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Expression Profile of Late Responsive Genes Induced by Spatial Learning Task: Involvement of Pathways in Locomotion and Memory

[1]Hui-Xin Teh, [1]Sue-Mian Then, [1]Indah Maisarah Shani, [2]Musalmah Mazlan, [1,2]Wan Zurinah Wan Ngah and [1]Rahman Jamal

[1]UKM Medical Molecular Biology Institute, Universiti Kebangsaan Malaysia, Malaysia;
[2]Biochemistry Department, Faculty of Medicine, Universiti Kebangsaan Malaysia, Malaysia.

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ABSTRACT

The altered molecular mechanisms by experimental therapies for neurodegenerative diseases itself could be overlapped with genes induced by the behavioral task. We employed the microarray platform to identify the differentially expressed late responsive genes in medial temporal lobes of four Morris Water Maze (MWM) trained and untrained BALB/c mice. After MWM training, the mice were sacrificed to obtain their brains' medial temporal lobes. The total RNA was extracted from the tissues and global mRNA gene expression analysis was performed using Affymetrix GeneChip ®Mouse Gene 1.0 ST Array. There were 3635 (62.2%) up-regulated genes and 2206 (37.8%) down-regulated genes at p-values of < 0.05. These genes were operationally defined as late memory-related genes and behavior-related genes indicating that behavioral learning has a significant impact on the gene expression of the medial temporal lobes. From the pathway analysis, the network of memory and locomotion genes, and the guanylate cyclase pathway were identified as one of the most interesting pathways. The qPCR validation showed that the genes NMDA receptor 2a (*Nmda2a*) and cAMP dependent protein kinase type I beta regulatory subunit (*Prkar1b*) were up-regulated while adenylate cyclase 5 (*Adcy5*) was down-regulated. We proposed that the involvement of guanylate cyclase pathway in the long-term potentiation lasted at least up to three days after the MWM test. Present study suggested that the molecular mechanisms followed by spatial learning task could be altered up to three days or even longer, it could be overlapped with genes induced by further invented experimental therapy.

INTRODUCTION

The molecular mechanism induced by behavioral task, such as Morris water maze (MWM) provides important information before proceeding to the experimental therapies. Many experimental therapies on neurological disorder are combined with behavioral study [1-4] however the altered molecular mechanisms by experimental therapies itself could be overlapped with genes induced by the behavioral task. These genes could be identified by comparing the medial temporal lobes of trained versus the untrained groups, as a basic reference before proceeding to the experimental therapies.

MWM is a hippocampal-dependent task, one of the most common behavioral study approaches on neuroscience research [5]. In this learning paradigm, a mouse learns to locate a submerged island in a large pool by creating a spatial map using extra-pool cues [6]. Previous studies has described the gene expression profile in the animal brain in response to different behavioral tasks [7-9] and a study described the temporal expression profile of mouse hippocampus followed by MWM task [10]. These studies designed to elucidate the memory-related genes, in contrast, overall altered gene expression profile (not only memory-related genes) followed by behavioral tasks which has been less investigated. Memory formation and consolidation induced by spatial exploration such as the MWM were not only confined to the hippocampus or the amygdala, but also involved a variety of brain regions [9]. Previous studies has shown that immediate early learning genes (e.g. c-Fos, c-Jun, Zif268 and Arc) were also expressed in various regions of the cortex and expression of these genes in the hippocampus often fails to differential between spatial and control groups in the MWM training [11-14]. Therefore, we examined the late responsive gene induced by MWM training in the medial temporal lobes

* Correspondence to: then.sue-mian@nottingham.edu.my
rahmanj@ppukm.ukm.my

which comprise of the hippocampus, the dentate gyrus and the subicular complex, together with the entorhinal, perirhinal and parahippocampal cortices [15] to represent the overall changes of gene expressions that occurred three days after the MWM training.

Although both early and late responsive gene expression profiles provide us the complete understanding of molecular mechanism in response to MWM test, we focused on the late responsive genes in an attempt to explore the lasting effects of these genes, assuming that their molecular mechanisms would greatly affect the other parameters employed compare to genes that were immediately expressed. The profile of the early and late responsive genes followed by spatial learning task might be different, in particular, as short-term memory is rapidly formed, outlasts training for minutes or hours, and is based on transient modification of pre-existing molecules by rapidly altering the synaptic transmission efficiency. Meanwhile long-term memory lasts from hours to days, weeks or even years and is dependent on *de novo* gene expression and protein synthesis [16-18]. However, it should be clear that the differential expressed genes in the present study include not only memory-related genes.

A number of behavioral studies have implicated events centering on neurotransmitter systems, increased synaptic strength and downstream signaling events. Biochemical and pharmacological studies have implicated that the Notch signaling pathway, calcium-mediated signaling, and glutamate signaling pathway [5] were involved in learning and memory. Previous study has proven that over-expression of NMDA receptor 2B (NR2B) in the forebrains of mice leads to enhanced activation of NMDA receptors which facilitates synaptic potentiation. These mice exhibit superior ability in learning and memory in various behavior tasks, showing that NMDA receptor is critical in learning and memory formation. However, a large-scaled genome wide study approach is required to uncover more behavioral-related pathway. Although previous studies have implicated individual genes or molecular pathway in learning and memory, they did not uncover the collective behaviors and patterns of the genes. From our genome-wide gene expression study, we aim to elucidate pathways related to learning and memory by investigating enriched gene sets that are involved in the late responsive genes induced by the MWM training.

MATERIALS AND METHODS

EXPERIMENTAL GROUPS

This study was approved by the animal ethics committee of Laboratory Animal Resource Unit (LARU) UKM (UMBI/2010/RAHMAN/20-JANUARY/292-JANUARY-2010-JUNE-2011). A total of 20 Balb/c mice were used for the behavioral study and all of the mice that were used in this study were six months old males weighing between 25-30g. The mice were housed in individual cages on a 12:12 hour light-dark cycle, and these mice were randomly selected for MWM task (n = 10) and untrained control (n = 10). All mice were maintained in accordance with the standards specified by the Laboratory Animal Resource Unit of UKM. Three days after the completion of the MWM test, four trained mice and four untrained mice were selected randomly for the genome-wide gene expression study.

