells with HPLC-EC is useful in the study of pharmacology as well as toxicolgy of the central serotonergic neurons.

Key Words: 5-Hydroxytryptamine, 5-Hydroxyindoleacetic acid, High performance liquid chromatography with electrochemical detection, Dissociated cultures of fetal rat brainstem

INTRODUCTION

Central serotonergic neurons that have cell bodies mainly in the raphe nuclei of the brainstem (Dahlstrom and Fuxe, 1964) and project to almost all levels of the central nervous system (Fuxe, 1965; Lidov *et al.*, 1980) have been implicated in such diverse activities as nociception (Yaksh and Wilson, 1979), sleep (Jouvet and Pujol, 1974), aggression (Soubrie, 1986), sexual behavior (McIntosh and Barfield, 1984) and depression (Takahashi *et al.*, 1981).

Serotonin (5-hydroxytryptamine, 5-HT) is formed from the 5-hydroxylation of L-tryptophan by tryptophan 5-hydroxylase (EC 1.14.16.4), and the subsequent decarboxylation of 5-hydroxytryptamine by aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28). And serotonin is oxidatively deaminated to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO, EC 1.4.3.4), preferentially by type A MAO (Beck *et al.*, 1987). Various drugs altering serotonin synthesis and metabolism are now available (for review, see Fuller, 1985).

In the present study, to establish an *in vitro* system for studying serotonin synthesis and metabolism as well as for screening potential drugs or toxins that affect central serotonergic neurons, we cultured dissociated brainstem cells and monitored the serotonergic neuronal growth by measuring 5-HT and 5-HIAA contents in the cells with high performance

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liquid chromatography with electrochemical detection (HPLC-EC). We studied also the effects of various drugs on the contents of 5-HT and 5-HIAA, confirming *in vivo* reports.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium/F12 (DMEM/F12), poly-D-lysine HBr (mol. wt.> 300,000), cytosine arabinofuranoside, L-tryptophan, 5-hydroxytryptophan, 5-HT, 5-HIAA, 3,4-dihydroxybenzylamine (DHBA), *p*-chlorophenylalanine (PCPA), pargyline were obtained from Sigma Chemical Co. (St. Louis, MO); NSD 1015 (3-hydroxybenzylhydrazine HCl) from Aldrich Chemical Co. (Milwaukee, MI); fetal bovine serum from Gibco (Grand Island, NY); reserpine from Aju Pharmaceut. Co. (Seoul); LY-51641 was a generous gift of Eli Lilly Co. (Indianapolis, IN) and all other reagents were of analytical grade.

Cell cultures

The procedure for obtaining timed-mated pregnant rats (Sprague-Dawley, the Hallym University Animal Center) has been described in detail in Kim *et al.* (1989). At the gestational day 14.5-15.5, rats were sacrificed and brainstem (from hypothalamus to medulla oblongata) were dissected from embryos aseptically under the dissecting microscope.

After careful removal of the meninges, approximately 30-50 brainstems were rinsed with DMEM/F12 and transfered to the nylon mesh bag. Dissociated cells were obtained by gently pressing the bag with a glass rod in DMEM/F12. After 2 additional washes with DMEM/F12 by spinning at 500g for 3 min, the final pellet was resuspended in DMEM/F12 supplemented with 10% fetal bovine serum. The cells were plated at a density of 10^5 cells/mm^2 onto 35 mm dishes or 24-multiwell Corning plates previously coated with polylysine (25 μg/ml). The cells were maintained at 37°C, in a water-saturated 5% CO_2:95% air atmosphere. The culture was treated with 10μM cytosine arabinofuranoside for 24 h on the 3rd day to suppress any growth of glial cells. Thereafter medium was changed every 3 days. The cultures were routinely examined with Olympus inverted phase-contrast microscope.

