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Microarray analysis of gene expression on herbal glycoside recipes improving deficient ability of spatial learning memory in ischemic mice

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Abstract

In order to reveal the mechanism of herbal glycoside recipes retrieving deficient ability of spatial learning memory in mice suffering from cerebral ischemia/reperfusion, a microarray system was used to analyze gene expression in those groups with increasing ability of spatial learning memory who were different from ischemic mice. In this work, we reported a comprehensive characterization of gene expression profiles of mouse hippocampus by the use of cDNA microarray system containing 1176 known genes in middle cerebral artery occlusion (MCAO) ischemic mice after treating with different dosage recipes of glycoside herbs (30, 90, and 270 mg/kg). The ability of spatial learning memory in ischemic mice was found to be decreased. The pathological process in ischemic mouse brain showed that a complex related to 100 genes' expression yielded 1.8-fold. Dose-dependent effects showed an improvement in the deficient ability and reduction in infarct volume when treated with glycoside recipes. Many genes (38–46) in expression were found greater than 1.8-fold in those effective recipes groups, including genes in cell cycle regulation, signal transduction, nerve system transcription factors, DNA binding protein, etc. Nine genes related to retrieving deficient ability of spatial learning memory treated with glycoside recipes were also found in this study. These results suggest that microarray analysis of gene expression might be useful for elucidating the mechanisms of pharmacological function of recipes.

Keywords: cDNA microarray, cerebral ischemia/ reperfusion, gene-related spatial learning memory, herbal remedy, messenger RNA, spatial learning memory.

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Drugs that enhance acquisition and/or recall of associative memory represent important goals in the therapy of cognitive disorders. Improving deficient ability of spatial learning memory has received extensive experimental attention because more and more people lose their learning and memory ability after stroke. Chinese herbs, such as Ginkgo biloba, baicalein and dioscin, have been used to enhance memory for thousands of year. Ginkgo biloba has been shown to be efficient on cognitive function in Alzheimer's disease (AD; Oken *et al*. 1998). Baicalein (5,6,7-trihydroxyflavone), a flavonoid isolated from the root of Chinese medicinal herb *Scutellaria baicalensis*, has been shown to exert anti-inflammatory and antioxidant effects, and is a well-known inhibitor of 12-lipoxygenase. Previous studies have shown that it may be useful as another template for the

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Abbreviations used: AICD, activation-induced cell death; CaMK, calcium/calmodulin-dependent kinase II; DMSO, dimethyl sulfoxide; EEG, electroencephalogram; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; INH2BP, inhibitor 5-iodo-6-amino-1,2-benzopyrone; LEO, Leonardo; LTP, long-term potentiation; MA, d-methamphetamine; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; ODN, oligodeoxynucleotide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SCI, spinal cord injury; SDS, sodium dodecyl sulfate; SSC, saline–sodium citrate.

development of better agents to prevent the pathological changes of atherosclerosis and restenosis (Huang *et al*. 1994). Current results (Kyoungho *et al*. 2003) suggest that baicalein selectively inhibits the nitric oxide (NO)-dependent apoptotic pathway of activated microglia by suppressing cytotoxic NO production. Also, the activation-induced cell death (AICD)-inhibiting effects of baicalein were specific for the inflammatory stimulus that activated microglia. Another glycoside, dioscin, exerted significant inhibitory effects on the growth of the human leukemia cell HL-60, inducing differentiation and apoptosis and affecting many cancer cells (Wang *et al*. 2001). Recipes composed of these glycosides have been used to retrieve the deficient ability of spatial learning memory in the stroke patient for thousands of years in traditional Chinese medicine (TCM) but its pharmaceutical mechanism was unclear.

Genomic analysis using cDNA microarray to detect the differential expression of mRNA assay permits rapid screening of hundreds or thousands of genes for involvement in pathogenesis of disease (Gary 2002). This approach has been applied to investigate aging (Heming *et al*. 2000), inflammation, metastasis, seizures, and neurodegenerative diseases such as multiple sclerosis (Lisman *et al*. 2002), mouse hippocampus development disorder (Mody *et al*. 2001), schizophrenia (Izquierdo *et al*. 1999), and brain gene expression disturbances (Brown *et al*. 2002). Recent study (Rao *et al*. 2002) evaluated the gene expression changes in rat cerebral cortex at 6 and 24 h of reperfusion following transient middle cerebral artery occlusion (MCAO) by GeneChip® analysis. Of other recent studies, Soriano *et al*. (2000) and Tang *et al*. (2002) studied the gene expression in the cerebral cortex of Sprague-Dawley (SD) rats suffering from permanent focal ischemia, and Jin *et al*. (2001) studied the gene expression in the hippocampus of SD rats following transient global ischemia. Leil *et al*. (2002) used a 9000-gene microarray to examine differences in hippocampal gene expression between two F1 hybrid mice strains that perform well in the Morris water maze and two inbred strains that perform poorly, and identified 27 differentially expressed genes. The results showed distinct temporal gene expression profiles associated with learning and memory (Cavallaro *et al*. 2002) and effects of environmental enrichment on gene expression in the brain (Rampon *et al*. 2000). But there were no reports that using the microarray method detected Chinese medicinal herbs improving deficient ability of spatial learning memory in ischemic mice. Here we report the results of microarray analysis of mRNA expression in mouse hippocampus – the groups that increased the ability of spatial learning memory were different from ischemic mice tested in a water maze – to reveal the mechanism of herbal glycoside recipes retrieving deficient ability of spatial learning memory and the related genes effected from glycoside recipes in this improving process.

Materials and methods

Animal model

Animal experiments were carried out in accordance with the Prevention of Cruelty to Animals Act 1986 and NIH guidelines for the care and use of laboratory animals for experimental procedures, and were approved by local committee review. Cerebral ischemia was induced in anesthetized mice by urethane [1.5 g/kg, intraperitoneally (i.p.)] to 38–48 g male or female mice by coagulation and transection of the vertebral arteries, ligation of the external carotid arteries, and occlusion of the common carotid arteries for 15 min twice with microvascular clips, followed by reperfusion for 10 min after the first occlusion. Sham-operated mice underwent identical procedures except for the common carotid artery occlusion. Blood pressure, blood gas, and glucose were monitored and rectal temperature was maintained at 37.0–37.5°C with a heating pad, and brain temperature was monitored with a 29-gauge thermocouple in the right corpus striatum and maintained at 36–37°C with a temperature-regulating lamp. Electroencephalogram (EEG) was monitored to ensure isoelectricity during ischemia.

