行政院國家科學委員會專題研究計畫　成果報告

花生四烯酸促進神經生長因子所引發之腎上腺親鉻母細胞瘤細胞株神經分化及其分子機制之研究

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-038-044-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：臺北醫學大學小兒科

計畫主持人：陳怡如

計畫參與人員：陳怡如，葉健全，賴音妤，劉大榮.

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 12 月 24 日

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

花生四烯酸促進神經生長因子所引發之腎上腺親鉻母細胞瘤細胞株神經分化及其分子機制之研究

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 91－ 2320－ B－ 038－ 044－
執行期間： 91 年 8 月 1 日至 92 年 7 月 31 日

計畫主持人：陳怡如
共同主持人：葉健全
計畫參與人：陳怡如, 葉健全, 賴音妤, 劉大榮.

成果報告類型(依經費核定清單規定繳交)：■精簡報告 □完整報告

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執行單位：臺北醫學大學醫學系小兒學科

中 華 民 國 92 年 10 月 30 日

花生四烯酸促進神經生長因子所引發之腎上腺親鉻母細胞瘤細胞株神經分化及其分子機制之研究

中文摘要：

花生四烯酸（AA）是多元不飽和脂肪酸的一種，會因各種外界的刺激或荷爾蒙的作用而由細胞膜中釋放出來，作為二級訊息傳遞物質。在中樞神經系統中，雖然腦部缺血缺氧時所釋放出的高濃度 AA 會造成細胞死亡，許多證據也顯示 AA 在神經系統的發育上，扮演著很重要的角色。本研究中發現，低濃度的 AA（5 μM）能顯著地促進神經生長激素 NGF 所引發的神經分化。此外，利用免疫化學染色法，我們證實以神經生長因子處理 PC12 細胞，能明顯提高膽鹼乙醯轉移酶和酪胺酸氫化酶的表現；而低濃度 AA 能近一步提高膽鹼乙醯轉移酶的表現，但對於酪胺酸氫化酶的表現則沒有顯著的影響。

Abstract:

Arachidonic acid (AA) is one of the polyunsaturated fatty acids, it can be released from the cell membrane phospholipid and serves as second messengers in response to extracellular stimuli or growth factors. In central nervous system, although high concentration of AA released during brain ischemia or hypoxia could induce cell death, evidences had also indicated the involvement of AA in the development of nerve system. In this study, we demonstrated that low concentration (5 μM) of AA can enhance the nerve growth factor (NGF)- induced neurite outgrowth in PC12 cells. Besides, using immunocytochemistry, we demonstrated that both NGF treatment increase the expression of both choline acetyltransferase and tyrosine hydroxylase, and that low concentration of AA can further enhance the expression of choline acetyltransferase (ChAT), but not tyrosine hydroxylase. Therefore, NGF can induce PC12 cells to differentiate toward both dopaminergic and cholinergic neuron.

(一)報告封面：請至本會網站（http：//www.nsc.gov.tw）下載製作（格式如附件一）。

(二)中、英文摘要及關鍵詞(keywords)。

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發現或其他有關價值等，作一綜合評估。

Introduction:

