行政院國家科學委員會專題研究計畫　成果報告

利用基因微陣列來探討增強記憶中藥之基因表現與分子訊息傳導

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Using microarry to explore the gene expression and molecular signal transduction of Chinese cognitive-enhancing herbs

計畫編號：NSC 91-2320-B-039-029
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一、中文摘要

乙醯膽鹼神經系統在學習記憶方面扮演著重要的角色，在相關的研究中已證實毒蕈素接受體作用劑，確能改善學習記憶障礙。為了探討乙醯膽鹼神經在學習記憶中涉及的分子層次機轉，本實驗應用 Cdna 微陣列之技術，經由乙醯膽鹼酯酶阻斷劑 scopolamine 所誘發學習記憶障礙模式，研究其對大鼠海馬回中表現異同之基因。

經由腦室投予 scopolamine 30 分鐘後，立即取其海馬回，萃取 mRNA 進行反轉錄，再與 microchip 雜交(hibridization)，利用此結果來比較投予 scopolamine 與投予生理食鹽水二者之基因表現差異。結果發現，有 42 個基因表現具有差異性，而這些具差異性的基因，再經由半定量的方式以老鼠的引子(primer)作 RT-PCR(reverse transcriptase polymerase chain reaction)來加以確認，結果有 28 個基因是正確無誤的。而這些基因的表現反映出經由 scopolamine 誘發學習記憶障礙之細胞分子訊息的傳遞；我們依照其功能，將 28 基因分為三大群，除了與毒蕈素接受體訊息調控機制相關的基因外，亦有與 Alzheimer's Disease 相關聯的基因,及功能不明確的基因存在。

本研究的成果應能為 scopolamine 所誘發學習記憶障礙模式，提供分子層次的研究依據，以及藉由此分子訊息來開發中草藥之智能增進劑。

關鍵詞：乙醯膽鹼、學習記憶、cDNA 微陣列、RT-PCR

Abstract

The molecular study of learning and memory was concentrated at muscarinic acetylcholine receptors and their associating signaling molecules. To explore the alternative pathways we applied human cDNA microarray and searched for differentially expressed genes in the hippocampus of scopolamine-treated rat. Thirty minutes after scopolamine treatment, rats displayed typical memory impairment. Interspecies hybridization using human cDNA microarray to analyze scopolamine-treated rat hippocampus exhibited a minor difference for the expression profile compared to normal control. Forty-two genes were selected by microarray based on the differential expression ratios. Twenty-eight genes were validated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) using primer pairs against rat orthologs. The broad spectrum of the differentially expressed genes indicated an overall cellular response upon scopolamine treatment. In addition to genes associated with muscarinic receptor signaling pathways, we have disclosed genes associated with novel pathways such as apoptosis, cytoskeleton reconstruction, protein trafficking, cell differentiation, and genes

without a clear role. Our result should provide an insight to the molecular study of scopolamine-induced memory impairment, and development of cognition-enhancing Chinese Herbs.

Keywords: Acetylcholine, Learning and memory, cDNA Microarray, RT-PCR

二、緣由與目的

在人類或動物，膽鹼神經系統在學習記憶性都扮演著重要的角色[1-3]。Scopolamine 是一個非選擇性的毒蕈素接受體阻斷劑，能夠造成學習與短期記憶障礙，並且被應用來當成抗痴呆藥物的篩選[4]。投與 Scopolamine 後會造成海馬回乙醯膽鹼濃度的下降，其造成學習記憶障礙涉及的神經化學機轉可能是經由阻斷毒蕈素接受體造成中樞膽鹼神經活性下降的結果[5-9]。所以本研究利用 Scopolamine 來作為中藥改善學習記憶障礙的篩選，藉此希望能找出有效的中藥智能增進劑。

現今已開發之智力增進劑，主要分成七大類：nootropics、vasodilators 及 metabolic enhancers、psychostimulants、cholinergic agents、biogenic amines drugs 及 neuropeptides 等[10]；其中已於臨床使用者為 vasodilators 及 metabolic enhancers 之藥物如 donepezil、dihydroergotoxine、pentofylline；cholinergic agents 之藥物如 tacrine、及 nootropics 類藥物如：piracetam、nefiracetam 等，此類藥物於 1972 年由 Giurgea 提出，主要作用在活化腦神經、促進腦內資訊聯匯及增強學習記憶，但相關開發藥物仍於動物試驗或臨床試驗階段。而自天然物開發智力增進劑上，已研發之藥物包括 huperzine A（臨床試驗階段）、gypenosides 等[11]，而其他中藥如人參、黨參、銀杏等亦發現具有智力增進之作用[12]；因此，從中藥開發智力增進劑，頗值得我們努力與期待。本研究計畫擬延續本研究室數年來所持續建立的研究模式、方向及成果：從行為藥理學、中樞神經傳遞物質乃至細胞內訊息傳遞及利用 cDNA Microarray 的技術探討核內基因表達[13-17]，藉此來研究中藥及其成分改善學習記憶障礙之作用機制。