Herbal therapy

Experimental animals were divided into five groups: sham-operated, vehicle (ischemic mice) and three herbal glycoside dosages (30, 90, and 270 mg/kg, 2 mL/kg, i.p, twice a day), respectively. The prescription was composed of baicalein and dioscin (ratio 1 : 1), the chemical structure of which is shown in Fig. 1. Herbal therapy started at 5 days before the operation and lasted for 45 days after operation. Sham-operated mice underwent identical procedures except therapy with vehicle [2 mL/kg; 100% dimethyl sulfoxide (DMSO)]. The course of treatment was 50 days.

Spatial maze tasks

Effects of herbs *in vivo* on spatial memory were evaluated in mice with the Morris water maze task. All mice were housed in a temperature-controlled (20–24°C) compartment for a week, allowed free access to food and water, and kept on a 12-h light/dark cycle. Mice were trained 40 days after being treated with herbs in the Morris water maze task to locate a hidden escape platform. On the first day of experiments, all mice were randomly assigned to swim for 2 min in a 1.5-m (diameter) × 0.6-m (depth) pool (22 ± 1°C). On the following day, mice were trained in a two-trial/day task for 4 consecutive days. Each training trial lasted for up to 2 min, during which mice learned to escape from water by finding a hidden platform that was placed at a fixed location and submerged about

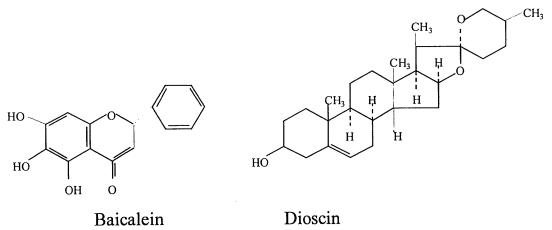

Fig. 1 Chemical structure of baicalein and dioscin. Prescription of chemicals in curing ischemic mice is composed of baicalein and dioscin (proportion being 1 : 1), which were extracted from herbs and belonged to glycoside.

1 cm below the water surface. The navigation of the mice was tracked by a videocamera. The escape latency and the route of mice swimming across the pool to the platform were recorded. The quadrant test (1 min) was performed after removing the platform, 24 h after the last training trial.

The data were expressed as mean ± SD. The statistical analysis for the differences in the parameters of a Morris water maze task such as escape latency and search time between vehicle (ischemic mice) and three dosage groups (30, 90, and 270 mg/kg) was performed by two-way ANOVA. When a significant overall F score was obtained, the difference among groups at each block was analyzed by Bonferroni's modified t-test as a post-hoc test. A value of $p < 0.05$ was considered to be statistically significant.

The percentage of the infarct volume

Forty-five days after operation, three mice from each group were killed by decapitation and their brain tissue was shifted into TTC solution (per 5 mL solution including 4% 2,3,5-triphenyltetrazolium chloride (TTC) 1.5 mL, 1 Mk_2HPO_4 0.1 mL) and incubated for 30 min at 37°C in darkness and then transferred into 10% formalin. Rose bengal areas mean non-ischemic tissue lumps and white areas mean ischemic lumps. The white lumps were carefully taken out and weighed. The percentage of the infarct volume was calculated by dividing the weight of right infarct lumps by the total weight of right cerebral hemisphere.

Microarray

The atlas of mouse brain tissue 1.2 array from Clontech (Palo Alto, CA, USA) containing 1176 genes was used to conduct the gene expression profiling. Genes were chosen for their possible role in mouse brain, including transporters, signal transduction, nervous system transcription factors, general DNA-binding proteins, cell-surface antigens, cell adhesion, cell-cycle regulators, ion channels, etc.

Probe synthesis and hybridization

Mouse brain hippocampus (subfields CA1–4 of Ammon's horn) was dissected, flash-frozen and stored at − 75°C. RNA extraction was performed using Atlas™ Pure Total RNA Labeling System (Clontech) according to the manufacturer's recommendations. Samples from three animals per group were pooled and homogenized in denaturing solution with a Polytron. Total RNA was isolated from tissue homogenates with phenol–chloroform extraction and dissolved in RNase-free water. DNase I-treated total RNA was precipitated by a second round of phenol–chloroform extraction and the quality of RNA was assessed by denaturing gel analysis and absorbance measurements at A_{260}/A_{280}. Total RNA samples exhibited A_{260}/A_{280} ratios of 1.8 or higher with no degradation visible by denaturing gel analysis. Poly-A^+ RNA was enriched by oligo(dT) separation followed by the cDNA probe synthesis using a gene-specific cDNA Synthesis Primer Mix (Atlas™ Pure Total RNA Labeling System) and incorporation of [α]-[^{33}P]dATP (YAFEI Life Science Products, Inc., Beijing, China) by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. Unincorporated nucleotides were removed by column chromatography according to the manufacturer's protocol. Membranes were hybridized in hybridization kit with continuous agitation at 68°C. After 24 h, 200 mL of solution 1 [0.1 × saline–sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS)] and solution (0.1 × SSC, 0.5% SDS), pre-warmed to 68°C, was used to wash the membrane under agitation at room temperature for 5 min. The X-ray film (Kodak BioMax MS film, Kodak, Rochester, NY, USA) was exposed at − 70°C with an intensifying screen.

Microarray scanning, quantitation, and statistical analysis

The hybridization was performed according to the manufacturer's recommendations and filters were exposed onto phosphor screen and scanned with STORM 860 PhosphorImager at 50 μm resolution. Radioactive intensity of each spot was linearly scanned to 65 536 gray-grade in a pixel of 50 microns in an Image Reader. After subtracting the background chosen from area where no PCR product was located, genes with intensity > 10 were considered as positive signals to ensure that they were distinguished from background with statistical significance (> 99.9%). The relative expression level of a given cDNA was assessed by comparing the signal obtained in one membrane (after normalizing to the global value of all the genes provided on the membranes) relative to the second membrane. Housekeeping genes in the two membranes presented similar hybridization signals. The differential expression was considered as significant between model and herb-treated mice when the ratio of signals between the same sites on different membranes was greater than ± 1.8 (Genespring software).