Assay of 5-HT and 5-HIAA

5-HT and 5-HIAA were separated by high performance liquid chromatography and quantitated by a Waters electrochemical detector (Model 460) with glassy carbon electrode (Waters Assoc., Milford, MA) (Saller and Salama, 1984). In brief, to 39 vols. of a buffer consisting of 7 vols. of 0.1M monobasic sodium phosphate (adjusted to pH 4.0 using a saturated solution of citric acid) containing 1mM disodium EDTA and 1mM sodium octanesulfonic acid and 3 vols. of acetonitrile, one additional vol. of this buffer was added containing internal standard, DHBA (2μg/ml). The solution containing DHBA was stored at −20°C. Then, the cells in each dish were homogenized in 50μl of this solution and aliquots (5-10μl) of this homogenized solution were used for protein determination (Lowry *et al.*, 1951) and the rest was centrifuged at 15,000g for 30 min at 4°C. The supernatant (20μl) was injected immediately onto a 5μm, 15cm×4.6mm C_{18} Yung-in Pak column (Ginsco, Seoul) using U6K injector (Waters Assoc., Milford, MA, U.S.A.). To protect the column from sample contaminants, a column inlet filter (type 73XX; Rheodyne, Corati, Calif.) was placed between the column and the injector. As a mobile phase, 100 vols. 0.1M monobasic sodium phosphate containing 1mM disodium EDTA and 1mM sodium octanesulfonic acid were adjusted to pH 4.0 with a saturated citric acid solution, and mixed with acetonitrile (10 vols.). Mobile phase flow-rate was 1 ml/min and the oxidation potential 0.65V. Sample values were calculated relative to the peak height of the internal standard, DHBA.

RESULTS

Shown in Fig. 1 are chromatograms of standards and cell extracts. Peaks corresponding to DA, 5-HIAA, and 5-HT were easily observed in 12 min in the chromatogram from cell extracts. No additional peaks were observed beyond the 5-HT peak.

Developmental patterns of 5-HT and 5-HIAA contents in cultures

In cultures from embryonic age 14 day (E14) fetus, contents of both 5-HT and 5-HIAA increased progressively upto 14 days *in vitro* (Fig. 2). In contrast, dissociated culture of embryonic age 19 day (E19)-brainstem showed small amounts of 5-HT and

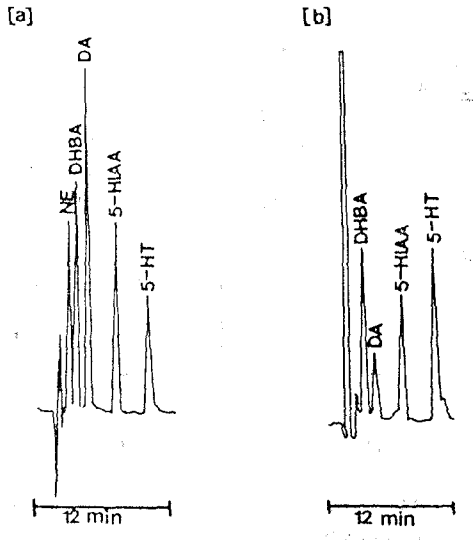

Fig. 1. Chromatograms of assay standards [a] and cultured cell extracts [b].

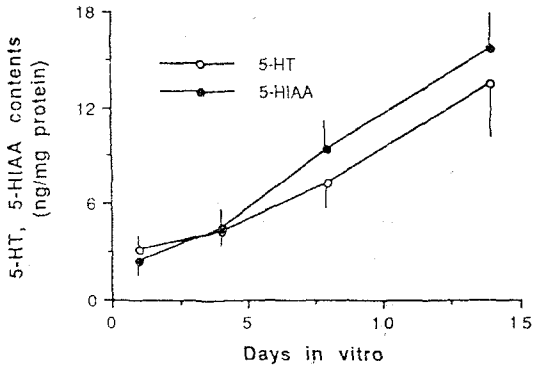

Fig. 2. Developmental patterns of 5-HT and 5-HIAA contents in cultures from embryonic age 14 day brainstem. Each point represents the mean ± SEM of four seperate cultures.

5-HIAA at the beginning of culture but as the culture-time proceeds the contents decreased.

After incubation of 14-day-old cultures from E14 fetus-brainstem with various drugs at the concentration of 0.1-1mM for 3 or 4 h, the 5-HT and 5-HIAA contents were measured (Fig. 3-6).

