bodies, degraded mitochondria and synaptic vesicles. Most of these share several critical subcellular alterations with AD. They suggested that plaque formation was initiated with local synaptic alterations induced by possible abnormalities in APP processing and Aβ formation. Furthermore Brion suggested that synaptic vesicle accumulation in dystrophic neurites might witness for axoplasmic flow disturbances in these neurites since synaptic vesicles would move along microtubules [3]. The c-Jun N-terminal kinase (JNK)-interacting protein-1b/islet-brain-1 (JIP-1) is also an adaptor protein which interacts with ApoER2 in the Reelin signaling pathway [19]. JIPs and APP also bind to the anterograde molecular motor kinesin [19]. Thus, apoER2 may be involved in the disturbance of the axonal flow leading to neurodegeneration.

ApoER2 antibody did not immunostain the tau-positive dystrophic neurites. One hypothesis has been suggested that Reelin/apoE-receptor/Dab1 complex may initiate a signal transduction cascade that controls tau phosphorylation [6]. Our results did not support this hypothesis. Our results indicate that apoER2 may play a role in disturbing axonal flow rather than paired helical filament formation.

Increasing evidence suggests that apoER2 may function as a signal transduction rather than receptor-mediated endocytosis. The first the binding affinity of RAP (the receptor-associated protein, a substrate for receptor-mediated endocytosis) to apoER2 was 25-fold lower than the binding affinity of Reelin [1]. The second ApoER2 contains one distinct structural feature in its cytoplasmic domain, encoded by a single exon, containing three PXXP motifs. PXXP motifs bind src homology domains, most of which are found in proteins involved in signal transduction pathways. In addition chimeras comprising the ectodomain and transmembrane domain of the LDL receptor fused to the cytoplasmic domain of apoER2 lacking the PXXP motifs are able to mediate clathrin-dependent endocytosis of LDL but not if the PXXP motifs is present in the protein [17]. Although the role of the Reelin pathway in the adult brain is not precisely known, there is one hypothesis that the binding of Reelin to apoER2 is inhibited by recombinant apoE [5]. Further studies will be needed to elucidate the underlying mechanism by which apoE4 predisposes its carriers to AD.

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ORIGINAL RESEARCH ARTICLE

Molecular characterization of bipolar disorder by comparing gene expression profiles of postmortem brains of major mental disorders

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We performed the oligonucleotide microarray analysis in bipolar disorder, major depression, schizophrenia, and control subjects using postmortem prefrontal cortices provided by the Stanley Foundation Brain Collection. By comparing the gene expression profiles of similar but distinctive mental disorders, we explored the uniqueness of bipolar disorder and its similarity to other mental disorders at the molecular level. Notably, most of the altered gene expressions in each disease were not shared by one another, suggesting the molecular distinctiveness of these mental disorders. We found a tendency of downregulation of the genes encoding receptor, channels or transporters, and upregulation of the genes encoding stress response proteins or molecular chaperons in bipolar disorder. Altered expressions in bipolar disorder shared by other mental disorders mainly consisted of upregulation of the genes encoding proteins for transcription or translation. The genes identified in this study would be useful for the understanding of the pathophysiology of bipolar disorder, as well as the common pathophysiological background in major mental disorders at the molecular level. In addition, we found the altered expression of *LIM* and *HSPF1* both in the brains and lymphoblastoid cells in bipolar disorder. These genes may have pathophysiological importance and would be novel candidate genes for bipolar disorder.
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Keywords: microarray; schizophrenia; depression; lymphoblastoid

Introduction

Bipolar disorder is a severe mental disorder characterized by recurrent manic and depressive episodes. Twin, adoption, family and linkage studies suggested that bipolar disorder is a complex disease caused by multiple genetic and environmental risk factors.[1] Pharmacological evidence suggests the involvement of monoaminergic systems and intracellular second messenger systems in bipolar disorder.[2,3] However, the etiology of bipolar disorder has not been established. The uncertainty of phenotype definition and complex mode of inheritance impede the understanding of bipolar disorder at the molecular level by conventional strategies.

Genome-wide gene expression analysis using DNA microarray, by which expression of thousands of genes can be monitored, has a great advantage to identify the genes or specific molecular cascades involved in the complex diseases, especially mental disorders.[4] Pioneering studies, which examined the postmortem brains of patients with schizophrenia by DNA microarray, revealed the downregulation of the genes encoding proteins functioning at the presynapses,[5] genes expressed in the oligodendrocytes,[6] neuropeptide Y,[7] or the upregulation of apolipoprotein genes in schizophrenia.[8] On the other hand, there is one DNA microarray study of bipolar disorder, which revealed the altered expression of the genes encoding the signal transduction proteins.[9]

Bipolar disorder shares signs and symptoms of depressive episodes with major depression, and also shares clinical features with schizophrenia, such as their chronic and relapsing course, and psychotic symptoms.[10] The molecular basis of the uniqueness of bipolar disorder and its similarity to the other two disorders remains to be studied. Here, we carried out the microarray analysis in bipolar disorder, major depression, schizophrenia, and control subjects using postmortem frontal cortices provided by the Stanley Foundation Brain Collection. The differentially expressed genes in bipolar disorder were compared with those in other mental disorders.

Among the genes whose differential expressions in the brains were confirmed by RT-PCR, the expressions of *LIM* and *HSPF1* were also altered in lymphoblastoid cells of patients with bipolar disorder. These genes may have some pathophysiological importance such as the genetic abnormality in bipolar disorder.