RT–PCR

A selection of candidate genes from the array data analysis was chosen for further investigation with RT–PCR based on the magnitude of their expression ratios or their functional similarity to highly regulated genes. After extraction of total RNA from mouse brain, reverse transcription was performed. Five micrograms of RNA were reverse transcribed for 1 h at 39°C with 0.5 μg random primers in the presence of 200 units of MMLV reverse transcriptase (Promega, Madison, WI, USA), 25 units of RNasin ribonulease inhibitor, and 500 μM final concentration of dNTP in a 25-μL reaction. The resulting single-strand cDNA was amplified by PCR, which was performed with specifically designed primers for the genes in the search. After an initial 1 min denaturing cycle at 95°C, an optimal number of cycles were performed as determined for the specific gene, including denaturation, annealing and polymerization, followed by a final 10 min elongation step at 72°C. In order to overcome the possible variation that may arise from the PCR machine, the reactions were performed in either duplicate or triplicate. In addition, a parallel PCR reaction was performed with a pair of sense- and gluceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers, as an internal control for normalizing variations in RNA aliquots taken for RT reactions and gel loading. PCR products were analyzed by electrophoresis on 2% agarose gel and stained with ethidium bromide. Densitometric analysis was then performed. Hybridization intensities were quantified from matching brain regions of herb-treated and identical vehicle control sections. The data were mean values of specific binding of all sections from the treatment group, and were presented as percentage of the mean specific binding of the corresponding control sections.

Western blot analysis

Three mice hippocampi were homogenized in 30 μL of modified radioimmunoprecipitation assay buffer (0.137 M NaCl, 20 mM Tris, pH 8, 10% glycerol, 0.1% SDS, and 0.1% sodium deoxycholate).

Samples were boiled for 5 min and centrifuged at 12 000 *g* for 10 min and, after addition of Laemli's buffer, 10 μg of protein was loaded per lane for SDS polyacrylamide gel electrophoresis (SDS–PAGE) and blotted using standard methods. The results were visualized with enhanced chemiluminescence and quantified using densitometry.

Results

The ability of spatial learning memory in different groups

We tested the effects of herbs on spatial learning in mice, using the hidden-platform water maze. As shown in Fig. 2(a), the latency to escape to the platform in all five groups of rats decreased following the training sessions. Statistical analysis revealed significant effects of groups ($F_{4,45} = 188.96$, $p < 0.01$), trials ($F_{7,307} = 1383.28$, $p < 0.01$), and groups × session of trials ($F_{28,315} = 26.07$, $p < 0.01$), indicating that spatial learning in mice treated with herbs was faster than in ischemic mice (vehicle). Moreover, a post-hoc analysis reveals a significant difference from the third to eighth trials ($p < 0.05$), confirming better learning in two groups (90 and 270 mg/kg). Quadrant tests 24 h after the last training trial revealed that the sham-operated, 90 and 270 mg/kg glycoside recipes groups ($F_{3,36} = 69.96$, $p < 0.05$; ANOVA and Newman–Keuls post-hoc test) spent more time searching in the target quadrant (quadrant 4) where the platform was previously placed and removed, compared with vehicle. The mice in two groups (90 and 270 mg/kg) exhibited a clearly greater preference for the target quadrant (by 33.7 ± 1.6%, $p < 0.05$; 40.1 ± 2.3%, $p < 0.01$; ANOVA; Figs 2f and g). The target quadrant ratios, target/average of the non-target quadrants, between the vehicle and the mice in the 90 and 270 mg/kg groups were significantly different ($F = 72.93$, $p < 0.05$, $p < 0.01$; Fig. 2b). Thus, the mice treated with the herbs in two dosage groups performed better than their vehicle counterparts in this spatial memory retention task. The average swim speeds for all eight trials, however, did not differ among all the groups (Fig. 2c; $p > 0.05$), indicating that herbal glycoside recipes and ischemia did not grossly affect their sensory or locomotor activities. During the experimental periods, no mice showed any apparent sign of discomfort or abnormal behavior such as hypo- or hyperactivity.

Infarct volume of different groups

Infarct volume in different degrees were visible (Fig. 3) in all groups except the sham-operated one 45 days after infarct operation and were decreased in all mice treated with herbs. Significant reductions in infarct volume were found in 90 and 270 mg/kg dosage groups to be different from vehicle group (ischemic mice; $F = 36.97$, $*p < 0.05$, $**p < 0.01$ vs. vehicle; mean ± SD, ANOVA, $n = 10$).

Gene expression profiling of mouse hippocampus in different groups

In all the groups (sham-operated, vehicle, three glycoside dosages; three microarrays for each group), 45.4–54.4%

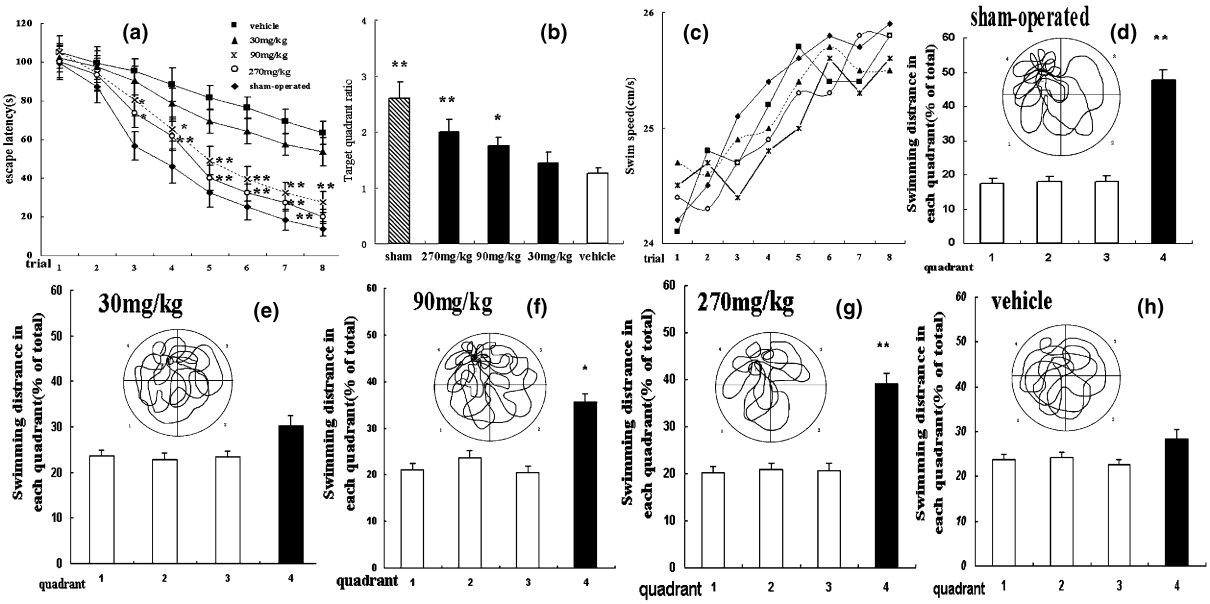

Fig. 2 Herbal glycoside recipes enhance ischemic mice performance in the hidden platform water maze task. The figure illustrates escape latency (means ± SEM; $n = 10$ for each group) in water maze training (a) across eight trials, swim speeds (c), and quadrant preference (d–h) conducted at the end of the eighth training session. Quadrant 4 is the target quadrant during training. Insets are paths taken by representative mice with quadrant numbers indicated. The target ratio is defined as the time searching in the target quadrant/the average of the non-target quadrants (b). Quadrant 4 is the target quadrant during training. $**p < 0.01$, $*p < 0.05$.