Effects of *p*-chlorophenylalanine (PCPA) on 5-HT and 5-HIAA contents

Incubation of cultures with 0.1-1mM PCPA, a

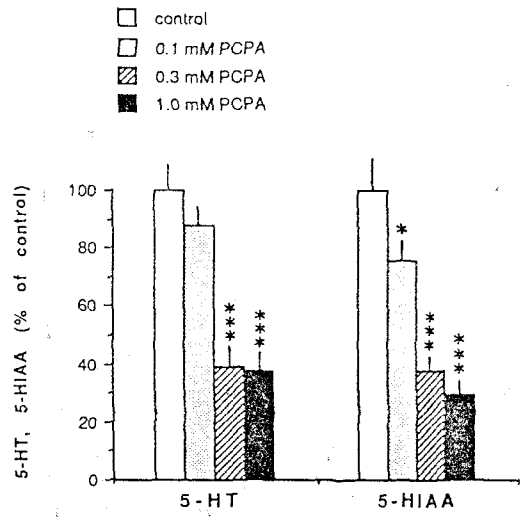

Fig. 3. Effects of *p*-chlorophenylalanine on 5-HT and 5-HIAA contents in cultures from embryonic age 14 day brainstem at 14 days *in vitro*. After incubation of cultures with *p*-chlorophenylalanine at the concentration of 0.1-1mM for 4 h, 5-HT and 5-HIAA contents were measured. Each value represents the mean ± SEM of four seperate cultures. Control values are 5.0 ± 1.5 ng/well for 5-HT content, and 2.2 ± 0.8 ng/well for 5-HIAA content. *t*-Statistical test with respect to controls: * $p<0.05$; *** $p<0.001$.

specific tryptophan 5-hydroxylase inhibitor, for 4 h dose-dependently decreased 5-HT and 5-HIAA contents (Fig. 3). After incubation of cultures with 0.1mM NSD 1015, an AADC inhibitor for 4 h, the 5-HT and 5-HIAA contents tended to decrease but the extents of decrease were not statistically significant.

Effects of tryptophan and 5-hydroxytryptophan on 5-HT and 5-HIAA contents

Incubation of cultures with 0.1mM tryptophan, which is the upper limit for physiologic concentration of tryptophan in the rat brain (Tyce *et al.*, 1964) for 3 h, increased 5-HT and 5-HIAA contents by 111% and 132%, respectively, over control levels with the increases largely blocked in the presence of 0.1mM PCPA (Fig. 4). Incubation of cultures with 0.1mM 5-hydroxytryptophan, the immediate precursor of 5-HT for 3 h, increased 5-HT and 5-HIAA contents to 6 and 12 fold, respectively, over control levels with the increases almost completely blocked

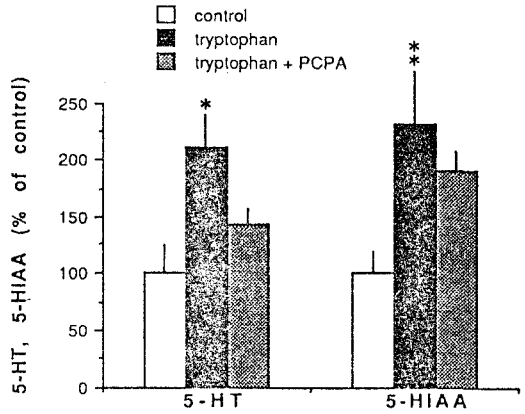

Fig. 4. Effects of tryptophan on 5-HT and 5-HIAA contents in cultures from embryonic age 14 day brainstem at 14 days *in vitro*. After incubation of cultures with 0.1mM tryptophan with or without 0.1mM *p*-chlorophenylalanine for 3 h, 5-HT and 5-HIAA contents were measured. Each value represents the mean ± SEM of four seperate cultures. Control values are 3.2 ± 0.6 ng/well for 5-HT content, and 0.5 ± 0.1 ng/well for 5-HIAA content. *t*-Statistical test with respect to controls: * $p<0.05$; ** $p<0.01$.