BEHAVIORAL OBSERVATION

In this learning paradigm, the trained mice learned to locate a submerged island in a large pool by creating a spatial map using extra-pool cues. The dimension of the water maze is 150 cm in diameter and 30 cm in height. An escape platform was hidden 1 cm below the water surface in a fixed location in one quadrant (quadrant 3) of the maze. The mice received 6 trials per test with 60 seconds intervals. On day zero, the mice would be allowed to swim freely for 60 seconds without a platform to acclimatize to the pool. In the place navigation task, the mice were trained to locate and escape onto the submerged platform from random release points around the pool on day one until day six. In the cued navigation task, the procedure is the same as place navigation task, but the platform is visible (2cm higher than the water level). If an animal could not find the platform in 60 seconds, it was placed on the platform for 10 seconds and then returned to the home cage. For the probe test, the platform was removed on day five and a 60 seconds probe trial was run to examine retention of spatial memory. The percentage of time spent in each quadrant was recorded. The untrained mice were not involved in any behavioral test.

TOTAL RNA ISOLATION

Three days after the training, mice were anesthetized with 90/10 mg/kg ketamine/xylazine and terminated by cardiac puncture. The whole brain of the selected mice were harvested, the medial temporal lobes were isolated and quickly frozen into liquid nitrogen. Total RNA were extracted from the medial temporal lobes of the mice brain using the RNeasy Plus Mini kit (Qiagen, USA). The concentration of RNA samples was ascertained and the quality of RNA was confirmed by detection of 18S and 28S bands using the Nano bioanalyzer (Agilent, USA).

AFFYMETRIX GENECHIP PROCESSING

cDNA was synthesized and amplified from the total RNA (Nugen Applause WT-Amp, USA). The amplified cDNA was purified by MinElute reaction cleanup kit (Qiagen, USA). The fragmented cDNA was then biotinylated and hybridized on the Mouse Gene 1.0 ST Array (Affymetrix, USA). The arrays were washed and stained by using a fluidics system with streptavidin-phycoerythrin, amplified with biotinylated anti-streptavidin antibody, and then scanned with a GeneChip® Scanner (Affymetrix, USA).

MICROARRAY DATA ANALYSIS

Quality control of probe array, normalization, filtering, and cluster analysis of the data were performed using the GeneSpring GX 10.0 software (Agilent, USA). The raw data from each array were normalized and each measurement for each gene was divided by the 75% percentile of all measurements. The adjusted intensity of the individual gene was analyzed by unpaired two-tailed t-test. All data are presented at statistical significance set at $p < 0.05$. Multiple testing correction was performed on the data obtained using the Benjamini-Hochberg procedure for false discovery rate. The Pathway Studio software 6.2 (Ariadne, USA) was used to perform gene set enrichment analysis (GSEA) using the Kolmogorov-Smirnov enrichment algorithm with 400 random permutations to determine statistical significance. The networks of differentially expressed genes were generated by Pathway Studio® software 6.2 from ResNet database of more than 500,000 functional relationships and the MedScan tool for automatic extraction of information from the scientific literature.

QUANTITATIVE REAL TIME PCR

Total RNA from medial temporal lobes of the mice brain were used to generate cDNAs using DyNamo ™ cDNA synthesis kit (Finnzymes, Finland). The qPCR was done using SYBR Green real time PCR master mix (Finnzymes, Finland) on a 7500 fast real time PCR system (Applied Biosystems, USA). The thermal cycling condition comprised of an initial step at 95°C for 15 min followed by 40 cycles of 94°C for 10 sec, 56°C for 25 sec and 72°C for 30 sec. All the primers were designed using Beacon Designer 7 software (Premier Biosoft International, USA). The primer sequences are shown in *supporting information 1*. The data were normalized by the quantity of beta actin (actb) mRNA. The cycle threshold values were used to calculate the fold change of gene expression using $2^{-\Delta\Delta ct}$ methods.

RESULTS

DIFFERENTIAL EXPRESSION PROFILE OF LATE RESPONSIVE GENES

The trained mice were successfully trained in the MWM task which included place navigation task, cued navigation task and probe test. All animals trained in the MWM task exhibited robust learning of platform location as indicated by the decrease in swim time to the platform in the trials after the first attempt (Figure 1).

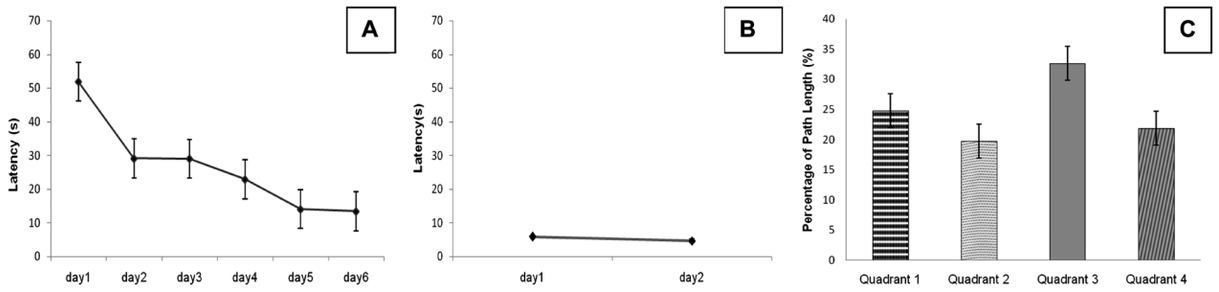

Figure 1: A) Place navigation task of trained mice. Mean (±SEM) latency for the trained mice to escape the hidden platform in the place navigation task (day 1-6). The trained mice improved after days. B) Cued navigation task of trained mice. Mean (±SEM) latency for the trained mice to escape the visible platform in the cued navigation task (day 7-8). The mice performed better in the cued navigation task compare to place navigation task. C) Path length in the target quadrant. Mean (±SEM) percentage of path length in the target quadrant (quadrant 3) during the probe test. MWM trained mice spent more time in the target quadrant