三、結果與討論

Expression profiling for the hippocampus of scopolamine-treated rat

腦室投予scopolamine 30分鐘後，萃取其海馬回mRNA反轉錄成cDNA, 經過雜交、顯色後，由軟體ScanAlyze 進行分析判讀，得到基因表現值，與投予saline之組別進行比較，得到一表現比值(ratio) （如fig.1）。由圖可知，經由scopolamine誘發後，大部分的基因分布在45度位置，表示scopolamine組與 saline組相比絕大多數的基因並無變化，經過統計，超過95% 以上的基因表現比值介於0.66~1.3，而大於1.5或小於0.5者不超過1%。我們挑選曲線上方表現值增加及曲線下方表現值低落的基因各1%，排除ESTs以及一些未知的基因得到48個與scopolamine誘導後具有相關基因。

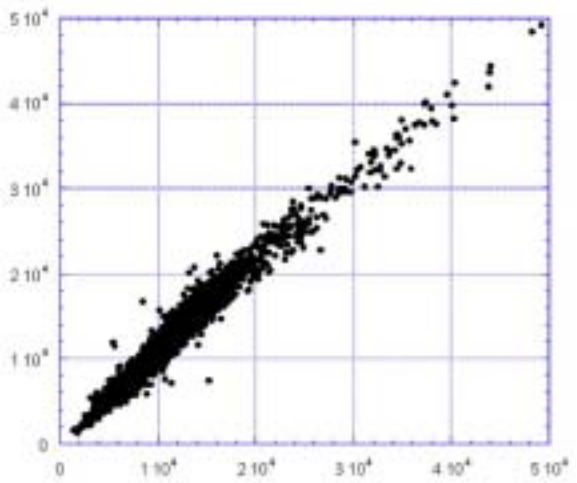

Fig. 1 Scatter plot of microarray dataset. Gene expression signal of scopolamine-treated rat hippocampus (Y-axis) was plotted against counterpart signal of control group (X-axis). The expression data was the average of three independent experiments. Unit for expression levels was obtained from image integration by Scanalyz and should be treated as referenced unit.

Validation of microarray datasets by semi-quantitative RT-PCR

為了確認基因晶片所得的結果是否無誤，我們以RT-PCR的技術來加以證明。實驗中以ribosomal protein L21 和L18來作標準對照，經由電泳跑膠得到28個RT-PCR的產物，如Fig. 2, 而這些表現差異的基因列於table 1。

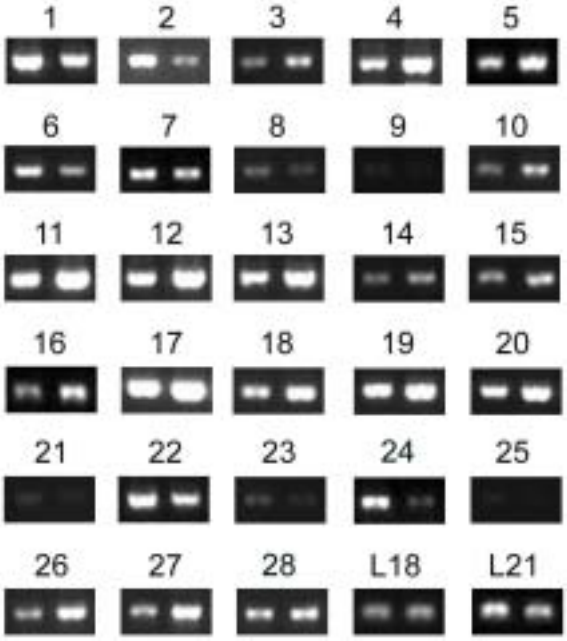

Fig. 4 Gel electrophoresis of the RT-PCR products of selected genes. Number above each gel slice corresponds to gene number in Table 1. In each gel slice, left lane is RT-PCR product from scopolamine-treated rat hippocampus, while right lane represents control rat. RT-PCR of ribosomal proteins L21 and L18 were performed as internal controls.