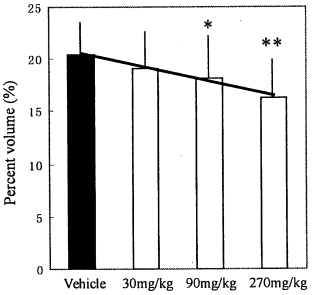

Fig. 3 Infarct volume (%) in mice brain of different groups. The black and white bar represents infarct volume in mice brain treated with the vehicle and herbs separately. Each bar plots mean and SD values from 10 mice brain. Stars indicate significant differences between vehicle and herbs with $*p < 0.05$, $**p < 0.01$(ANOVA).

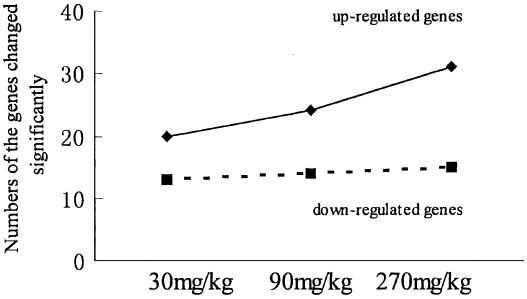

Fig. 5 Numbers of the changed genes divided into upregulated and downregulated in different groups. The upper line represents the quantity of upregulated genes and the lower line those downregulated ones.

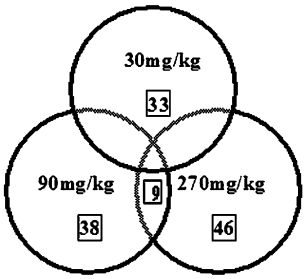

Fig. 4 A schematic presentation of relationship gene expression induced greater than 1.8-fold in different groups as determined by cDNA-based microarrays. Numbers in blocks represent the genes of expression changed significant in corresponding group. Nine genes expression were been found greater than 1.8-fold in in two (90 and 270 mg/kg) dosage groups commonly.

transcripts (534–640 out of 1263) were observed to be present. Fold induction is calculated as the normalized and averaged intensity of all gene expression in microarray (mean ± SD) in three dosage groups (30, 90, and 270 mg/kg) separately versus vehicle which intensity versus sham-operated mice (three microarrays were taken in every groups). One hundred and eighty, 100, 33, 38, and 46 gene expression induced greater than 1.8-fold were found obviously in the sham, vehicle, 30, 90, and 270 mg/kg groups (see Fig. 4).

Among those 100 genes in the vehicle group (different expression from sham-operated mice), 49 genes were upregulated and 51 downregulated. In the 30, 90, and 270 mg/kg dosage groups, the signal values of hybridization when compared with the vehicle mice were 20, 24, and 31 genes, respectively, showing upregulation and 13, 14, and 15 genes revealing downregulation, respectively (see Fig. 5). The higher the dosage, the more the genes were found to have been changed in their expression. But with dosage increasing, the intensity of affected gene expression decreased eventually. A sequence of up to five major genes in different mice groups is shown in Table 1.

According to the phenotype of mice, performance in the water maze and the expression of nine genes was found to be induced greater than 1.8-fold in two groups (90 and 270 mg/kg) compared with vehicle mice. Interestingly, changes of gene expression have the same direction in mice from this group. When the expression was downregulated in one group of mice, the gene expression would decrease in another group of mice, so as to upregulate genes. Those genes are listed in Table 2.

RT–PCR and western blot

RT–PCR analysis of gene expression of 5-HT receptor and 14-3-3 protein

The herb-induced increase or decrease in 14-3-3 and 5-HT obtained in the cDNA microarray was further verified by RT–PCR. RT–PCR analysis gene expression of 5-HT receptor in herb-treated mouse brain shows a progressive decrease dependent on dosage from 30 to 270 mg/kg. The expression of 14-3-3 protein mRNA was significantly increased by herbs, as examined by RT–PCR analysis.

Independent measurements of selected gene expression levels by RT–PCR (Fig. 6a) and western blot (Fig. 6b) were carried out. Expression levels measured by RT–PCR were normalized to GAPDH and plotted relative to the level in vehicle. To determine the reliability of these results, this experiment was repeated, as shown in Fig. 6; the expression profiles measured by these three independent methods (RT–PCR, microarray, western blot) were very similar, even though the absolute value for each mRNA level might be different among the three measurements.

The effects of spatial learning in normal mice treated with herbs

The effects of herbs on spatial learning in normal mice and normal ones treated with herbs (90 mg/kg, i.p., twice a day for 30 days) were tested using the hidden-platform water maze. As shown in Fig. 7, the latency to escape to the

Table 1 Sequence of five major genes in different mice groups

Group	Name	Ratio	GenBank	SwissProt
Sham	Transthyretin precursor	7.87 ± 1.11	D89076	P07309
	Apolipoprotein E precursor	6.09 ± 1.07	M12414	P08226
	Calbindin 2	5.54 ± 0.73	X73985	Q60964
	14-3-3 protein eta	4.06 ± 0.56	U57311	P70198
	7B2 neuroendrocrine protein	3.78 ± 0.46	X15830	P12961
Vehicle	Methyl-CpG-binding protein2	12.27 ± 3.28	Af072251	Q9Z2D6
	Frizzled homolog7(FZD7)	11.40 ± 2.17	U43320	Q61090
	Myelin proteolipid protein(PLP)	− 11.22 ± 2.32	M16472	P06905
	5-hydroxytryptamine receptor	8.67 ± 2.03	S49542	P35363
	84-kDa heat shock protein(HSP84)	− 8.67 ± 2.38	M36829	M36829
30 mg/kg	40S ribosomal protein SA	− 5.71 ± 1.82	J02870	P14206
	Spinal cord axial homeobox proein 1	4.44 ± 1.57	X75384	P42580
	Axonal membrane protein GAP-43	− 3.89 ± 1.17	J02809	P06837
	Non-muscle cofilin(CFL1)	− 3.81 ± 0.87	D00472	P18760
	LIM domain kinase	3.45 ± 0.65	U15159	P53668
90 mg/kg	5-hydroxytryptamine receptor	− 7.19 ± 1.59	S49542	P35363
	Kinesin like protein KIF38	− 5.88 ± 1.74	D26077	Q61771
	HER2 protein tyrosine kinase	− 5.01 ± 1.36	L47239	P70424
	MCM5 DNA Replication licensing factor	− 4.72 ± 1.35	D26090	P49718
	Myelin-associated oligodendrocytic basic protein	4.52 ± 1.26	U81317	O35713
270 mg/kg	H-ras proto-oncogene	3.92 ± 0.93	Z50013	Q61411
	Thyroid homone receptor alpha 1	3.80 ± 1.13	X51983	P16416
	7B2 neuroendrocrine protein	3.32 ± 1.26	X15830	P12961
	Sim transcription factor	3.26 ± 0.83	U42554	Q61079
	Cyclin C(G1-specific)	− 2.92 ± 0.94	U62638	Q62447