The gene expression profiles of the medial temporal lobes of the trained and untrained mice brain were measured using microarrays. The Box-Whisker plot of the log2-transformed data of all the 8 arrays showed that the means of the distribution of feature intensities were all equal to zero by using the GeneSpring 10.0 software (*Supporting Information 2*). Quality control of all the array samples was examined by values in Principal Component Analysis (PCA) mapping. The PCA map showed that the genes of two groups of samples (trained and untrained) were well distributed into their respective axes indicating that the expression pattern of genes from the two groups had significantly different trends (Figure 2). The expression pattern of genes of all MWM trained samples was more homogenous compare to the untrained samples, indicating that the MWM training resulted in significant changes in gene expression pattern of the medial temporal lobes of the brain. There was no outlier in the samples.

A hierarchical clustering algorithm was used to arrange the differentially expressed genes into up-regulated (red) and down-regulated (green) patterns. From the unpaired t-test with asymptotic computation and Benjamin-Hochberg multiple testing corrections, there were 3635 up-regulated genes (62.2%) and 2206 down-regulated genes (37.8%) at a cut-off p value of < 0.05 (Figure 3), the gene list is available as *supporting information 3* These differentially expressed genes were grouped into 3 clusters as shown in the top, middle and bottom of the heatmap under the supervised

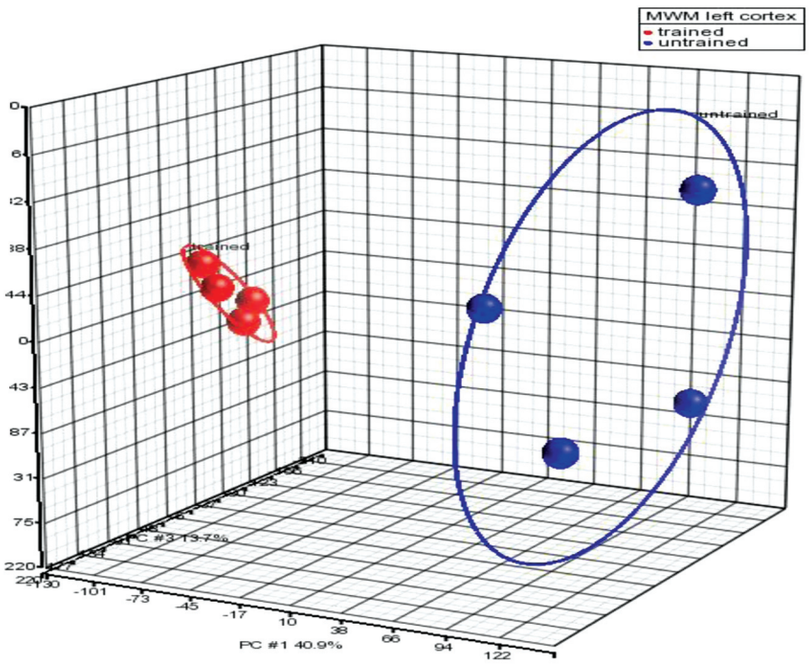

Figure 2: PCA of trained and untrained mice. The PCA mapping showing that the genes expressed from the medial temporal lobes of MWM of the trained (red) and untrained (blue) mice brain were well distributed into their respective axes. This indicated that the gene expression pattern from these two groups would be significantly different. The gene expression patterns of all MWM trained samples were more homogenous compared to the untrained samples, which indicate that the MWM training induced changes to the gene expression pattern of the medial temporal lobes of the brain

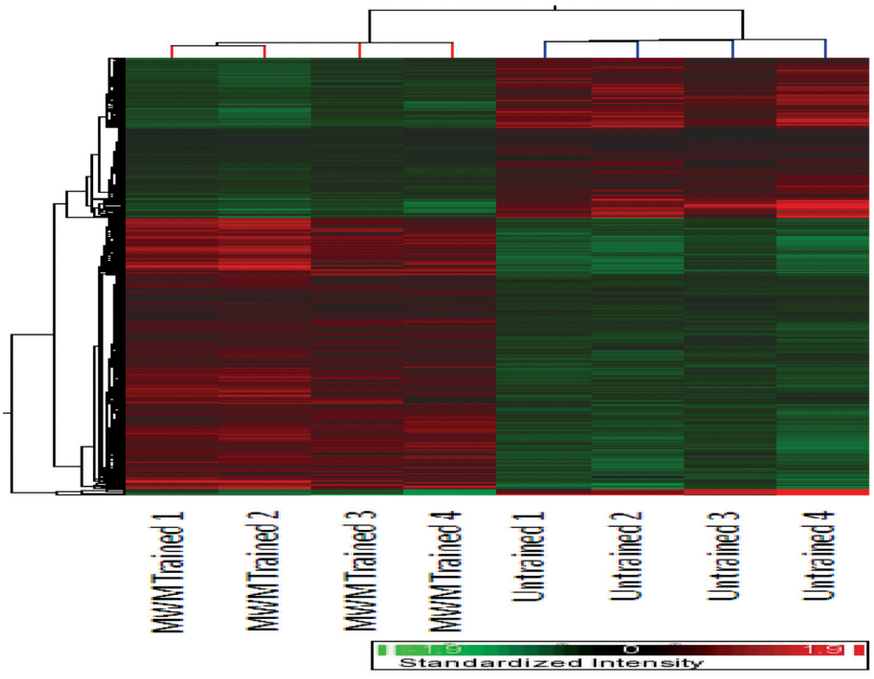

Figure 3: Heatmap of trained and untrained mice. Gene expression profiles of medial temporal lobes in trained and untrained mice were measured three days after completion of the training using microarray contained differentially expressed genes defined as memory and physical activity-related genes. The data represented the gene expression pattern of 4 biological replicates from two separate experimental groups (trained vs untrained). A hierarchal clustering algorithm was used to order the differential expressed genes into up (red) and down (green) regulated patterns. Each row represents a single gene and each column represents a biological replicate. The colour intensity reflects the magnitude of deviation from the median (see scale at the bottom)