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Materials and methods

Brain samples

Samples of postmortem prefrontal cortex (Broadmann's Area 10) were donated by the Stanley Foundation Brain Collection. They were derived from patients with bipolar disorder, major depression, schizophrenia, and control subjects. Each group consisted of 15 subjects. Diagnoses had been made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition.[11] A summary of the demographic information of subjects used in this study is shown in Table 1. Detailed information of the original set of subjects was described elsewhere.[12]

Microarray procedure

Total RNA was extracted from 0.1 g of frozen tissues using Trizol (Invitrogen, Groningen, The Netherlands). After cleaning up using an RNeasy column (Qiagen, Hilden, Germany), the purity and integrity of total RNA was evaluated by OD measurements and denaturing agarose gel electrophoresis, respectively. Microarray analysis was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Briefly, 8–10 μg of total RNA was used to synthesize cDNA. This was used to generate biotinylated cRNA. cRNA was fragmented and first applied to the Test2Chip (Affymetrix) to assess the sample

Table 1 Summary of the demographic variables of subjects used in this study

	N	*Age (years)*	*Gender*	*PMI (h)*	*Medication*	*Cause of death*
			Male : female		*Medicated : nonmedicated*	*Suicide : nonsuicide*
Postmortem brains						
Control subject	15	48±11	9M:6F	24±10	0M:15NM	0S:15NS
Bipolar disorder	11	39±12	8M:3F	32±16	9M:2NM	8S:3NS
Major depression	11	46±10	6M:5F	27±12	9M:2NM	4S:7NS
Schizophrenia	13	44±14	8M:5F	33±15	10M:3NM	4S:9NS
Lymphoblastoid cells						
Control subject	11	51±10	8M:3F	—	—	—
Bipolar disorder	14	53±11	9M:5F	—	—	—

Table 2 Primer sequences used in this study

	Size (bp)	*Forward primer (5′ to 3′)*	*Reverse primer (5′ to 3′)*
GAPDH	107	ATCATCAGCAATGCCTCCTGC	ATGGCATGGACTGTGGTCATG
Beta actin	152	ATTGCCGACAGGATGCAGAA	TTGCTGATCCACATCTGCTGG
Cofilin	73	CGCCCCTTAAGAGCAAAATG	TGCAATTCATGCTTGATCCC
HSP27	113	GTCCCTGGATGTCAACCACTTC	AGATGTAGCCATGCTCGTCCTG
IFITM3	90	TCGTCTGGTCCCTGTTCAACA	TCCTGTCCCTAGACTTCACGGA
CEBPD	101	CATCGACTTCAGCGCCTACAT	CCTTGTGATTGCTGTTGAAGAGGT
APM2	96	TGACGACTCCACAGATACCCC	GCTGACACGGCTTCCTGG
AQP4	144	TGTCTTCTACATCGCAGCCCA	TCAACCAGGAGACCATGACCA
HEPH	237	AGGAAAATGTGGCAACCCATG	TCTCGCCATTCCGATAGAGGA
LIM	111	TCCTTGGAGAAGTCATCAATGC	CACCATCCTCCAAGTGAAAAAC
KIAA0133	137	AGTATGGAAGCGTCTTCCCGA	CCCTTCCCGCATAACTGAAAA
CACNA1A	90	ACCTCAGTACCATCTCAGACACCAG	CCAGCGAGTAATCGTCCAGG
GRM1	99	TCTGTGAAGGCATGACAGTGCG	TGTCTGCCCATCCATCACTTC
GRIK1	86	GGCGGTTAGAGATGGATCAACA	TGCTGCTCATGAAAGCCCA
DTNA	134	TGTCTTCCATCCGGTTGAGTG	TATGAGAACCACCGGCATGTC
HSP40	139	TGATGTCATTTATCCTGCCAGG	AGGAACTTTTCGCCGCATG
DKFZp564H203	84	GCAGACAGAAAACCTCAACCCC	AACAAGCACTTCCTGTGCCCAG
COL16A1	89	GACATTGGTATTGGCATTGCAG	GTTGCACCCATCTTGCCAT
SPRAC	113	AATGACAAGTACATCGCCCTGG	GAGAATCCGGTACTGTGGAAGG
BTN3A3	144	TACGCTGCAACAGAGCAAGAA	CACATCCGCAGGTTTGAAGA
TAF6L	81	GGACTTGCAGACGAACTCCAA	TGGCTTACAGATTTCACCCCA
GOLGA4	128	GGACACCTTACAAAGGTGGCAA	TGCCATGGTCTTAGTCTCACGA
KIAA0645	154	GACCCTGGATGTTGACGTGAA	CAACCTTGCACCATCTCGAAG
KDR	94	AGAGCCGGCCTGTGAGTGTAAA	CCACTGTCCGTCTGGTTGTCAT

quality, and then applied to the HU95A chip (Affymetrix), which contains probes for about 12 000 genes. The hybridization signal on the chip was scanned using a scanner (HP GeneArray scanner, Hewlett-Packard, Palo Alto, CA, USA), and was processed by GeneSuite software (Affymetrix).

Microarray data analysis

The gene expression data generated by microarray analysis were imported into GeneSpring 5.0. software (SiliconGenetics, Redwood, CA, USA). Data of each array were then normalized by dividing the median of its gene expression value. Data were then filtered based on the following criteria: (i) genes marked as present (detected) in at least half of the samples in each diagnostic group, (ii) genes that showed their expression changes were not associated with aging, postmortem interval (Pearson's correlation coefficient, $P \geq 0.05$), and gender (Student's *t*-test, $P \geq 0.05$). Of approximately 12 000 genes, 5138 genes passed these filtering procedures. Differentially expressed genes compared with the control group were defined based on the following criteria: (i) 1.3-fold or greater change in the mean expression level, (ii) $P < 0.05$ in the two-tailed Student's *t*-test. Statistical analysis was performed using SPSS 10.0J software (SPSS Co. Ltd, Tokyo, Japan).