Differentially expressed genes indicated alternative pathways

經由 scopolamine 誘發之學習記憶障礙，在老鼠海馬回的基因表現改變涉及許多路徑（如 table 1），其中包括毒蕈素接受體訊號傳遞的基因、 Alzheimer's Disease 相關的基因，如 amyloid, protein tau, glutamine synthetase, presenilins, 我們亦發現可能與學習記憶障礙相關且尚未報導過的基因。

Muscarinic receptor signal transduction

毒蕈素m1, m3, m5接受體，訊息傳遞路徑可能是由於IP3接受體過分表現所致，而毒蕈素m2, m4接受體是與Ras-like GTPase superfamily有密不可分的關係且受cAMP相關性的蛋白質所影響，如Epac1 (cAMP-GEFI) 和 RagA；在本實驗中，經由scopolamine誘發之空間性記憶障礙可能經由毒蕈素接受體m1活化L-type的鈣離子通道有關。

我們也發現 Arginase 的還原作用、cytochrome c oxydase 的誘導與 glutamine synthetase 的過表現，可能與粒線體發生障礙或者是 NO 自由基產生有關。至於，programmed cell death 4 (PDCD4), MAP-kinase activating death domain (MADD), endothelial differentiation sphingolipid G-protein-coupled receptor 1, and eukaryotic translation initiation factor-2, 這幾個為促使 MAP kinase 活化的基因。

Alzheimer's disease

在 Alzheimer's Disease 的病人身上，可能會發現 amyloid-beta protein (A beta), presenilin 2, and microtubule-associated protein tau, 以及和 amyloid-beta 分泌有關的 ATP-binding cassette (ABC) transporter, 這幾個基因發生變化，在本研究中也是如此。許多報導指出，Alzheimer's Disease 的病人，學習能力低落、記憶障礙的可能原因為中樞乙醯膽鹼神經活性降低，其中又以毒蕈素 m2, m4 接受體的變化最為明顯，所以毒蕈素接受體作用劑被認為是有效的治療藥物；但是針對 Alzheimer's Disease 變異的基因加以治療可能才是未來的趨勢。

Table 1 Differentially expressed genes affected by scopolamine-treatment

Gene number	Name	Folds expression
	Alzheimer's disease	
1	microtubule-associated protein tau	0.5*
2	presenilin 2 (Alzheimer disease 4)	0.1
3	ATP-binding cassette, sub-family G,member 1 (Abcg1)	4.2
4	amyloid precursor-like protein 2	3.5
5	amyloid beta (A4) precursor protein-binding, family A, member 1 (X11)	5.2
	Muscarinic receptor	
6	protein kinase, interferon-inducible doublstranded RNA dependent	0.3
7	arginase type II	0.6
8	cAMP-regulated guanine nucleotide exchange factorI (cAMP-GEFI)	0.2
9	ATP-binding cassette, sub-family B (MDR/TAP), member 1	0.1
10	calcium channel, voltage-dependent, L type, alpha1D subunit	5.8
11	cytochrome c oxidase subunit Vb	2.1
12	endothelial differentiation sphingolipid G-protein-coupled receptor 1	2.2
13	ras-related GTP-binding protein ragA	2.3
14	MAP-kinase activating death domain	2.0
15	programmed cell death 4	2.5
16	testis-specific heat shock protein-related gene hst70	2.9
17	glutamine synthetase	1.8
18	inositol 1,4,5-triphosphate receptor	2.2
19	protein tyrosine phosphatase, non-receptor type substrate 1	1.9
20	eukaryotic translation initiation factor 2, subunit 1	2.1
	Other	
21	coagulation factor C homolog (Limulus polyphemus)	0.3
22	phosphoglycerate mutase 1	0.5
23	Burkitt lymphoma receptor 1	0.4
24	glypican 3	0.2
25	epidermal growth factor receptor	0.2
26	coronin, actin binding protein 1B	3.2
27	secretory carrier membrane protein 2	3.5
28	deiodinase, iodothyronine, type II	2.1

*The intensity of RT-PCR bands was quantified using densitometer. Folds expression was obtained by dividing band intensity of scopolamine-treated hippocampus with controls. Final value was the average of three independent RT-PCR.

四、計畫成果自評

在人類或是動物上，乙醯膽鹼神經系統在學習與記憶都扮演著重要的角色。在我們的這個研究找到一些基因發現除了毒蕈素接受體外亦有一些值得進一步研究的基因。雖然，目前為止與學習記憶直接關係的基因尚未建立清楚，造成記憶的分子機轉也未明確，但希望我們的研究能夠讓學習記憶進入分子層次新的領域。

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