hierarchical clustering. The clustered genes showed entirely different expression patterns between trained and untrained mice (Figure 2). Some differentially expressed genes, such as nitric oxide synthase (*Nos*), inositol 1,4,5-triphosphate receptor type 1 (*Itpr1*), microtubule-associated protein 2 (*Map2*) and $Ca^{2+/}$calmodulin-dependent protein kinase II (*CamkII*), synaptotagmins, syntaxins and (GABA) A and B type receptors (Table 1) were also found to be overlapped with early responsive memory-related genes previously shown in previous study [10] though the previous study compare the trained and the swimming control groups, but not the untrained group.

Table 1: List of significant differentially expressed genes associated with memory, locomotion and the guanylate cyclase pathway when trained mice were compared with untrained mice

Genbank number	*Gene symbol*	Gene name	P-value	Regulation	Absolute fold change
locomotion					
BC036127	*Atp1a2*	ATPase, Na+/K+ transporting, alpha 2 polypeptide	0,04	down	1,42
AF053471	*Atp2b2*	ATPase, Ca++ transporting, plasma membrane 2	0,04	up	2,34
BC094242	*Chrm1*	cholinergic receptor, muscarinic 1, CNS	0,04	down	1,28
BC144722	*Cln6*	ceroid-lipofuscinosis, neuronal 6	0,04	down	1,53
BC010588	*Ets1*	E26 avian leukemia oncogene 1, 5' domain	0,03	down	2,28
BC071233	*Psen1*	presenilin 1	0,01	up	1,71
U57324	*Psen2*	presenilin 2	0,01	down	1,60
Memory					
BC067025	*Accn2*	amiloride-sensitive cation channel 2, neuronal	0,04	down	1,95
BC020177	*Atp1a3*	ATPase, Na+/K+ transporting, alpha 3 polypeptide	0,04	up	2,08
AF360543	*Chst10*	carbohydrate sulfotransferase 10	0,04	down	2,43
D10028	*Nmda1*	glutamate receptor, ionotropic, NMDA1 (zeta 1)	0,03	down	2,96
D10217	*Nmda2a*	glutamate receptor, ionotropic, NMDA2A (epsilon 1)	0,03	up	1,70
BC053031	*Itga3*	integrin alpha 3	0,02	up	1,22
AB110830	*Prkcz*	protein kinase C, zeta	0,01	down	1,69
BC138682	*Rasgrf1*	RAS protein-specific guanine nucleotide-releasing factor 1	0,01	down	1,86
BC051950	*Srf*	serum response factor	0,01	down	4,06
Guanylate cyclase pathway					
BC050125	*Adcy1*	adenylate cyclase 1	0,04	down	1,06
BC057316	*Adcy3*	adenylate cyclase 3 | centromere protein O	0,04	down	1,48
M93422	*Adcy6*	adenylate cyclase 6	0,04	up	1,42
U30602	*Adcy9*	adenylate cyclase 9	0,04	up	1,18
AY061807	*Calml4*	calmodulin-like 4	0,04	up	1,76
BC014825	*Camk1*	Ca^{2+}/calmodulin-dependent protein kinase I	0,04	up	1,20
BC141413	*Camk1d*	Ca^{2+}/calmodulin-dependent protein kinase ID	0,04	up	1,31
AF428262	*Camk1g*	Ca^{2+}/calmodulin-dependent protein kinase I gamma	0,04	down	1,16
BC031745	*Camk2a*	Ca^{2+}/calmodulin-dependent protein kinase II alpha	0,04	down	1,79
BC080273	*Camk2b*	Ca^{2+}/calmodulin-dependent protein kinase II, beta	0,04	down	1,60
AF117384	*Camkk1*	Ca^{2+}/calmodulin-dependent protein kinase kinase 1, alpha	0,04	up	1,83
BC111033	*Camkv*	CaM kinase-like vesicle-associated	0,04	up	1,78
BC022632	*Gsk3a*	glycogen synthase kinase 3 alpha	0,03	down	1,68
BC003271	*Itpr1*	inositol 1,4,5-triphosphate receptor 1	0,02	down	1,54
D14552	*Nos1*	nitric oxide synthase 1, neuronal	0,02	up	1,53
BC089615	*Nppa*	natriuretic peptide precursor type A	0,02	down	6,64
BC042470	*Npr2*	natriuretic peptide receptor 2	0,02	up	1,31
BC054533	*Prkacb*	protein kinase, cAMP dependent, catalytic, beta	0,01	down	2,41
BC011424	*Prkar1b*	protein kinase, cAMP dependent regulatory, type I beta	0,01	down	2,41
Others					
BC054735	*Gabbr1*	gamma-aminobutyric acid (GABA-B) receptor, 1	0,03	up	1,70
BC099939	*Gabrg1*	gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 1	0,03	up	1,32
D45858	*Syt3*	synaptotagmin III	0,01	up	2,83
BC056949	*Stx3*	syntaxin 3	0,01	down	2,53
10479685	*Oprl1*	opioid receptor-like 1	0,02	up	2,13

GENE SET ENRICHMENT ANALYSIS (GSEA) UNVEILED GENE NETWORKS RELATED TO MEMORY AND LOCOMOTION

Gene set enrichment analysis (GSEA) was performed to group the differentially expressed genes according to their gene sets (Table 2). A total of 23276 differential expressed genes filtered by the criteria of false discovery rate of less than 0.05 in the GSEA (*Supporting Information 4*). The late responsive genes in this study were grouped into 762 gene sets (*Supporting Information 5*). The complete GSEA table generated by the Pathway Studio® software revealed that there were co-expressions of gene sets involved in learning and memory, physical activity and also stress response. Among the highly enriched and significant gene sets ($p < 0.05$), three gene sets discussed here are the memory, locomotion and guanylate cyclase pathway gene sets with their normalized enrichment scores of 1.31, 1.58 and 1.46 respectively. From the Pathway Studio®, a network of regulatory molecules and their target genes in memory and locomotion were generated with a total of 57 genes found in the memory gene set and 21 genes in the locomotion gene set.