Consideration of the effects of medication status, alcohol abuse, and drug abuse on the gene expressions in bipolar disorder

The possible effect of each class of medication, as a confounding factor for genes differentially in bipolar disorder, was considered by dividing all subjects regardless of their diagnoses ($N=50$; see the Results and discussion section) into medicated and nonmedicated groups. Two-tailed Student's *t*-test was employed in the statistical analysis ($P<0.05$ was considered to be significant). The medication status considered was as follows: lithium-treated ($N=6$) and nontreated groups ($N=44$), anticonvulsants treated ($N=4$) and nontreated groups ($N=46$), antidepressants treated ($N=13$) and nontreated groups ($N=37$), antipsychotics treated ($N=16$) and nontreated groups ($N=34$), benzodiazepine treated ($N=6$) and nontreated groups ($N=44$). In case of the consideration of the effects of alcohol abuse and drug abuse, all subjects were divided into two groups according to the qualitative ratings (on a 1–5 scale) of severity of abuse: moderate to severe use (ratings 3–5) of alcohol ($N=10$) and none or light use (ratings 1–2) groups ($N=34$), moderate to severe use of drugs ($N=12$) and none or light use ($N=38$) groups. Some subjects whose status of alcohol use was not available were excluded from the analysis.

a

accession	gene	*R*	*P*	*N*
AF060538	vesicle-associated membrane protein 1 (synaptobrevin 1)	0.356	0.011	50
M63138	cathepsin D	0.332	0.019	50
J04177	collagen, type XI, alpha 1	-0.324	0.022	50
AF038660	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2	-0.309	0.029	50

b

accession	gene	symbol	locus	male	*N*	female	*N*	M/F ratio	*P*
L25270	Smcx homolog, X chromosome	SMCX	Xp11	1.08	31	1.47	19	0.74	<0.001
AF000994	ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	UTY	Yq11	0.42	31	0.03	16	13.19	<0.001
U79247	protocadherin 11 X-linked	PCDH11X	Xq21	0.57	31	0.18	19	3.21	<0.001
AA669799	acetylserotonin O-methyltransferase-like	ASMTL	Xp22	1.54	31	1.10	19	1.40	<0.001
U52191	Smcy homolog, Y chromosome	SMCY	Yq11	1.01	31	0.11	18	9.48	<0.001
M88934	DNA segment, numerous copies, expressed probes (GS1 gene)	DXF68S1E	Xp22	0.41	31	0.64	19	0.63	<0.001
AF000984	DEAD/H box polypeptide, Y chromosome	DBY	Yq11	1.20	31	0.03	16	34.26	<0.001
AB018328	Ac-like transposable element	ALTE	Xp22	3.03	31	2.57	19	1.18	<0.001
AF000982	DEAD/H box polypeptide 3	DDX3	Xp11	1.31	31	1.76	19	0.75	<0.001
Y14391	Pseudoautosomal GTP-binding protein-like	PGPL	Xp22	2.39	31	1.73	19	1.38	<0.001
M16279	antigen identified by monoclonal antibodies 12E7, F21 and O13	MIC2	Xp22	1.55	31	0.73	19	2.13	<0.001
M58459	ribosomal protein S4, Y-linked	RPS4Y	Yp11	3.70	31	0.05	16	77.40	<0.001
Y15801	protein kinase, Y-linked	PRKY	Yp11	0.32	31	0.22	19	1.45	0.001
M58458	ribosomal protein S4, X-linked	RPS4X	Xq13	15.94	31	17.73	19	0.90	0.002
Y15521	acetylserotonin O-methyltransferase-like	ASMTL	Xp22	1.30	31	0.85	19	1.53	0.003
U78575	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	PIP5K1A	1q22-q24	0.89	31	1.10	19	0.82	0.004
X89887	HIR histone cell cycle regulation defective homolog A	HIRA	22q11	0.44	31	0.59	19	0.73	0.004
AB002354	KIAA0356 gene product	KIAA0356	17q21	0.51	31	0.67	19	0.77	0.004
AF051160	protein tyrosine phosphatase type IVA, member 1	PTP4A1	6q12	2.67	31	3.26	19	0.82	0.005
M58525	catechol-O-methyltransferase	COMT	22q11	1.20	31	1.46	19	0.82	0.005

Figure 1 Genes whose expressions were influenced by aging or gender. (a) Representative examples of the aging-influenced gene expressions, which were previously identified by microarray analysis of aging.[14] *R*, Pearson's correlation coefficient; *P*, probability value; *N*, number of the samples used for calculation. (b) List of the top 20 genes whose expressions were influenced by gender. The pairs of NRY gene and its X homologue are colored. The mean expression levels in males and females are shown. The values less than 1 in the M/F ratio means downregulation in males. The complete lists are available on request (kato@brain.riken.go.jp).

Lymphoblastoid cells

Lymphoblastoid cell lines of control subjects and patients with bipolar disorder were established by standard protocols.[13] A summary of demographic information of subjects is shown in Table 1. Briefly, lymphocytes were separated from peripheral blood using Ficoll-Paque (Pharmacia-Upjohn, Peapack, NJ, USA), and cultured with RPMI 1640 medium containing 20% fetal bovine serum (FBS), penicillin, streptomycin, cyclosporin A, and filtered supernatant of the B95-8 cell culture infected by Epstein–Barr Virus. The cells were subcultured every week until the cell line was established. Thereafter, the cells were subcultured three times a week using a similar medium, except for the addition of 10% FBS and no cyclosporin A. The cells were kept frozen until the experiment. After reculturing, total RNA of the cells was extracted using Trizol reagent and then was treated with DNase I. This study was approved by the Ethical Committee of the Brain Science Institute, RIKEN, and written informed consent was obtained from each subject.