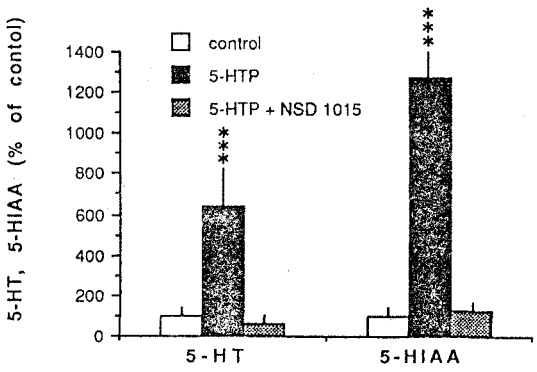

Fig. 5. Effects of 5-hydroxytryptophan on 5-HT and 5-HIAA contents in cultures from embryonic age 14 day brainstem at 14 days *in vitro*. After incubation of cultures with 0.1mM 5-hydroxytryptophan with or without 0.1mM NSD 1015 for 3 h, 5-HT and 5-HIAA contents were measured. Each value represents the mean ± SEM of four seperate cultures. Control values are 2.8 ± 0.6 ng/well for 5-HT content, and 7.5 ± 0.6 ng/well for 5-HIAA content. *t*-Statistical test with respect to controls: *** $p<0.001$.

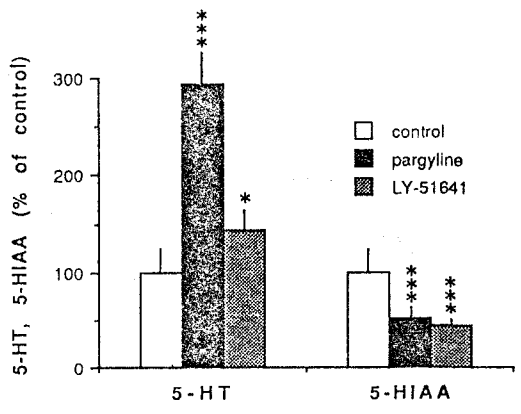

Fig. 6. Effects of pargyline and LY-51641 on 5-HT and 5-HIAA contents in cultures from embryonic age 14 day brainstem at 14 days *in vitro*. After incubation of cultures with 0.1mM pargyline or 0.1mM LY-51641 for 3 h, 5-HT and 5-HIAA contents were measured. Each value represents the mean ± SEM of four seperate cultures. Control values are 5.1 ± 1.0 ng/well for 5-HT content, and 2.9 ± 0.9 ng/well for 5-HIAA content. *t*-Statistical test with respect to controls: * $p<0.05$; *** $p<0.001$.

in the presence of 0.1mM NSD 1015 (Fig. 5).

Effects of reserpine, pargyline, and LY-51641 on 5-HT and 5-HIAA contents

Incubation of cultures with 0.1mM reserpine, a depletor of catecholamines and 5-HT for 3 h, decreased the 5-HT content to 18% of the control level with no effect on 5-HIAA content. Incubation of cultures with 0.1mM pargyline, a nonspecific MAO inhibitor for 3 h, increased 5-HT content to 3 fold and decreased 5-HIAA content to 50% of the control levels. A similar but less effect was obtained by incubation with 0.1mM LY-51641, a specific type A MAO inhibitor for 3 h (Fig. 6). The 5-HT turnover rate of about 17 pmol/mg protein/h was estimated from the accumulation of 5-HT by inhibition of MAO with 1mM pargyline on the assumptions that MAO was completely inhibited by 1mM pargyline, that the rate of 5-HT synthesis was not slowed down by the accumulation of 5-HT, that all accumulated 5-HT was synthesized in the cells, and that pargyline produced a linear increase of 5-HT (Neff and Tozer, 1968).

DISCUSSION

The first dissociated culture of central serotonergic neurons was from embryonic (E14-15) rat brainstem (Yamamoto *et al.*, 1981). These cells were shown to synthesize ^{3}H-serotonin from ^{3}H-tryptophan and release ^{3}H-serotonin. Azmitia and Whitaker-Azmitia (1987) used ^{3}H-serotonin uptake as a quantitative index of maturation of dissociated raphe cells. Our study indicates that endogenous 5-HT and 5-HIAA levels in the cells measured with HPLC-EC method are also useful biochemical parameters of serotonergic neuronal maturation in culture. This HPLC-EC method allows simultaneous dectection of 5-HT and 5-HIAA in only 12 min with each sample. The predictable changes in 5-HT and 5-HIAA levels by various drugs altering serotonin synthesis and metabolism (Fig. 3-6) indicate that the most portions of 5-HT and 5-HIAA were made in the cultured cells instead of uptake of 5-HT and 5-HIAA from the culture media containing 10% fetal bovine serum.