Arachidonic acid (AA) is one of the polyunsaturated fatty acids, which are structural compartment of membrane phospholipids. In response to extracellular stimuli or growth factors, AA can be released from the cell membrane phospholipid by phospholipase A2 or sequentially by phospholipase C (to relese diacylglycerol, DAG) and DAG lipase, serving as second messengers. AA is metabolized through three major pathways, namely cyclooxygenase, lipoxygenase and epoxygenase pathways, producing prostaglandin or thromboxane, leukotrienes, and epoxides, respectively (refer to [6] for review). AA can also be converted to anandamide, a natural ligand for cannabinoid receptor [7, 8]. Besides, AA can activate protein kinase C (PKC) [9, 10], and potentiate the NMDA receptor current [11]. In central nervous system, although high concentration of AA released from cellular membrane during brain ischemia or hypoxia is toxic and could induce cell death [12, 13], evidences had also indicated the involvement of AA in the development of nerve system. It had been reported that the contents of AA and other polyunsaturated fatty acids were changed in the nerve growth cone during the synaptogenesis [14]. Besides, nerve growth factor (NGF) increases the release of AA [15] and the capacity to metabolize AA [16] in PC12 cells, which may be important in nerve fiber growth. The release of AA is also a major step in the signaling pathway of fibroblast growth factors (FGF)- induced neurite outgrowth in cerebellar neurons [17] and cell adhesion molecules (CAMs)- stimulated neurites outgrowth [18]. Exogenously added AA could directly induced the FGF- or CAMs- induced neurite extension (1 to 100 贡M AA) [17, 18], significantly supported the survival and promoted the neurite elongation in cultured hippocampal neurons (1 贡M AA) [19], or enhance the neurite outgrowth in NG108-15 neural hybrid cells (1 贡M AA) [20]. Accordingly, AA, at certain concentration, shall have positive effect on the differentiation of neuronal cells, yet the optimal AA concentration required for promoting neuronal differentiation might vary between cell types.

PC12 is a rat pheochormocytoma cell line; it can undergo neuronal differentiation in response to NGF and was hence widely used to study the mechanism of neuronal differentiation. Dehaut et.al [21] reported that AA between 1 to 100 贡M increase the length of neurite in PC12 cells at 7 day after NGF treatment, and in contrary, Ikemoto et al. [22] demonstrated suppression of NGF-induced neurite outgrowth by 60 贡M AA in PC12. It should be noted that the concentration of AA used in these study might be cytotoxic [23], and only single time point [21] or single high concentration of AA (60 贡M) [22] was used in these studies. In this study, we demonstrated that 5 贡M of AA can significantly increase the length of neurite in NGF- treated PC12 cells. Besides, NGF treatment in PC12 cells can significantly increase the expression of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH).

Material and Methods:

Cell culture:

PC12 cells will be plated on petri dishes pre-coated with 10 贡g/ml poly-D-lysin, and cultured in Dulbecco's modified Eagle's medium (DMEM) with 7.5 % fetal bovine serum (FBS), 7.5 % horse serum, 100 U/ml penicillin and 100 ng/ml streptomycin at 37°C in

humidified CO2 incubator. For NGF/AA treatment, the cells will be cultured in the above media plus 50 ng/ml of NGF (NGF) or 50 ng/ml of NGF plus 1 or 5 贡M AA (NGF/AA), and the media will be replaced daily. To block the metabolism or action of AA, one or several of the following inhibitors: 1mM aspirin, 1 贡M miconazole, 10 贡M NDGA, 50 贡M H-7, 20 贡M H-89, 0.5 贡M calphostin C, 10 贡M TCP, or 10 贡M AM281, will be added 2 hours prior to the addition of AA.

Evaluation of PC12 cell differentiation:

Three parameters, including percentage of differentiated cell, average neurite length and choline acetyltransferase (ChAT) activity, will be used to evaluate the extend of neuronal differentiation PC12. At various days during NGF/AA treatment, photography of the cells will be taken from three pre-marked areas of the petri dishes under the light microscope, and the percentage of differentiated cells and the average neurite length per cell are measure from these photographs. The differentiated cell is defined as cells which had extended neurites. The percentage of differentiated cells are calculated by dividing the number of differentiated cells by the number of total cells, and the average neurite length is calculated by dividing total neurite length by the number of differentiated cells.