Genes induced greater than 1.8-fold by herbs in different groups as determined by cDNA-based microarrays. Fold induction is calculated as the normalized intensity in herb-treated versus control treated mice. The average and standard deviation of three mice brain preparations are shown. Positive and negative number representative gene expression increased and decreased compare with vehicle mice, separately (vehicle mice for which different from sham-operated mice).

Table 2 Nine genes induced greater than 1.8-fold in two dosage groups

Gene	Sham	Vehicle	30 mg/kg	90 mg/kg	270 mg/kg
Apolipoprotein E precursor	6.09 ± 1.07	2.58 ± 0.39	− 1.13 ± 0.36	− 2.01 ± 0.34	− 2.36 ± 0.41
14-3-3 protein eta	4.06 ± 0.56	− 3.45 ± 0.43	1.2 ± 0.16	2.53 ± 0.32	2.77 ± 0.22
Glutathione S-transferase Pi 1	3.12 ± 0.42	− 2.72 ± 0.56	1.23 ± 0.31	2.13 ± 0.28	2.30 ± 0.24
5-hydroxytryptamine receptor	1.23 ± 0.22	8.67 ± 2.03	− 1.67 ± 0.32	− 2.59 ± 0.39	− 2.85 ± 0.35
Calmodulin-binding protein p-57	1.43 ± 0.36	2.89 ± 0.33	− 1.77 ± 0.42	− 2.13 ± 0.52	− 2.24 ± 0.39
Cathepsin D	3.57 ± 0.47	− 2.48 ± 0.59	1.33 ± 0.46	2.25 ± 0.37	2.61 ± 0.35
Kinesin like protein KIF38	1.11 ± 0.12	3.20 ± 0.52	− 1.33 ± 0.31	− 5.88 ± 0.55	− 1.88 ± 0.27
7B2 neuroendrocrine protein	3.78 ± 0.46	− 3.60 ± 0.63	1.53 ± 0.47	2.09 ± 0.46	3.32 ± 1.26
Methyl-CpG-binding protein	1.28 ± 0.32	12.27 ± 3.28	− 1.5 ± 0.32	− 1.83 ± 0.26	− 2.46 ± 0.32

Genes induced greater than 1.8-fold by herbs in two dosage groups (90 and 270 mg/kg) as determined by cDNA-based microarrays. Fold induction is calculated as the normalized intensity in herb-treated versus vehicle (ischemic mice). The average and standard deviation of three mice brain preparations are shown. Positive and negative number representated gene expression increased and decreased compare with vehicle mice, separately.

platform in all five groups of rats decreased following the training sessions. Statistical analysis revealed no significant effects of groups ($F_{1,15} = 2.34$, $p > 0.05$) or groups × session of trials ($F_{7,126} = 1.67$, $p > 0.05$; Fig. 7a), indicating that spatial learning in mice treated with herbs was not better than in normal mice. The target quadrant ratios also did not differ between the two groups (Fig. 7b; $p > 0.05$). RT–PCR mRNA analysis of 14-3-3 between the two groups showed

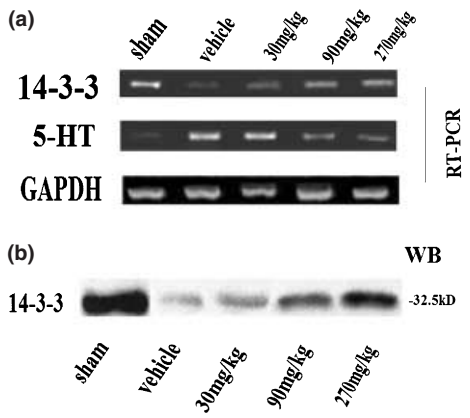

Fig. 6 RT–PCR mRNA analysis and western blotting of 5-HT receptor, 14-3-3 protein and GAPDH in mice hippocampus of different groups. Densitometry values for 14-3-3 and 5-HT receptor from sham, vehicle to three dosage groups, standardized for GAPDH, are 4.03, 1.22, 1.56, 30.5, 3.27; 1.03, 8.28, 5.31, 5.01, and 3.21, respectively (a). 14-3-3 protein expressed in all groups was analyzed by western botting (b), similar results of RT–PCR.

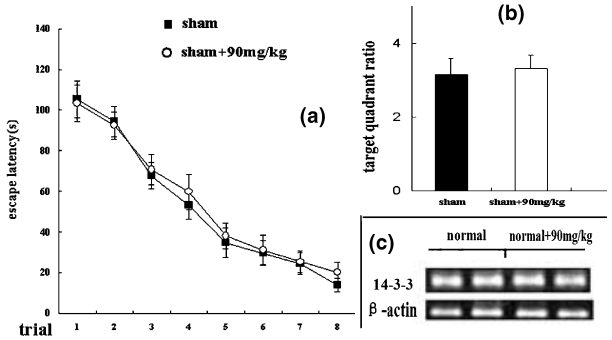

Fig. 7 Herbal glycoside recipes affect normal mice performance in the hidden platform water maze task. The figure illustrates escape latency (means^{+}-standard errors of the means; $n = 10$ for each group) in water maze training (a), the target ratio (b), and RT–PCR mRNA analysis of 14-3-3 (c) between normal mice and normal ones treated with herbal glycoside recipes were carried out no found significantly changes ($p > 0.05$).

no significant change; β-actin was used as a positive control (Fig. 7c).