Real-time quantitative PCR

A measure of 1–5 μg of total RNA was used for cDNA synthesis by oligo(dT) and SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR using SYBR/GREEN I dye (Applied Biosystems, Foster City, CA, USA) was performed with ABI PRISM 7700 or 7900HT (Applied Biosystems). The comparative Ct method was employed for quantification of transcripts according to the manufacturer's protocol (User Bulletin #2, Applied Biosystems). In addition to the two housekeeping genes conventionally used for normalization (beta actin and GAPDH), cofilin 1, which encodes actin-binding protein, was selected as a control gene by identifying a gene showing the constant expression levels by DNA microarray analysis across the samples (data not shown). Measurement of delta Ct was performed at least in triplicate. Amplification of the single product in RT-PCR was confirmed by monitoring the dissociation curve and by agarose gel electrophoresis. One-tailed and two-tailed Student's *t*-tests were employed in the statistical analysis of the results of postmortem brains and lymphoblastoid cells, respectively ($P<0.05$ was considered to be significant). The genes examined by RT-PCR were as follows: *HSP27*, *IFITM3*, *CEBPD*, *APM2*, *AQP4*, *HEPH*, *LIM*, *KIAA0133*, *CACNA1A*, *GRM1*, *GRIK1*, *DTNA*, *HSPF1*, *DKFZp564H203*, *COL16A1*, *SPARC*, *BTN3A3*, *TAF6L*, *GOLGA4*, *KIAA0645*, and *KDR*. They were chosen for the quantitative PCR analysis, since they were also identified as the differentially expressed genes by the preliminary data analysis based on different normalization procedures (data not shown). Primer sequences used for RT-PCR are listed in Table 2.

Results and discussion

Filtering procedures

Of the 60 samples initially analyzed, 10 were not suitable for DNA microarray analysis estimated by denaturing agarose gel electrophoresis or Test2Chip analysis. Thus, we could obtain gene expression profiles from 50 samples. These included 11 patients with bipolar disorder, 11 with major depression, 13 with schizophrenia, and 15 control subjects. Although there were no significant differences in the demographic variables among four groups, we removed the genes whose expressions were associated with aging, gender or postmortem interval (PMI). The genes previously reported to be altered with aging were confirmed, such as aging-dependent upregulation of cathepsin D and downregulation of collagen, type XI (Figure 1a).[14] The genes influenced by gender mainly consisted of the genes on the sex chromosomes (Figure 1b), including three nonrecombining region of the Y chromosome (NRY) class I genes. X homologues of NRY class I genes were also highly expressed in females, which is compatible with the finding that they are not subject to X

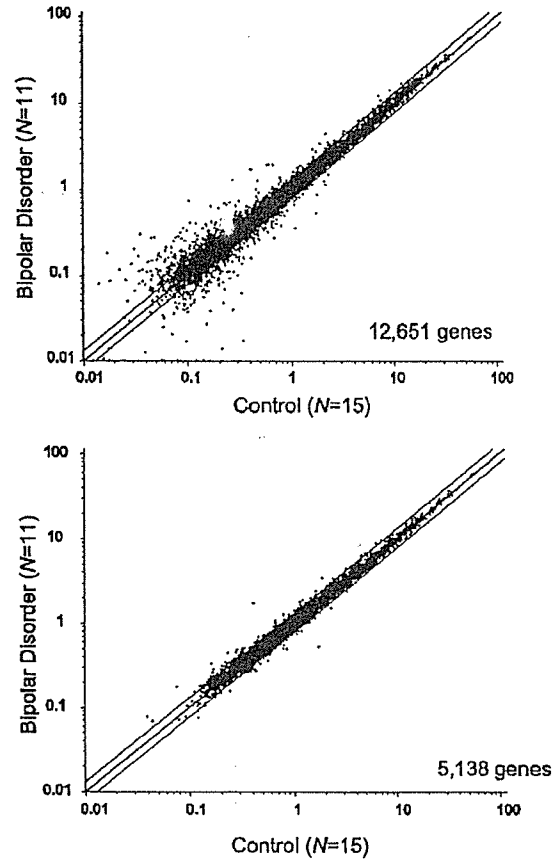
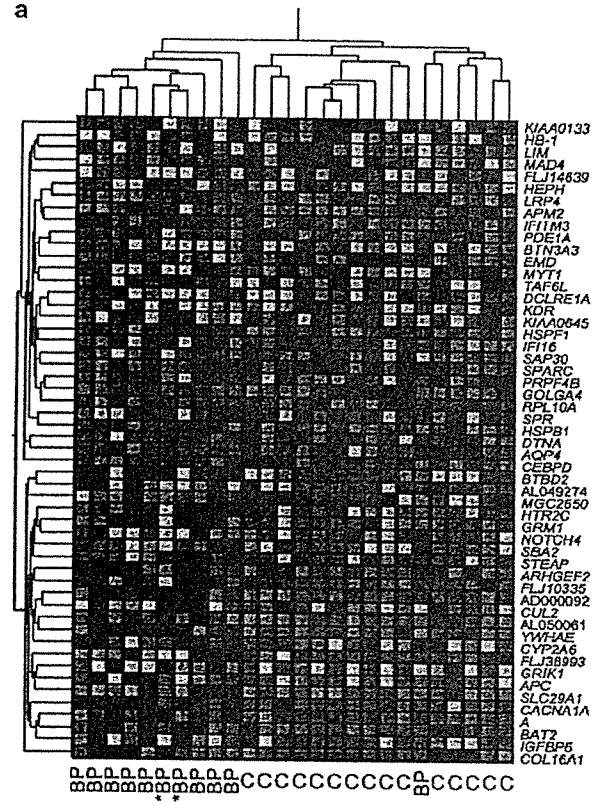
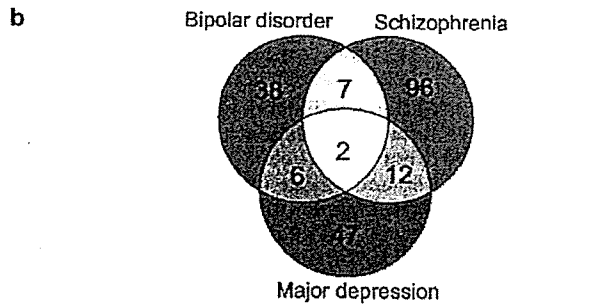