Immunocytochemistry:

The PC12 cells will be cultured in 6-well plates containing poly-D-lysine coated coverslide, with cell density about 3×10^5 cells/well and subjected to treatment of NGF and AA. After various days, the cover slides will be taken out, rinsed with PBS, and fixed with 4% paraformaldyhyde(PFA)for 30 minutes. The slides will be incubated for 10 minutes in 1ml of 0.3 % H_20_2, then blocked with 200 μl of blocking buffer (1.5- 3 % rabbit or goat serum) for 1 hour. After incubating in 200μl of anti- ChAT or TH当 antibodies (1/200 dilution in blocking buffer) for 2 hours, and in 200μl of biotinylated secondary antibody (1/200 dilution in blocking buffer) for 1 hour, 200μl of ABC reagent (2 drops of reagent A and 2 drops of reagent B added in 5ml of PBS, prepared 30 minutes before use) will be added and incubated for another 1 hour. The slides will then be stained with DAB solution (DAB chromogen 20μl added to 1 ml buffered substrate), then dehydrated subsequently with 50%, 75%, 80%, 85%, 90%, 95%, 100% Ethanol and xylene. Between each treatment, the slides should be rinsed 3 times with PBS.

Results and discussion:

As shown in figure 1, our results demonstated that in NGF- treated PC12 cells, 5 μM of AA can significantly increase the length of neurites at day 5, but the difference diminished at day 7, indicating that at earlier stage, AA may accelerate the elongation of neurites induced by NGF, however, gradually caught up by cells treated with NGF only. Immunocytostaining of the NGF-induced differentiated PC12 cells with ChAT or TH antibodies showed different level of staining (figure 2). 1 or 5 μM AA treatment significantly increase the number of medium level statining cells at day 5 (figure 3A), and low level ChAT staining in undifferentiated cells at day 7 (figure 3B). The staining of TH, on the other hand, is not significantly affected by AA treatment (figure 4).

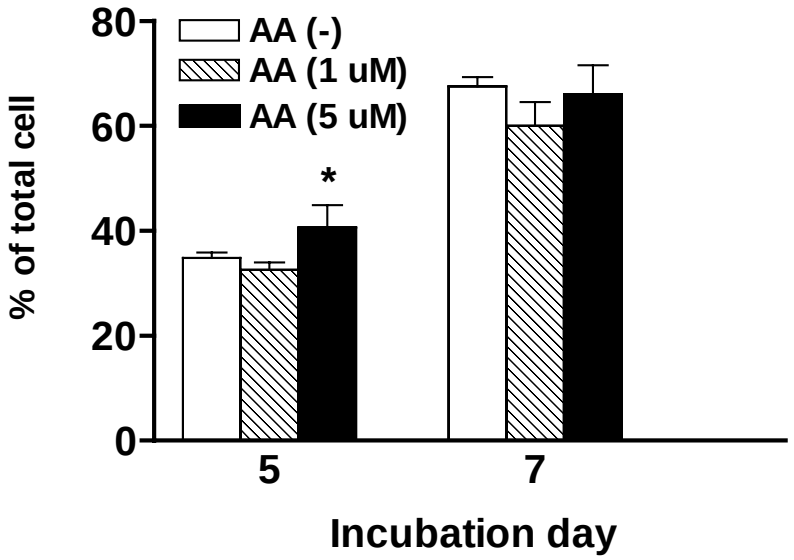


Figure1: The effect of low concentration AA on the differentiation of NGF- treated PC12 cells at 5 and 7 days after treatment.