The 5-HT content (13 ng/mg protein) and 5-HT turnover rate (17 pmol/mg protein/h) at 14 days *in vitro* are in good agreement with those reported for the adult rate brain (Bogdanski *et al.*, 1975; Smith *et al.*, 1978). As *in vivo*, the 5-HT content was more easily depleted with PCPA, a tryptophan hydroxylase inhibitor than with NSD 1015, an AADC inhibitor in our *in vitro* system (Fig. 3). The fact that incubation with tryptophan or 5-hydroxytryptophan increased the rate of serotonin formation (Fig. 4&5) implys that neither tryptophan hydroxylase nor AADC is saturated with its amino acid substrate in this *in vitro* system, confirming *in vivo* report (Moir and Eccleston, 1968). The 5-HT content was depleted by reserpine. The 5-HT and 5-HIAA contents were increased and decreased, respectively, by MAO inhibitors (Fig. 6). All the above results indicate that the biochemical properties of the central serotonergic neurons in this culture system reflect reliably those of central serotonergic neurons *in vivo*. And we suggest that measuring the 5-HT and 5-HIAA contents in the primary cultured dissociated brainstem cells with HPLC-EC is useful in studying serotonin synthesis and metabolism as well as in screening potential drugs or toxins that affect the central serotonergic neurons.

REFERENCES

Azmitia EC and Whitaker-Azmitia PM: *Target cell stimulation of dissociated serotonergic neurons in culture. Neuroscience 20(1):47-63, 1987*

Beck O, Lundman A and Jonsson G: *5-Hydroxytryptophol and 5-hydroxyindoleacetic acid levels in rat brain: effects of various drugs affecting serotonergic transmitter mechanisms. J Neural Transm 69:287-298, 1987*

Bogdanski DF, Weissbach H and Udenfriend S: *The distribution of serotonin, 5-hydroxytryptophan decarboxylase and monoamine oxidase in brain. J Neurochem 1:227-228, 1957*

Dahlstrom A and Fuxe K: *Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in cell bodies of brain neurons. Acta Physiol Scan 62(Suppl):1-55, 1964*

Fuller RW: *Drugs altering serotonin synthesis and metabolism. In Neuropharmacology of Serotonin (ed. Green AR) P. 1-20. Oxford University Press, Oxford, 1985*

Fuxe K: *Evidence for the existence of monoamine neurons in the central nervous system. IV. Distribution of monoamine terminals in the central nervous system. Acta Physiol Scan 64(supp 247):39-84, 1965*

Jouvet M and Pujol JF: *Effcts of central alterations of serotonergic neurons upon the sleep-waking cycle. Adv Biochem Psychopharmac 11:199-209, 1974*

Kim YH, Song DK, Wie MB, Suh YH and Park CW: *Ontogeny of phenylethanolamine-N-methyltransferase catalytic activity in primary neuronal culture derived from the embryonic rat brainstem. Korean J Pharmacol 25:157-162, 1989*

Lidov HGW, Grzanna R and Molliver ME: *The serotonin innervation of the cerebral cortex in the rat-an immunocytochemical analysis. Neuroscience 5:207-227, 1980*

Lowry OH, Rosebrough NJ, Farr AR and Randall RJ: *Protein measurement with the Folin phenol reagents. J Biol Chem 193:265-275, 1951*

McIntosh TK and Barfield RJ: *Brain monoaminergic control of male reproductive behavior. Behav Brain Res 12:255-281, 1984*

Moir ATB and Eccleston D: *The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles. J Neurochem 15:1093-1108, 1968*

Neff NH and Tozer TN: *In vivo measurement of brain serotonin turnover. Adv Pharmacol 6A:97-109, 1968*