Figure 2 Scatterplots of the mean expression levels of bipolar disorder *vs* control groups. The plots were drawn based on all genes on the microarray (*left*) or the genes after the filtering procedures (*right*). The middle line in each plot indicates that the mean expression levels of the genes on this line are not different between the two groups. The upper and lower lines indicate the 1.3-fold up- and downregulation, compared with the control group, respectively.

inactivation in females.[15] These results imply the reliability of our microarray analysis. Although these genes might offer some insight into the significance of the pathophysiology, they were eliminated from the analysis since the sample size was not large enough to be subject to subgroup analysis.

Among the previously reported DNA microarray studies of mental disorders, Hakak *et al* reported the differentially expressed genes in schizophrenia, using a microarray system comparable with ours.[6] Comparison of the microarray results by the clustering analysis revealed that schizophrenics could be rela-





c

Acc no.	Symbol	Gene product	Fold change		
Channel/Receptor/Transporter			BP	MD	SZ
U49516	*HTR2C*	serotonin receptor 2C	-1.55	-	-1.46
U79666	*CACNA1A*	calcium channel, P/Q type, alpha 1A	-1.47	-	-
L76627	*GRM1*	glutamate receptor, metabotropic 1	-1.44	-	-
L19058	*GRIK1*	glutamate receptor, ionotropic, kainate 1	-1.43	-	-
U47924	*A*	protein "A"	-1.41	-	-
M62402	*IGFBP6*	insulin-like growth factor binding protein 6	-1.39	-	-
AB014551	*ARHGEF2*	rho/rac guanine nucleo. exchange factor 2	-1.37	-	-
U61375	*SLC29A1*	solute carrier family 29, member 1	-1.34	-	-
U95299	*NOTCH4*	Notch homolog 4	-1.34[b]	-	-
AC005053	*STEAP*	six transm. epithelial antigen of the prostate	-1.33	-	-
U90548	*BTN3A3*	butyrophilin, subfamily 3 member A3	1.32	-	-
AF035121	*KDR*	kinase insert domain receptor	1.35[b]	-	-
AB011540	*LRP4*	LDL receptor-related protein 4	1.35	1.36	-
U34846	*AQP4*	aquaporin 4	1.52[b]	1.45	-
Stress response/Chaperon			BP	MD	SZ
Z23090	*HSP27*	heat shock 27kDa protein 1	1.32	-	1.30
M63838	*IFI16*	interferon, gamma-inducible protein 16	1.35	1.52	-
D85429	*HSPF1*	heat shock protein 40	1.36[c]		-
X57352	*IFITM3*	interferon induced transm. protein 3	1.39	-	-
Transcription/Translation			BP	MD	SZ
AL022721	*RPL10A*	ribosomal protein L10a	1.31	-	-
AF099735	*TAF6L*	PCAF-associated factor, 65kDa	1.34	-	1.43
AB028973	*MYT1*	myelin transcription factor 1	1.38	-	1.34
AB011108	*PRPF4B*	PRP4 homolog B	1.38	1.36	1.31
AF055003	*SAP30*	sin3-associated polypeptide, 30kDa	1.42[a]	1.38	-
D42045	*DCLRE1A*	DNA cross-link repair 1A	1.42	1.83	-
AF040963	*MAD4*	MAX dimerization protein 4	1.44	-	-
M83667	*CEBPD*	C/EBP, delta	1.56	-	1.74
Signal Transduction			BP	MD	SZ
M73548	*APC*	adenomatosis polyposis coli	-1.35	-	-
AA923149	*SBA2*	SS box-containing WD protein	-1.32	-	-
U54778	*YWHAE*	14-3-3 Protein, Epsilon	-1.30	-	-
U40370	*PDE1A*	PDE1A, calmodulin-dependent	1.40[a]	-	-
AF061256	*LIM*	LIM protein	1.56	1.43	1.48
U26742	*DTNA*	dystrobrevin, alpha	1.71	-	-
Others/Unknown			BP	MD	SZ
AC005306	*BTBD2*	BTB domain containing 2	-1.73	-	-
AL049274		cDNA DKFZp564H203	-1.60	-	-
AI885381	*MGC2650*	hypothetical protein MGC2650	-1.56	-	-
AF070524	*FLJ38993*	hypothetical protein FLJ38993	-1.45	-	-
AI962638	*COL16A1*	collagen, type XVI, alpha 1	-1.44	-	-1.58
AL043470	*FLJ10335*	hypothetical protein FLJ10335	-1.37	-	-
AD000092		EST	-1.35	-	-
M33316	*CYP2A6*	CYP2A, polypeptide 6	-1.35	-	-
AL050061		cDNA DKFZp566J123	-1.33	-	-
U83410	*CUL2*	cullin 2	-1.32	-	-
M33509	*BAT2*	HLA-B associated transcript 2	-1.30	-	-
X82834	*GOLGA4*	golgi autoantigen, golgin subfamily a, 4	1.31[b]	-	-
M76231	*SPR*	sepiapterin reductase	1.34	-	1.48
J03040	*SPARC*	osteonectin	1.34[a]	-	-
AB014545	*KIAA0645*	KIAA0645 gene product	1.34	-	-
X96810	*EMD*	emerin	1.37	1.36	-
AI381790	*APM2*	adipose specific 2	1.39		-
AA152202	*FLJ14639*	hypothetical protein FLJ14639	1.47	-	-
AB014598	*HEPH*	hephaestin	1.56	-	-
AF103884	*HB-1*	minor histocompatibility antigen HB-1	1.92	-	-
D50923	*KIAA0133*	KIAA0133 gene product	2.05[b]	-	-