Discussion

It is known that much gene-related spatial learning memory has been identified individually or systematically. It was believed that protein kianse C (PKC), PKA or MAPK (mitogen-activated protein kinase) led separate signaling pathways in long-term memory formation (Izquierdo and Medina 1997). Remarkable progress has been made in understanding the role of CaMKII in long-term potentiation (LTP). There is thus little doubt that CaMKII is activated during LTP induction and that this activation is necessary and sufficient for LTP. It is also clear that CaMKII can strengthen synaptic transmission by multiple mechanisms. CaMKII autophosphorylation and dephosphorylation indicates that this kinase could serve as a molecular switch capable of long-term memory storage. Consistent with such a role, mutations that prevent persistent activation of CaMKII block LTP, experience-dependent plasticity, and behavioral memory. Lisman *et al*. (2002) made CaMKII a leading candidate in the search for the molecular basis of memory, and Stefan and Roger 1998) found that activation and Thr286 autophosphorylation of CaMKII following Ca^{2+} influx via NMDA-type glutamate receptors was essential for hippocampal LTP.

The present study is the first report of altered expression of several gene-related herbal glycoside recipes retrieving deficient ability of spatial learning memory in mice following transient MCAO. From phenotype significant changed, such as performed better and decreased infarct volume than their vehicle, gene expression data from two groups (90 and 270 mg/kg) of mice treated with the herbs were analyzed together regardless of how different these two groups were in gene expression. The genes inducing greater than 1.8-fold in two dosage groups were methyl-CpG-binding protein 2, kinesin-like protein KIF38, 7B2 neuroendocrine protein, calmodulin-binding protein p-57, cathepsin D, glutathione S-transferase Pi 1, 14-3-3 protein eta 5-HT receptor, apolipoprotein E precursor. Some of these genes and their protein products played a significant role in stroke-induced neuronal damage and could be targeted to develop new treatments. To test this concept, we analyzed the role of 14-3-3 upregulation and 5-HT downregulation in post-ischemic learning memory damage from recent studies.

14-3-3 proteins

14-3-3 proteins were first identified as abundant, acidic, soluble brain proteins with a molecular weight of 25–32 kDa (Moore and Perez 1967). They have been shown to play diverse roles in many biological processes, such as the activation of PKC (Toker *et al*. 1990; Dai and Murakami 2003), the stimulation of calcium-dependent exocytosis (Morgan and Burgoyne 1992), involvement in mitochondrial and chloroplastic import (Alam *et al*. 1994; May and Soll 2000), the regulation of proton-pumping mechanisms via the plant plasma membrane H^{+}-ATPase (Oecking *et al*. 1994) and of key plant metabolic enzymes (Moorhead *et al*. 1996; Toroser *et al*. 1998), involvement in controlling regulation of G protein signaling pathways (Niu *et al*. 2002), upregulation in amyotrophic lateral sclerosis spinal cord (Malaspina *et al*. 2000), and the ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors (Fu and Subramanian 2000). So, the 14-3-3 proteins have an overall inhibitory effect on cell cycle progression and apoptosis, whereas in signal transduction they may act as stimulatory or inhibitory factors

(van Hemert *et al*. 2001). Lately, studies have revealed a 14-3-3 protein associated with memory acquisition. Satchell *et al*. (2003) found ribosylation of 14-3-3γ was completely inhibited by the dose of INH2BP that produced profound memory disturbances. Studies by Philip *et al*. (2001) indicated that monomers and homodimers of either LEO isoform and/or heterodimers with D14-3-3ϵ were essential for learning and memory. 14-3-3 protein etc. and calmodulin-binding protein p-57 may be involved in the CaMKII and MAPK pathways in spatial memory (Tang *et al*. 1998; Heming *et al*. 2000).

5-HT

The neuromodulator serotonin (5-HT) has been associated with mood disorders such as depression, anxiety, impulsive violence, and behavioral phenotypes. Some recent studies have indicated that the serotonergic (5-HT) system played important roles in memory function. Because acetylcholine (ACh) is a crucial mediator of learning and memory (Blokland 1995), drugs that reduce cholinergic function, such as the muscarinic receptor antagonist scopolamine, caused profound memory impairments in animals and humans (Deutsch and Rocklin 1967; Deutsch 1971). On the other hand, pre-synaptic 5-HT_{1A} receptor stimulation and post-synaptic 5-HT_{1A} receptor blockade can improve scopolamine-induced deficits (Carli *et al*. 1998; Galeotti *et al*. 1998; Malleret *et al*. 1999), indicating that a partial 5-HT_{1A} receptor agonist, which would be expected preferentially to stimulate pre-synaptic autoreceptors, may also reverse scopolamine-induced memory deficits. These findings suggest that a combined σ and partial 5-HT_{1A} receptor agonist may increase ACh release and improve memory independently of effects on AchE. Drugs that stimulate pre-synaptic 5-HT_{1A} receptors, such as 5-HT_{1A} receptor partial agonists, may be useful in the symptomatic treatment of human memory disturbances associated with loss of cholinergic innervation to the hippocampus (Carli *et al*. 2000). Post-synaptic 5-HT_{1A} receptors localized in the hippocampal formation have a negative influence on explicit memory function, which raises the possibility that the antagonistic effect of post-synaptic 5-HT_{1A} receptors in the hippocampus leads to improvement of human memory function. Drugs that work as antagonists on post-synaptic 5-HT_{1A} receptors may be favorable for improved control of memory impairment (Yasuno *et al*. 2003).

The stimulation of 5-HT_{1B} receptors in the CA1 field of the dorsal hippocampus impaired the performance of rats in a spatial learning task (Buhot *et al*. 1995). A study by Williams *et al*. (2002) showed that pre-frontal 5-HT_{2A} receptors have a hitherto unrecognized role in the cognitive function of working memory, which involves actions at both excitatory and inhibitory elements within local circuitry. Activation of central 5-HT_4 receptors may enhance cognitive performance. Fontana *et al*. (1997) studies supported a role of 5-HT_4 receptors in rat spatial learning and memory. In all accounts, 5-HT receptors have been shown associated with spatial memory whatever subunit 5-HT has.

Other gene-related spatial memory in the present study

Mutation of methyl-CpG-binding protein 2 was related to Rett syndrome and mental retardation (Shahbazian and Zogbi 2001). Neuroendocrine protein 7B2 was downregulated greater than 50% in spinal cord injury (SCI) tissue (Tachibana *et al*. 2002). More research is needed to reveal its function in spatial memory process. We are currently characterizing the function of a number of these genes in transgenic animals.