Table 2: Gene Set Enrichment Analysis (GSEA) by Pathway Studio® for trained vs untrained mice. For convenience, 64 out of total of 762 gene sets were shown. Five of the gene products were listed in each gene set, the complete list of gene set and gene products of each gene set is available as *Supporting Information 4*

Gene sets	Genes	P-value	Normalized enrichment scores
Actin Cytoskeleton Regulation	GNGT2, IPP, FAM50A, ITGBL1, ACTL7B	<0.01	1,40
axon guidance	BOC, C1orf187, Enah, Prrxl1, RAC1	<0.01	1,67
axonogenesis	LPPR4, SLITRK3, SLITRK5, DSCAML1, SLITRK4	<0.01	1,88
calcium ion binding	AOC2, CACNB1, CACNG1, CALML3, ENTPD6	<0.01	1,53
Focal Adhesion Regulation	ITGBL1, PIK3R5, TAOK3, AIF1L, SHC4,	<0.01	1,63
Gonadotrope Cell Activation	CACNB1, CACNG1, CALML3, AP2S1, CLTB	<0.01	1,60
Guanylate Cyclase Pathway	ATP1B3, CALML3, AP2S1, CLTB, GABRR2	<0.01	1,46
inositol-polyphosphate 5-phosphatase activity	Inpp5j, INPPL1, INPP5B, INPP5D, INPP5A, SYNJ2	<0.01	1,61
ionotropic glutamate receptor activity	GRID1, GRIK4, GRIN1, GRIK3, GRIK2	<0.01	1,72
locomotory behavior	CHRNB4, C1QL1, SOBP, Grm6, NRG1	<0.01	1,57
neurotransmitter secretion	NRXN2, SNAP25, SYT1, NSF, NOS1AP	<0.01	1,67
neurotransmitter transport	SLC6A16, SLC6A15, SLC6A17, Slc6a20b, Slc17a7	<0.01	1,79
NK Cell Activation	CALML3, AP2S1, CLTB, IFNA2, TSPAN31,	<0.01	1,63
Skeletal Myogenesis Control	CALML3, PIK3R5, TAOK3, PLCXD1, ITPKC,	<0.01	1,39
synaptic transmission	CHRNA5, GABRR2, GABRG3, GRIK4, TAAR5	<0.01	1,67
Gap Junction Regulation	GPR183, GNGT2, GPR18, Gucy1b2, SGSM3	<0.01	1,34
Wnt receptor signaling pathway	FBXW4, TCF7L1, RSPO4, CCDC88C, Peg12	<0.01	1,53
integrin binding	EGFL6, MFGE8, TIMP2, VCAM1, ICAM1	<0.01	1,61
regulation of synaptic plasticity	ADORA1, BDNF, PLAT, GRIN1, ADORA2A	<0.01	1,71
learning	GRIN1, JUN, FOSL1, PARK2, COMT, PTN	<0.01	1,65
Notch signaling pathway	DTX4, DTX3, Aph1c, NRG1, ADAM17	0,01	1,57
neurological system process	Nkx1-1, CHRNA4, CACNA1A, ATG7, GPR98	0,01	1,69
glutamate receptor activity	GRIK3, GRM1, GRM4, GRM8, GRM5	0,01	1,60
amyloid precursor protein catabolic process	ABCG1, PSEN1, CLN3, PSEN2, NCSTN	0,01	1,56
memory	PTGS2, IL1B, PTEN, NGF, IGF1	0,01	1,55
post-translational protein modification	UBE2W, UBE2O, AKTIP, UBE2Z, UBE2Q2	0,02	-1,47
locomotion	PSEN1, ATP1A2, ATP2B2, PSEN2, GRIN2A	0,02	1,58
neurotransmitter metabolic process	CCDC92, CACNA1A, CLN3, DYNLL1, GCHFR	0,02	1,52
GABA-A receptor activity	GABRR2, GABRG3, GLRA2, GABRQ, Glra4	0,02	-1,52
regulation of synaptogenesis	GRIN1, SNAP25, PVRL1, GHSR, CHRNB2	0,03	1,46
syntaxin binding	NAPB, CAV1, NAPA, NSF, SPTAN1	0,03	1,49
regulation of synaptic transmission	PARK2, NTF3, DRD2, PPP3CA, CSPG5	0,04	1,49
glutamate signaling pathway	GRIK4, GNAQ, GRIK3, GRIK2, GRIN2B, SSTR1	0,04	1,44
neuropeptide signaling pathway	GLRA2, LPHN3, ELTD1, GPR112, GPR113	0,04	1,32
synaptogenesis	GLRA2, NRXN2, PCDHB5, PCDHB14, PCDHB13	0,05	1,46
glutamate metabolic process	CCDC92, GCLC, NAGS, GCLM, SLC1A3	0,05	1,45

We then took a closer look into each entity by applying regulation, expression and direct regulation parameters, the network interaction of the regulatory molecules and their target genes from these two biological processes, memory and locomotion was generated (Figure 4). From the network generated, a total of 59 genes were found to be connected in this network after excluding the unconnected genes. A total of 218 relations were formed by these 59 genes. The complete relation table of each gene was available in *Supporting Information 6*, suggesting that this network is related to the late responsive genes and their interaction is essential for control of spatial learning task. A full list of entities involved and the differential expression values in this network is available in *Supporting Information 7*. In this network, five entities that showed higher connectivity level (those which formed more than five relations with other entities in this network) were Glutamate N-methyl D-aspartate receptor, dopamine receptors, insulin-like growth factor 1 (*Igf1*), interleukin-1 beta (*Il1b*) and nerve growth factor (*Ngf*).