Figure 3 Differentially expressed genes in bipolar disorder. (a) Two-way hierarchical clustering analysis based on the expression profiles of the differentially expressed genes in patients with bipolar disorder. Each column represents the gene expression levels in individual samples and each row represents the individual gene. The expression value of the individual gene is normalized by dividing its median value. Blue and red colors indicate that the values are less than and greater than the mean, respectively. Gene symbols are given in the right. BP, patients with bipolar disorder; *, medication-free patients; C, control subjects. (b) Venn diagram drawn based on the differentially expressed genes in bipolar disorder, major depression, and schizophrenia, compared with controls. (c) List of the differentially expressed genes. The genes were functionally classified using the NetAffix database (http://www.affymetrix.com/index.affx, Affymetrix). The fold change was determined by comparing the mean expression level of each disease and control subject. Negative value in the fold change column means fold decrease. Genes showing upregulations compared with controls are highlighted in red. BP, bipolar disorder; MD, major depression; SZ, schizophrenia. The dash indicates statistically not significant. [a,b] and [c] indicate that significant effects of anticonvulsants, antipsychotics, and drug abuse, respectively, were found.

tively well separated from control subjects, despite independent samples being used (Figure 4). In addition, the reduced expression of neuropeptide Y in schizophrenia[7] was also observed in this study (data not shown). These would suggest the consistency and reproducibility of the microarray analyses using postmortem samples performed by independent studies.

Gene expression changes in bipolar disorder

The scatterplot drawn based on the genes after the filtering procedures showed that the gene expressions of most of the genes were similar between bipolar disorder and control groups from high to low expression levels (Figure 2). We defined the differentially expressed genes based on the fold change (1.3-fold or greater) and the *P*-value ($P<0.05$) of the mean expression values. The number of genes that met our criteria was 53. As expected, the hierarchical clustering analysis based on the expression level of these genes could separate patients well from control subjects (Figure 3a).

Importantly, some of the genes encoding channels, receptors, or transporters were downregulated, suggesting the impairment of the diverse neuronal functions within the prefrontal cortex in patients with bipolar disorder, similar to the results of microarray studies of schizophrenia.[5,6] On the other hand, genes encoding the stress response proteins or molecular chaperones, whose expressions were usually induced by environmental insult, were upregulated in bipolar disorder (Figure 3c). Several lines of evidence suggest their neuroprotective roles by preventing the formation of protein aggregations[16] or the neuronal cell death induced by injury.[17] Their altered expressions might be involved in part of the pathophysiology of bipolar disorder.

Many lines of evidence suggest that the dysregulation of calcium-dependent signal transductions are involved in bipolar disorder.[18] The downregulation of channels or receptors, *CACN1A*, *HTR2C*, *GRM1*, *GRIK1*, and the upregulation of *LIM* would directly affect the intracellular calcium ion concentration. The glutamatergic system is the main excitatory neurotransmitter system responsible for fundamental brain functions, and hypofunction of this system has been postulated in schizophrenia[19] and bipolar disorder.[20] The downregulation of two glutamate receptors, *GRIK1* and *GRM1*, would be involved in the hypofunction of this system in bipolar disorder. In addition to *GRIK1*, *HTR2C* and *NOTCH4* have been the candidate genes for association studies of schizophrenia and bipolar disorder, although consistent results have not been obtained.[21–28] It may be interesting to note the upregulation of dystrobrevin alpha gene, *DTNA*, in bipolar disorder, since genetic variation of *DTNBP1* (dystrobrevin-binding protein 1) was recently reported to be associated with schizophrenia.[29,30] DTNA and DTNBP1 are the components of dystrophin-associated protein complex (DPC),[31] and the altered function of DPC may cause the dysregulation of maturation and maintenance of the synapses.[32]

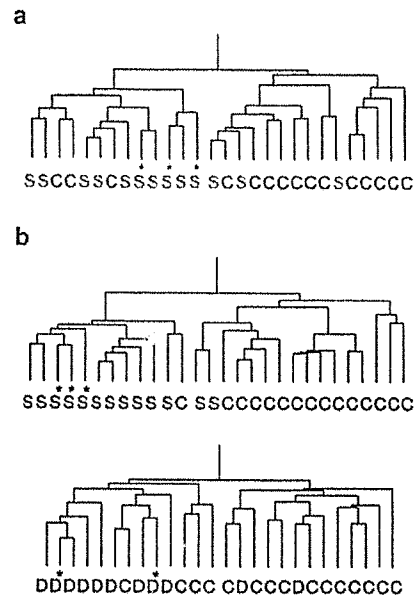


Figure 4 Clustering analysis. (a) Hierarchical clustering analysis based on the expression profiles of the previously reported 89 genes by microarray analysis in schizophrenia.[6] (b) Hierarchical clustering analysis based on the expression profiles of the differentially expressed genes in patients with schizophrenia (*upper*; 117 genes) or major depression (*lower*; 67 genes). S, patients with schizophrenia; D, patients with major depression; *, medication-free patients; C, control subjects.