Saller CF and Salama AI: *Rapid automated analysis of biogenic amines and their metabolites using reversed-phase high-performance liquid chromatography with electrchemical detection. J Chromatogr 309:287-298, 1984*

Smith JE, Co C and Lane JD: *Turnover rates of serotonin, norepinephrine and dopamine measured in seven rat*

brain regions. Prog Neuropsycopharmacol 2:359-367, 1978

Soubrie P: *Reconciling the role of serotonin neurons in human and animal behavior. Behav Brain Sci 9:319-363, 1986*

Takahashi R, Tateishi T, Yoshida H, Narayama H and Tachiki KH: *Serotonin metabolism of animal model of depression. Adv Exp Med Biol 133:603-625, 1981*

Tyce GM, Flock EV and Owen CA: *Tryptophan metabolism in the brain of the developing rat. In Himwith WA and Himwith HE (eds.) The developing brain. Prog Brain Res 9:198-203, Elsevier, Amsterdam, 1964*

Yaksh TL and Wilson PR: *Spinal serotonin terminal system mediates antinociception. J Pharm Exp Therap 208:446-453, 1979*

Yamamoto M, Steibusch HWM and Jessell TM: *Differentiated properties of identified serotonin neurons in dissociated cultures of embryonic rat brain stem. J Cell Biol 91:142-154, 1981*

=국문초록=

흰쥐 태아 뇌간의 일차 세포배양에서 Serotonin의 합성 및 대사에 대한 연구

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김영희 · 송동근 · 위명복 · 송준호 · 최연식

본 연구에서는 흰쥐 태아 뇌간의 일차 세포배양을 이용하여 중추 serotonin(5-HT)신경세포의 연구에 적합한 *in vitro* 모델을 확립하고, 여기에서 5-HT 대사의 전반적인 과정을 살펴보고자 하였다. 배양세포의 5-HT 및 5-hydroxyindoleacetic acid (5-HIAA)함량을 high performance liquid chromatography-electrochemical detection (HPLC-EC)방법으로 측정함으로써 흰쥐 태아(태령 14-15일)뇌간으로 부터 얻은 5-HT 신경세포가 배양상에서 2주까지 발달함을 추적할 수 있었고 아울러 이들 세포에서 5-HT의 합성, 저장, 대사의 각 과정을 몇가지 약물들을 이용하여 확인하였다. 배양 14일에 5-HT의 함량이 13ng/mg protein으로서 성숙한 흰쥐 뇌속에 존재하는 5-HT의 값과 유사하였다. 5-HT 합성의 속도조절효소인 tryptophan hydroxylase(TPH)의 상경적 억제제인 p-chlorophenylalanine (PCPA)처리시 aromatic L-amino acid decarboxylase (AADC)억제제인 3-hydroxybenzylhydrazine NSD 1015보다 5-HT 및 5-HIAA 함량을 더 많이 감소시켰다. TPH의 기질인 tryptophan은 5-HT의 합성을 현저히 (200%) 증가시켰으며 이 증가는 PCPA로 상당히 둔화되었다. 5-HIAA의 변화도 유사한 양상을 보였다. 5-hydroxytryptophan처리시 5-HT 및 5-HIAA 함량이 현저히 증가하였으며 이 증가는 NSD 1015로 거의 차단되었다. 5-HT 대사 경로인 monoamine oxidase (MAO)의 비특이적 억제제인 pargyline과 MAO A의 특이적 억제제인 LY-51641을 처리한 결과 5-HT 함량은 각각 대조군의 295% 및 140%로 증가하여 pargyline이 LY-51641보다 현저한 작용을 나타내었다. 그러나 5-HIAA의 함량은 반대로 현저히 감소하여 pargyline 및 LY-51641에 의해서 각각 대조군의 50% 및 40%의 함량을 나타내었다.

이상의 결과로 보아 5-HT 신경세포는 일차 세포배양상에서 활발한 5-HT대사가 이루어짐을 HPLC-EC 방법으로 확인할 수 있었으며 따라서 본 *in vitro* system은 중추 5-HT 신경세포에 대한 전반적인 약리 및 독성 연구에 매우 유용하게 사용될 수 있다고 사료된다.