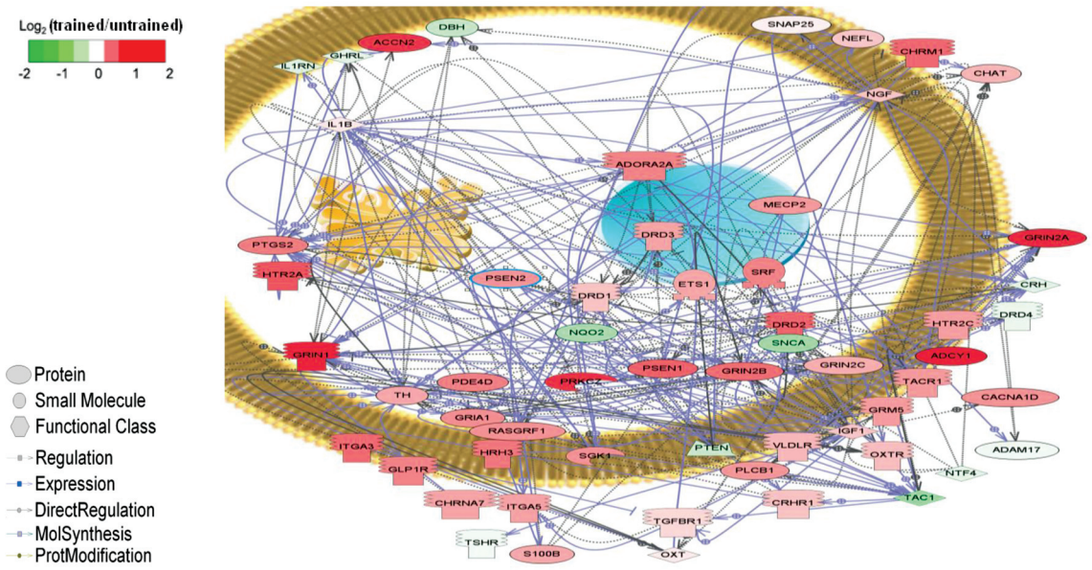

Figure 4: Network of memory and locomotion gene sets. Pathway analysis from the Pathway Studio® software revealed interaction between regulatory molecules and their target genes from two biological processes, which were memory and locomotion gene sets. These interaction includes regulation, expression and direct regulation parameters. The genes showing the highest connectivity level in memory and locomotion network were *Nmda1*, *Nmda2b*, *Drd2*, *Igf1*, *Il1b* and *Ngf*. Dark red represent genes with higher increased expression and light red represent genes with lower increased expression and grey represent genes that were not spotted in the microarray

THE INVOLVEMENT OF LATE RESPONSIVE GENES IN GUANYLATE CYCLASE PATHWAY

Our study also revealed the involvement of the commonly studied guanylate cyclase pathway in the late responsive gene expression profile. The interactions between the regulatory molecules in the guanylate cyclase pathway and their target genes are shown in Figure 5. In our study, there were 21 down-regulated and 41 up-regulated genes in the guanylate cyclase pathway (*Supporting Information 8*). These genes include adenylate cyclases (*Adcy*), *Camk*, *Itpr*, *Nos* and cAMP dependent regulatory protein kinases / Protein Kinase A (Pka).

VALIDATION OF MICROARRAY DATA BY QRT-PCR

In order to verify the microarray data, qRT-PCR assay was performed on three candidate genes namely, NMDA receptor 2a (*Nmda2a*), cAMP dependent protein kinase type I beta regulatory subunit (*Prkar1b*), and adenylate cyclase 5 (*Adcy5*) (Figure 6). The results of the validation assay showed the up-regulation of *Nmda2a* (representative of glutamate ionotropic receptors members), and *Prkar1b* (representative of PKAs) which confirmed the microarray results. However, the qRT-PCR showed the down-regulation of *Adcy5*, which was shown to be up-regulated in the present microarray data.

DISCUSSIONS

The differentially expressed genes which were operationally defined as late responsive genes indicated that spatial learning task has a significant impact on the gene expression of the brain's medial temporal lobes. The differentially expressed genes in hippocampus reported from a previous study, [10] namely *Nos*, *Itpr1*, *Map2*, synaptotagmins, syntaxins and (GABA) A and B type receptors, were similarly expressed in this study suggesting that the expression of these genes might be sustained up to