Table 3 Correlation between microarray and RT-PCR data using the three control genes

	N	*Mean of R*	*Dev.*
Beta actin	21 (10)	0.360 (0.465)	0.213 (0.246)
GAPDH	21 (10)	0.300 (0.437)	0.222 (0.231)
Cofilin	21 (10)	0.340 (0.417)	0.199 (0.231)

Pearson's correlation coefficients were calculated in each of the 21 genes tested. Correlation coefficients of each gene were derived from 26 (bipolar disorder and control), 37 (bipolar disorder and major depression and control), 39 (bipolar disorder, schizophrenia and control), or 50 comparisons (all). The mean of the correlation coefficients of the 21 genes and its standard deviations are given. Results of the 10 genes, whose differential gene expressions in microarray were confirmed by RT-PCR using all the three control genes for normalization, are shown in brackets. *N*, number of the genes; *R*, correlation coefficient.

Uniqueness of bipolar disorder and its similarity to major depression and schizophrenia at the molecular level

We compared the differentially expressed genes in bipolar disorder with those in major depression and

schizophrenia (Figure 3b). The hierarchical clustering analysis revealed that the differentially expressed genes identified in major depression and schizophrenia were useful for the separation of patients from control subjects (Figure 4b), suggesting the appropriateness of data analysis in major depression and schizophrenia. Interestingly, most of the differentially expressed genes in bipolar disorder, as mentioned above, were not shared with these two diseases, suggesting that they are distinctive diseases at the molecular level. Unexpectedly, despite bipolar disorder and major depression sharing symptomatic similarities and genetic background, these were clearly different with regard to gene expression patterns. Most of the genes showing altered expressions in two or more disorders turned out to encode the proteins responsible for transcription or translation (Figure 3c). This finding may imply that the common pathophysiological background underlies the different mental disorders to some extent.

Among the not many commonly altered gene expressions in bipolar disorder and the other two mental disorders, altered expression of *AQP4*, *HTR2C*, and *LIM* would be of particular interest. AQP4 is associated with DPC by interacting syntrophin, a component of DPC,[33] and is involved in the water permeability across the blood–brain barrier and cerebrospinal fluid–brain interface.[34] Impaired water homeostasis due to the upregulation of *AQP4* could be involved in the pathophysiology common to bipolar disorder and depression such as white matter hyperintensity observed by magnetic resonance imaging,[35] although the effects of medication could not be excluded.

Considering the wide-ranging physiological roles of serotonin including regulation of mood, appetite, and sexual behavior, and the mechanisms of drugs such as the antipsychotic properties of serotonin–dopamine antagonists, and the hallucinogenic effects of serotonin agonists,[36] downregulation of *HTR2C* would contribute to the pathophysiology of common clinical features of bipolar disorder and schizophrenia such as psychotic symptoms.

LIM protein, initially identified as a protein kinase C (PKC)-beta-1-binding protein,[37] was recently found to regulate the N-type calcium channel activity by

Table 4 Summary of the results of the quantitative RT-PCR analysis

	Control gene					
	Beta actin		*GAPDH*		*Cofilin*	
	Fold change[a]	*P-value*[b]	*Fold change*	*P-value*	*Fold change*	*P-value*
Postmortem brains[c]						
GRIK1	−1.39	0.0344	−1.43	0.0209	−1.47	0.0307
HSP27	1.65	0.0238	1.63	0.0116	1.63	0.0146
APM2	1.60	0.0298	1.73	0.0173	1.49	0.0199
HSPF1	1.91	0.0035	1.77	0.0041	1.79	0.0164
KIAA0133	1.99	0.0002	1.93	0.0015	2.12	0.0012
IFITM3	1.86	0.0091	1.96	0.0047	1.80	0.0080
LIM	2.10	0.0102	2.05	0.0070	2.06	0.0089
AQP4	2.46	0.0011	2.34	0.0019	2.47	0.0060
HEPH	2.88	0.0021	2.78	0.0021	2.64	0.0035
CEBPD	3.04	0.0002	3.21	0.0002	2.81	0.0001
Lymphoblastoid cells						
GRIK1	nd		nd		nd	
HSP27	1.10	0.5934	1.04	0.6513	1.09	0.6513
APM2	1.04	0.9142	−1.09	0.9927	1.00	0.9927
HSPF1	1.85	**0.0016**	1.74	**0.0010**	1.84	**0.0010**
KIAA0133	−1.09	0.4850	−1.18	0.3304	−1.13	0.3304
IFITM3	1.10	0.4621	1.00	0.5384	1.08	0.5384
LIM	−2.81	**0.0003**	−3.11	**0.0004**	−3.04	**0.0004**
AQP4	nd		nd		nd	
HEPH	nd		nd		nd	
CEBPD	1.54	0.1772	1.40	0.2415	1.45	0.2415

[a]Fold change was determined by comparing the mean expression level of bipolar disorder and control groups. Negative value means fold decrease.
[b]The *P*-values are derived from one-tailed (postmortem brains) or two-tailed (lymphoblastoid cells) Student's *t*-test. $P<0.05$ in the lymphoblastoid cells are denoted in bold.
[c]Among the 21 genes tested, genes whose differential expressions were confirmed by three different control genes are shown.
nd, RT-PCR product was not detected in the lymphoblastoid cells.