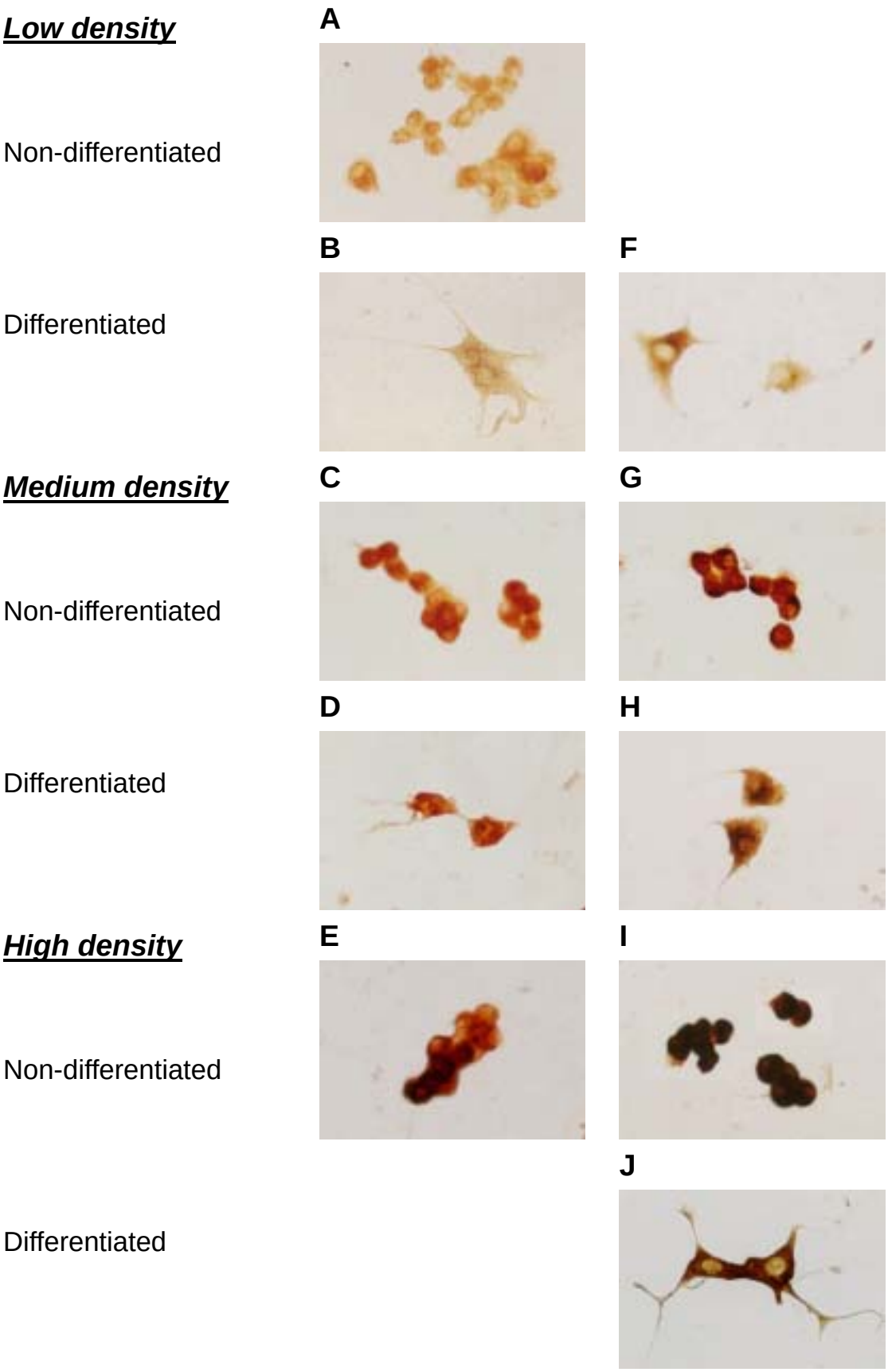
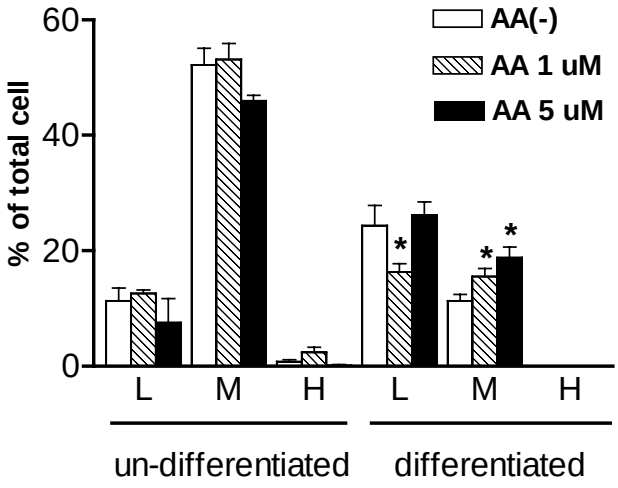


Figure 2: Immunocytostaining of NGF-treated PC12 cells with antibodies against **(A~E)** choline acetyltransferase or **(F~J)** tyrosine hydroxylase. **A, B, F** represent cells with low density staining; **C, D, G, H** represent cells with medium density of staining; **E, I, J** represent high density of staining.