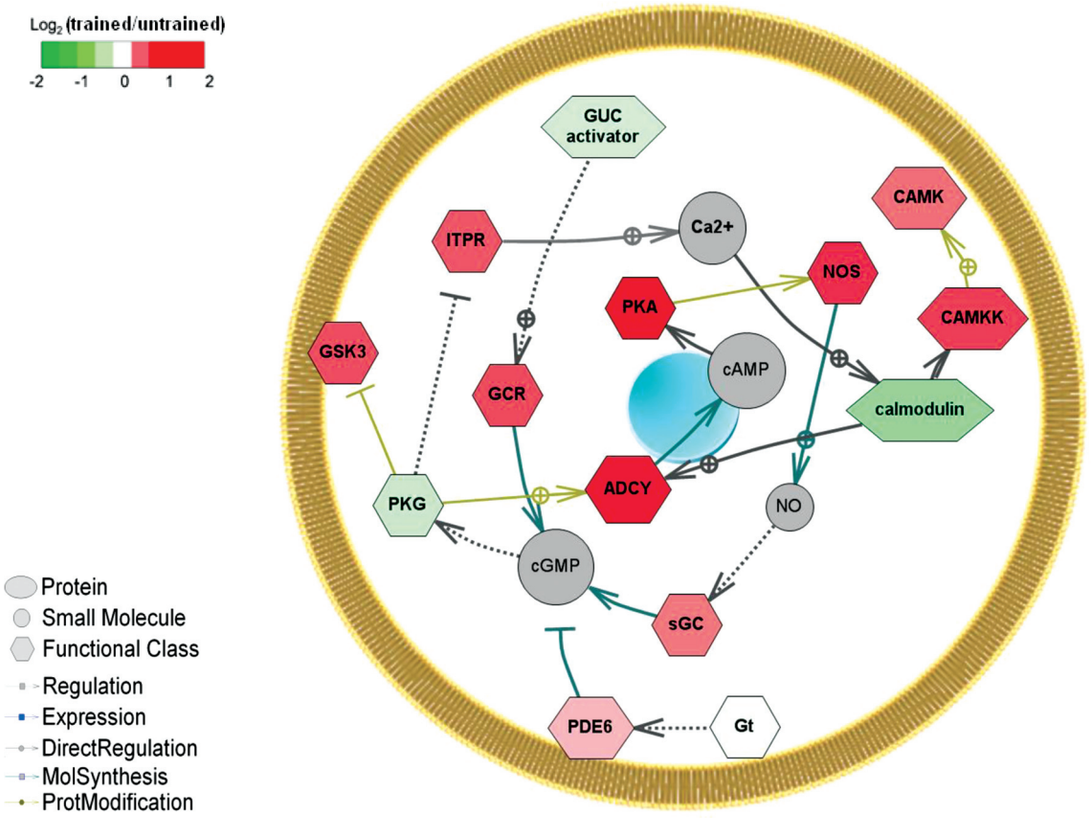

Figure 5: Guanylate cyclase pathway. Pathway analysis revealed the involvement of guanylate cyclase pathway in late responsive gene expression profile. The network showed interaction between the regulatory molecules, including NOS, PKA, cGMP-dependent protein kinase (PKG), ITPR, soluble guanylate cyclase (sGC), ADCY, NO, CAMK, guanylate cyclase activator (GUC activator), G protein alpha, transducing activity (Gt), guanylyl cyclase receptor (GCR), phosphodiesterase 6 (PDE6), glycogen synthase kinase 3 (GSK3), CaM kinase kinase (CAMKK) and their target genes. Dark red represent genes with higher increased expression and light red represent genes with lower increased expression and grey represent genes that were not spotted in the microarray

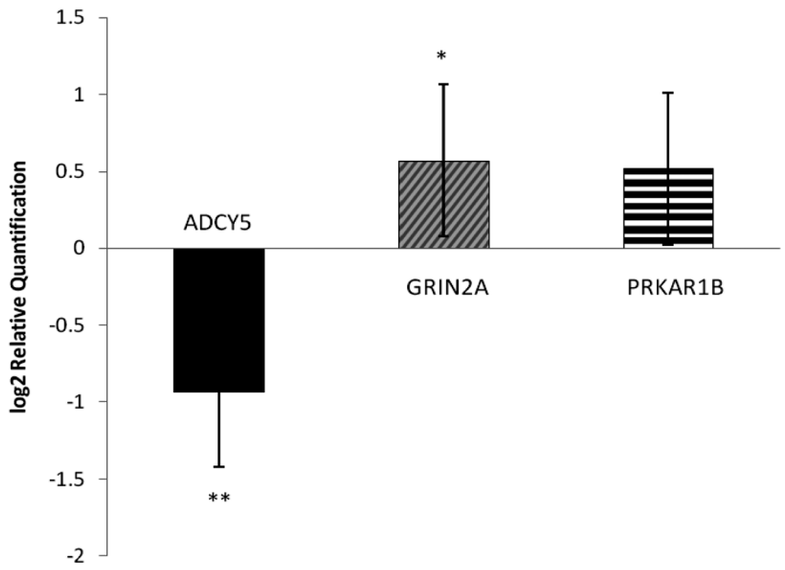

Figure 6: qRT-PCR confirmation of microarray results. Four biological replicates were analyzed per group for each gene by qRT-PCR, with a technical replicate of three for each sample. The graph shows the average $\log_2$ ratios obtained for each gene for the trained vs untrained samples analyzed by qRT-PCR. Data are presented as means of ± SEM. *Denotes $P < 0.05$ compared to untrained mice while ** denotes $P < 0.01$ compared to untrained mice

few days or even longer after the MWM training. However, there were also some differences in our study compared to the previous study: we found that the gene expression opioid receptor-like receptors increased after three days of MWM training while from the previous study, the gene expression of the opioid receptor-like receptor was down-regulated one hour after the MWM training [10]. Our results suggest that the MWM training induced a similar set of genes which may change over a period of time; certain genes which were down-regulated at the earlier time point right after the training (e.g. 1 hr or 24 hr) may be up-regulated after longer period of after three days of MWM training and vice versa.

The correlation between locomotion and memory formation has been accepted for the last few decades [19-21]. Previous studies have reported that physical exercises induce neuronal growth and synaptogenesis in animal models [22-24]. Locomotion serves as an intrinsic feedback mechanism by signaling to the brain, hence contributing to memory formation [24]. From our findings, one of the entities involved in this network and which formed extensive relationship with other entities is the glutamate ionotropic receptors. In this study, NMDA receptor 1 and NMDA receptor 2 genes were both found to be up-regulated three days after the MWM training. The NMDA receptor is one of the major glutamate receptor types that play an important role in neuronal plasticity by mediating most of the excitatory synaptic transmission in the central nervous system [25-27]. From a previous study on hippocampus, NMDA receptor 1 gene was found to be down-regulated 1 hour after MWM training, where as NMDA receptor 2 gene was up-regulated after 24 hour [10]. However Tang et al. (1999) reported that over-expression of NMDA receptor 2B (*Nmda2b*) gene in the forebrains of mice led to enhanced activation of NMDA receptors and they exhibited superior ability in performing various behavioral tasks. Our microarray and qRT-PCR results showed that the mRNA expression of *Nmda2a*, which is representative of NMDA receptors, was up-regulated. This concurred with the results from a previous study which reported that the over-expression of different NMDA subunits play a role in specific form of spatial memory [29].