The present study observed increased or decreased expression of 14-3-3 or 5-HT measured by three independent methods (RT–PCR, microarray, and western blot) and other neuroprotective genes as a putative attempt to retrieve spatial learning memory following an ischemic insult, and has not found a significant effect of herbs on spatial learning and mRNA analysis of 14-3-3 between normal mice and normal ones treated with herbs (90 mg/kg). These results suggest that glycoside recipes act upon its pharmacological functions as many pathway or modes, which may be useful for elucidating the complex pharmaceutical mechanisms of herbs.

The results described above demonstrated the ability of microarray analysis to detect nine genes related to spatial learning memory in ischemia reperfusion-induced changes in gene expression that some have been documented before using other methods, but the greater potential value of this approach lies in its capacity to identify genes not previously known to be involved in the memory process in the spatial memory and herbal therapy. An interesting finding was that nine genes recovered deficient spatial learning memory in middle and high dosage groups. In this study, these nine gene expressions were downregulated or upregulated in the same direction, leaving the question open of whether this phenomena simply represents an altered hippocampus function or whether it could be involved in herbal pharmacogenomics. Another interesting findings of dose-dependent gene expression on learning and memory alteration, in contrast, may cover a number of regulatory pathways responsible for the control of cell proliferation, cell signaling and extracellular communication, transcription factors, general DNA-binding proteins, cell-cycle regulators, ion channels, metabolic pathway, post-translation modification, folding, oncogenes, apoptosis, DNA synthesis, repair and recombination, cell skeleton protein.

Drugs based on known mechanisms of memory-related plasticity have been developed. The effects of neonatal D-methamphetamine (MA) treatment on cued and spatial learning and memory were investigated, and deficits of spatial learning and memory were found to have a selective effect of neonatal methamphetamine treatment irrespective of other

learning and performance variables (Charles *et al*. 2000). Yamazaki *et al*. (1995) concluded that FR121196 (a potential antidementia drug) ameliorates the memory deficits of rats with cholinergic dysfunction through the action on the hippocampal monoaminergic (possibly serotonergic) neurons. Issues now arise about appropriate applications of candidate drugs and optimal cellular targets for future development. Thus, certain memory-enhancing agents may prove more useful when implemented early in the course of a disease such as stroke, and they also may enjoy a wide application for the treatment of the memory decline associated with stroke.

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References

Alam R., Hachiya N., Sakaguchi M., Kawabata S., Iwanaga S., Kitajima M., Mihara K. and Omura T. (1994) cDNA cloning of mitochondrial import stimulation factor (MSF) purified from rat liver cytosol. *Biochem. J.* **1116**, 416–425.

Blokland A. (1995) Acetylcholine: a neurotransmitter for learning and memory? *Brain. Res. Rev.* **21**, 285–300.

Brown V. M., Ossadtchi A., Khan A. H., Cherry S. R., Leahy R. M. and Smith D. J. (2002) High-throughput imaging of brain gene expression. *Genome Res.* **12**, 244–254.

Buhot M. C., Patra S. K. and Naili S. (1995) Spatial memory deficits following stimulation of hippocampal 5-HT_{1B} receptors in the rat. *Eur. J. Pharmacol.* **285**, 221–228.

Cavallaro S., D'Agata V., Manickam P., Dufour F. and Alkon D. L. (2002) Memory-specific temporal profiles of gene expression in the hippocampus. *Proc. Natl Acad. Sci. USA* **99**, 16279–16284.

Carli M., Balducci C. and Samanin R. (2000) Low doses of 8-OH-DPAT prevent the impairment of spatial learning caused by intrahippocampal scopolamine through 5-HT_{1A} receptors in the dorsal raphe. *Br. J. Pharmacol.* **131**, 375–381.

Carli M., Bonalumi P. and Samanin R. (1998) Stimulation of 5-HT_{1A} receptors in the dorsal raphe reverses the impairment of spatial learning caused by intrahippocampal scopolamine in rats. *Eur. J. Neurosci.* **10**, 221–230.

Charles V. V., Sandra L. I. W., LaRonda L. M., Harry W. B., Masao F. and Mary S. M. (2000) Adult learning deficits after neonatal exposure to D-methamphetamine: selective effects on spatial navigation and memory. *J. Neurosci.* **20**, 4732–4739.

Dai J. G. and Murakami K. (2003) Constitutively and autonomously active protein kinase C associated with 14-3-3ζ in the rodent brain. *J.Neurochem.* **84**, 23–34.

Deutsch J. A. (1971) The cholinergic synapse and the site of memory. *Science* **174**, 788–794.

Deutsch J. A. and Rocklin K. W. (1967) Amnesia induced by scopolamine and its temporal variations. *Nature* **216**, 89–90.

Fontana D. J., Daniels S. E., Wong E. H., Clark R. D. and Eglen R. M. (1997) The effects of novel, selective 5-hydroxytryptamine (5-HT) 4 receptor ligands in rat spatial navigation. *Neuropharmacology* **36**, 689–696.

Fu H., Subramanian R. R. and Masters S.C. (2000) 14-3-3 Proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.* **40**, 617–647.

Galeotti N., Ghelardini C. and Bartolini A. (1998) Role of 5-HT_4 receptors in the mouse passive avoidance test. *J. Pharmacol. Exp. Ther.* **286**, 1115–1121.

Gary L. (2002) Memory enhancement: the search for mechanism-based drugs. *Nat. Neurosci.* **5**, 1035–1038.

van Hemert M. J., Steensma H. Y. and van Heusden G. P. (2001) 14-3-3 Proteins: key regulators of cell division, signalling and apoptosis. *Bioessays* **23**, 936–946.

Heming X., Shaosong Z., Carla W., Attila K. and Anthony J. M. (2000) 14-3-3 Protein block apoptosis and differentially regulate MAPK cascades. *EMBO J.* **19**, 349–358.

Huang H. C., Wang H. R. and Hsieh L. M. (1994) Antiproliferative effect of baicalein, a flavonoid from a Chinese herb, on vascular smooth muscle cell. *Eur. J. Pharmacol.* **251**, 91–93.

Izquierdo I. and Medina J. H. (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol. Learn. Mem.* **68**, 285–316.

Izquierdo I. and Medina I. H. (1999) Separate mechanisms for short and long-term memory. *Behav. Brain Res.* **103**, 1–11.

Jin K., Mao X. O., Eshoo M. W., Nagayama T., Minami M., Simon R. P. and Greenberg D. A. (2001) Microarray analysis of hippocampal gene expression in global cerebral ischemia. *Ann. Neurol.* **50**, 93–103.