(A)



(B)

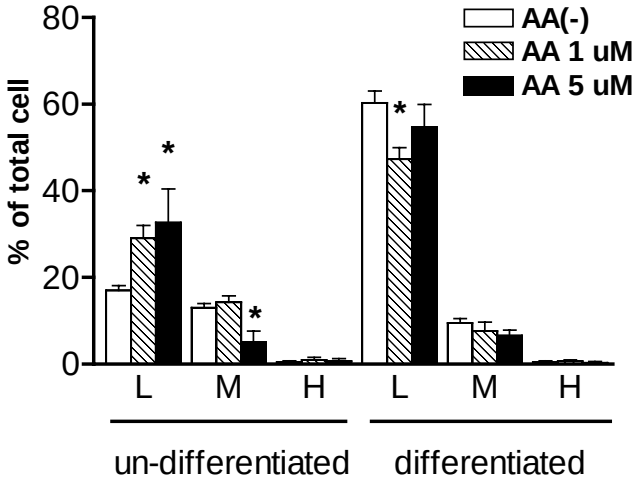
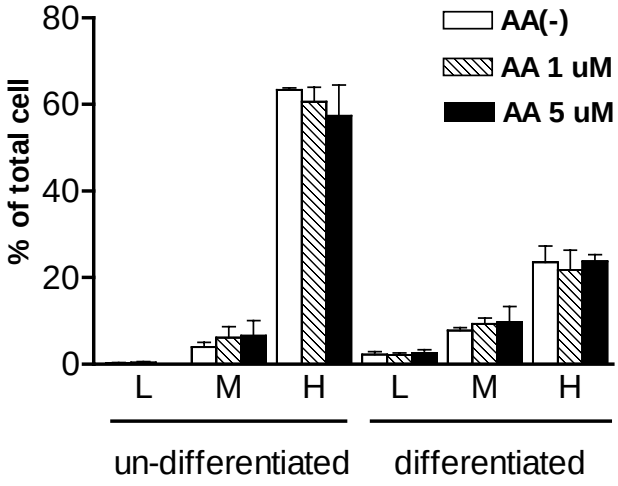


Figure 3: The effect of low concentration AA on the expression of choline acetyltransferase in PC12 cells at **(A)** 5 day and **(B)** 7 days of NGF/ AA treatment. **L**：low density；**M**：medium density；**H**：high density. Data are mean±SEM of three experiments.
***** $p < 0.05$, one-way ANOVA with paired t test

(A)



(B)

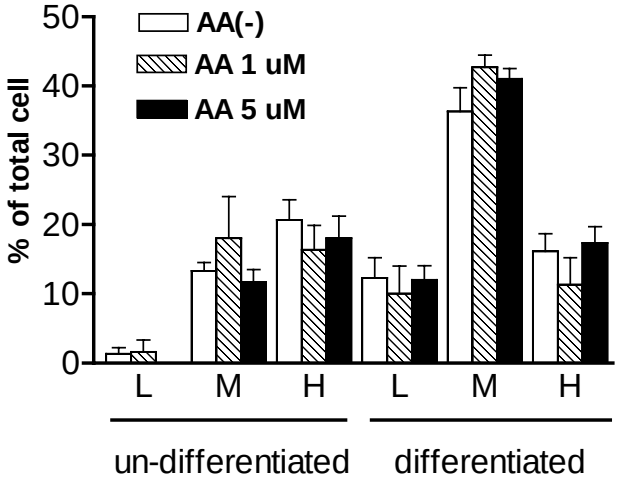


Figure 4: The effect of low concentration AA on the expression of tyrosine hydroxylase in PC12 cells at **(A)** 5 day and **(B)** 7 days of NGF/ AA treatment. **L**：low density；**M**：medium density；**H**：high density. Data are mean±SEM of three experiments.