Another entity in the network is the dopamine receptors, which was also found to be up-regulated after the MWM training. It is well established that dopamine receptors play a role in hippocampal synaptic plasticity and are involved in long term potentiation and memory formation [30-32]. As with the other dopamine receptors, dopamine receptor 2 (DRD2) connects with their second messenger generating enzymes and ion channels via the G-protein [33, 34]. DRD2 play a role in characterizing the action of dopamine, a neurotransmitter on synaptic plasticity, hence contributing to the long term synaptic potentiation [31]. *Igf1* which was up-regulated in this study has a role in the proliferation and the survival of neurons and oligodendrocytes numbers, promotion of neuritic outgrowth, synaptogenesis and myelination [35]. The up-regulation of *Il1b gene expression* found in this study is consistent with a previous study that showed IL1B improving the performance of passive avoidance conditioning test in the rodents [36]. IL1B is synthesized and released in the brain by glial cells and neurons, and affects neural plasticity and memory processes [37]. The *Ngf* which was up-regulated in this study is the most important target-derived trophic factor for basal forebrain cholinergic neurons [38] and is crucial for the improvement of spatial learning memory in rodents [39, 40].

The guanylate cyclase pathway plays an important role in long term potentiation, which is a stable process and causes a long-lasting increase in the efficiency of synaptic transmission [41, 42]. Our study revealed the increased mRNA expression of *Nos1*, *Prkar1b* and *Camk*, suggesting that the *de novo* expressions of these genes are crucial in the guanylate cyclase pathway. In this pathway, nitric oxide (NO) is synthesized post-synaptically by NOS, a Ca^{2+}/calmodulin-dependent enzyme [43] which is also found in several parts of the brain such as the cerebellum, hypothalamus, striatum, cerebral cortex, and hippocampus [44]. NO is a powerful activator of the cyclic GMP/cGMP-synthesizing enzyme soluble guanylyl cyclase [43] and it has been suggested that the activation of soluble guanylyl cyclase may be a major pathway for the NO messenger function in the brain [45]. In this pathway, the protein kinase A (PKA) stimulate the voltage-gated Ca^{2+} influx, leading to the increase in intracellular Ca^{2+} concentration and subsequently the stimulation of NOS [46, 47]. The qPCR validation on *Prkar1b* as representative of PKA showed the over-expression of *Prkar1b* hence suggesting that it is a late responsive gene. Previous studies have reported that PRKAR1B is required for long term depression (LTD) and depotentiation which contribute to synaptic plasticity of the brain [48, 49].

Meanwhile, the ITPR which is present on the endoplasmic reticulum functions as a Ca^{2+} releasing channel [50]. CAMK which responds to the physiological changes of intracellular Ca^{2+} concentration is involved in the regulation of diverse functions such as post-synaptic responses and neurotransmitter synthesis [51, 52]. We also noted other entities which were up-regulated in this pathway namely *Adcy*, *Itpr* and *Camk*. There are multiple isoforms of ADCYs which are uniquely regulated by protein kinases (such as PKA and PKC), G proteins, Ca^{2+} and calmodulin. For example, ADCY1 and ADCY8 are Ca^{2+}-stimulated enzymes, where as Ca^{2+} inhibit the ADCY3 and the ADCY5 [53-55]. Validation by qRT-PCR in this study showed the down-regulation of Adcy5 in MWM trained mice (Figure 5). Previous studies also showed the inhibition of ADCY5 by increased Ca^{2+} concentration [56, 57] which could be induced by physical activity [58]. In the present study, we believed that increased Ca^{2+} level induced by spatial learning task results in the inhibition of ADCY5, causing the down-regulation of ADCY5.

Taken together, present study suggested that the molecular mechanisms followed by spatial learning task

could be altered up to three days or even longer, it could be interfered or overlapped with genes induced by further invented experimental therapy. Although guanylate cyclase was previously reported to be an important pathway of long term potentiation [59], the duration of this molecular pathway after the spatial learning task was uncertain. In this study we have demonstrated the involvement of the guanylate cyclase pathway as well as the locomotion and memory networks of the medial temporal lobes of the brain for up to three days or even longer after MWM behavioral task. Although in this study we analyzed the whole medial temporal lobes of the brain (rather than extracting tissues from specific regions in the brain, such as hippocampus, cerebral cortex or frontal lobe), significant pathways related to behavior induced by the MWM test were uncovered. This suggested that the altered molecular mechanisms contributed by spatial learning task may not be just confined to hippocampus but also other parts of the medial temporal lobes. The molecular pathways presented in this study revealed novel connectivity of the genes related to locomotion and memory and verified the involvement of previously mapped guanylate cyclase pathway.

SUPPORTING INFORMATION

SUPPORTING INFORMATION 1

https://docs.google.com/spreadsheet/ccc?key=0AlQwYGmBlZi4dFQtNVFZMXdYMTVwUTEtajZxU2dCR3c&usp=sharing

SUPPORTING INFORMATION 2

https://docs.google.com/spreadsheet/ccc?key=0AlQwYGmBlZi4dHZ3NVd6UDlWY1VNNnRTR0JSOFJYYUE#gid=0

SUPPORTING INFORMATION 3

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SUPPORTING INFORMATION 6

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SUPPORTING INFORMATION 7

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SUPPORTING INFORMATION 8

https://docs.google.com/spreadsheet/ccc?key=0AlQwYGmBlZi4dGY2M3lkaE9yTk92VHNKd25QRW14SEE&usp=sharing

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