Kyoungho S., Heasuk L., Sang S. K., Gyeong J. C. and Wan S. C. (2003) Flavonoid baicalein attenuates activation-induced cell death of brain microglia. *J. Pharmacol. Exp. Ther.* **21**, 1124–1131.

Leil T. A., Ossadtchi A., Cortes J. S., Leahy R. M. and Smith D. J. (2002) Finding new candidate genes for learning and memory. *J. Neurosci. Res.* **68**, 127–137.

Lisman J., Schulman H. and Cline H. (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat. Rev.* **3**, 175–190.

Malaspina A., Kaushik N. and Belleroche J. (2000) A 14-3-3 mRNA is upregulated in amyotrophic lateral sclerosis spinal cord. *J.Neurochem.* **75**, 2511–2520.

Malleret G., Hen R., Guillou J. L., Segu L. and Buhot M. C. (1999) 5-HT_{1B} receptor knock-out mice exhibit increased exploratory activity and enhanced spatial memory performance in the Morris water maze. *J. Neurosci.* **19**, 6157–6168.

May T. and Soll J. (2000) 14-3-3 Proteins form a guidance complex with chloroplast precursor proteins in plants. *Plant Cell.* **12**, 53–63.

Mody M., Yanxiang Cao Y. *et al.* (2001) Genome-wide gene expression profiles of the developing mouse hippocampus. *Proc. Natl Acad. Sci. USA* **98**, 8862–8867.

Moore B. W. and Perez V. J. (1967) Specific acidic proteins of the nervous system. In: *Physiological and Biochemical Aspects of Nervous Integration* (Carlson F. D., ed), pp. 343–359. Prentice Hall, Englewood Cliffs, NJ.

Moorhead G., Douglas P., Morrice N., Scarabel M., Aitken A. and MacKintosh C. (1996) Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. *Curr. Biol.* **6**, 1104–1113.

Morgan A. and Burgoyne R. D. (1992) Interaction between protein kinase C and Exo 1 (14-3-3 protein) and its relevance to exocytosis in permeabilized adrenal chromaffin cells. *Biochem. J.* **286**, 807–811.

Niu J., Scheschonka A., Druey K. M. *et al.* (2002) RGS3 interacts with 14-3-3 via the N-terminal region distinct from RGS domain. Biochem. J. Immediate Publication, DOI: 10.1042/BJ20020390.

Oecking C., Eckerson C. and Weiler E. W. (1994) The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. *FEBS Lett.* **352**, 163–166.

Oken B. S., Storzbach D. M. and Kaye J. A. (1998) The efficacy of Ginkgo biloba on cognitive function in Alzheimer's disease. *Arch. Neurol.* **55**, 1409–1415.

Philip N., Acevedo S. F. and Skoulakis E. M. C. (2001) Conditional rescue of olfactory learning and memory defects in mutants of the 14-3-3ζ gene Leonardo. *J. Neurosci.* **21**, 8417–8425.

Rampon C., Jiang C. H., Helin Dong H., Ya-Ping Tang Y. P., Lockhart D. J., Schultz P. G., Joe Z., Tsien J. and Hu Y. H. (2000) Effects of environmental enrichment on gene expression in the brain. *Proc. Natl Acad. Sci. USA* **97**, 12880–12884.

Rao V. L. R., Bowen K. K., Dhodda V. K., Song G., Franklin J. L., Gavva N. R. and Dempsey R. J. (2002) Gene expression analysis of spontaneously hypertensive rat cerebral cortex following transient focal cerebral ischemia. *J.Neurochem.* **83**, 1072–1086.

Satchell M. A., Zhang X. P., Kochanek P. M., Dixon C. E., Jenkins L. W., Melick J., Szabó C. and Clark R. S. B. (2003) A dual role for poly-ADP-ribosylation in spatial memory acquisition after traumatic brain injury in mice involving NAD^+ depletion and ribosylation of 14-3-3 [Reinsert graphic]. *J. Neurochem.* **85**, 697–708.

Shahbazian M. D. and Zogbi H. Y. (2001) Molecular genetics of Rett syndrome and clinical spectrum of MECP2 mutations. *Curr. Opin. Nuerol.* **14**, 171–176.

Soriano M. A., Tessier M., Certa U. and Gill R. (2000) Parallel gene expression monitoring using oligonucleotide probe arrays of multiple transcripts with an animal model of focal ischemia. *J. Cereb. Blood Flow Metab.* **20**, 1045–1055.

Stefan S. and Roger J. C. (1998) Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the *N*-methyl-D-aspartate receptor. *J. Biol. Chem.* **273**, 20689–20692.

Tachibana T., Noguchi K. and Ruda M. A. (2002) Analysis of gene expression following spinal cord injury in rat using complementary DNA microarray. *Neurosci. Lett.* **327**, 133–137.

Tang S. J., Suen T., McInnes R. R. and Buchwald M. (1998) Association of the TLX-2 homeodomain and 14-3-3 eta signaling protein. *J. Biol. Chem.* **273**, 15356–15363.

Tang Y., Lu A., Aronow B. J., Wagner K. R. and Sharp F. R. (2002) Genomic response of the brain to ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia. *Eur. J. Neurosci.* **15**, 1937–1952.

Toker A., Ellis C. A., Sellers L. A. and Aitken A. (1990) Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. *Eur. J. Biochem.* **191**, 421–429.

Toroser D., Athwal G. S. and Huber S. C. (1998) Site-specific regulatory interaction between spinach leaf sucrose-phosphate synthase and 14-3-3 protein. *FEBS Lett.* **435**, 110–114.

Wang Z., Zhou J., Ju Y., Zhang H., Liu M. and Li X. (2001) Effects of two saponins extracted from the polygonatum zanlanscianense pamp on the human leukemia (HL-60) cells. *Biol. Pharm. Bull.* **24**, 159–162.

Williams G. V., Rao S. G. and Goldman-Rakic P. S. (2002) The physiological role of 5-HT_{2A} receptors in working memory. *J.Neurosci.* **22**, 2843–2854.

Yamazaki M., Matsuoka N., Maeda N., Kuratani K., Ohkubo Y. and Yamaguchi I. (1995) FR121196, a potential antidementia drug, ameliorates the impaired memory of rat in the Morris water maze. *J. Pharmacol. Exp. Ther.* **272**, 256–263.

Yasuno F., Suhara T., Nakayama T. *et al.* (2003) Inhibitory effect of hippocampal 5-HT_{1A} receptors on human explicit memory. *Am. J. Psychiatry* **160**, 334–340.