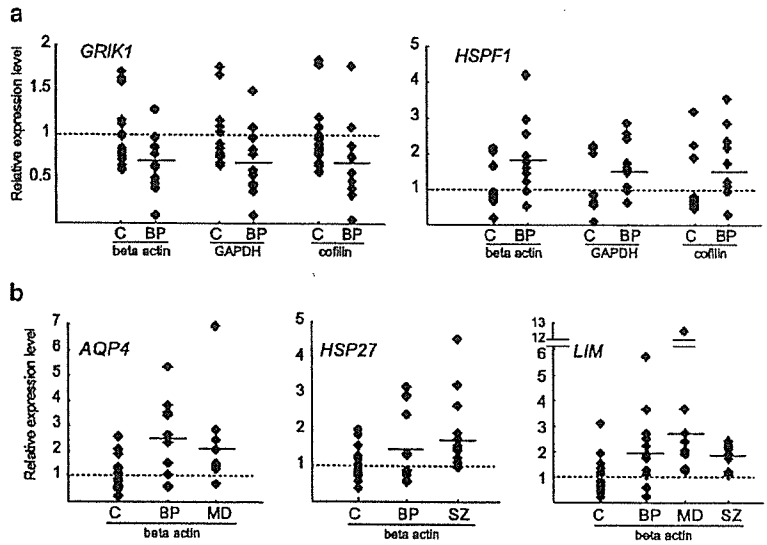


Figure 5 Differentially expressed genes examined by quantitative RT-PCR in postmortem brains. Representative examples of the altered gene expressions in bipolar disorder (a) and the commonly altered gene expressions between and among the mental disorders (b) are shown. Expression value is normalized by dividing the mean of the value of control subjects (C, $N=15$), which is indicated by the dotted line in each plot. Bars indicate the mean of the values of patients (BP, bipolar disorder, $N=11$; MD, major depression, $N=11$; SZ, schizophrenia, $N=13$). In each analysis, expression values were determined by using three different control genes. For simplicity, only the data normalized by beta actin are shown in (b). The differences shown here are significant (one-tailed Student's *t*-test, $P<0.05$). See Table 4 for the *P*-values with respect to bipolar disorder. The *P*-values with respect to major depression are $P=0.0156$ (*AQP4*) and $P=0.0150$ (*LIM*), and those with respect to schizophrenia are $P=0.0034$ (*HSP27*) and $P=0.018$ (*LIM*). The *P*-value after excluding a subject with major depression showing extremely high-level expression of *LIM* (12.63) was still significant ($P=0.0122$).

interacting with both calcium channel and PKC.[38] Considering that PKC activity is altered in bipolar disorder, and antibipolar drugs such as lithium and valproate regulate the PKC signaling cascade,[3,18] upregulation of *LIM* may be involved in the pathophysiology of bipolar disorder, and may also be related to the common pathophysiology of major mental disorders.

Effects of medication status, alcohol abuse, and drug abuse on the gene expressions in bipolar disorder

Among the 53 genes differentially expressed in bipolar disorder patients, the expressions of nine genes were significantly affected by anticonvulsants, antipsychotics, or drug abuse (Figure 3c). No significant effects of lithium, antidepressants, and alcohol use were found. Since patients were usually treated with multiple drugs and had complex histories of medication status, alcohol, and drug abuse, the effects of these confounding factors on gene expressions are difficult to define. However, it would be interesting to note that two drug-free patients were clustered within one patient branch (Figure 3a). This suggests that the altered gene expressions identified in this study largely reflect the pathophysiological conditions of bipolar disorder, rather than the effects of medication. More detailed studies will be needed to examine the effects of these factors as well as other confounding factors such as duration of illness and terminal condition of subjects.

Quantitative RT-PCR analysis

To consider the general consistency of microarray analysis, we compared the mRNA expression levels of each of the representative 21 genes measured by microarray and quantitative RT-PCR (see Materials and methods section). Although the correlations between the results by the two methods were highly variable and dependent on the gene tested, many of them were significantly correlated with each other (Table 3). We then examined whether the differential expressions identified by microarray analysis were confirmed by RT-PCR. Among the 21 genes tested, the altered expressions in bipolar disorder were confirmed in 10 genes using three control genes for normalization (Table 4; Figure 5). This relatively low success rate of confirmation may be caused by multiple reasons such as strict criteria of quantitative RT-PCR analysis using three control genes, relatively small level of fold change in microarray analysis, and the type of genes we examined. In general, the downregulated genes encoding receptors or channels were difficult to obtain the consistency by quantitative RT-PCR analysis. This may be partly attributable to the complexity of their transcripts in the brain.[39]

We also confirmed the gene expression changes observed in bipolar disorder and in other disorders; *AQP4* in bipolar disorder and major depression, *HSP27* in bipolar disorder and schizophrenia, *LIM* in three mental disorders (Figure 5b).

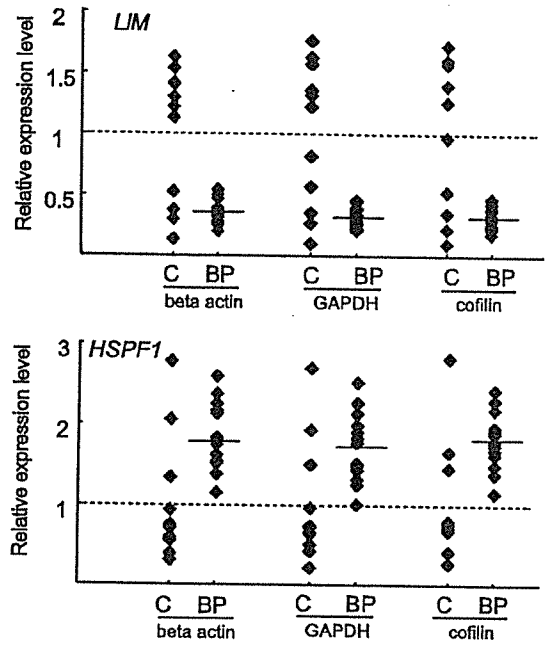


Figure 6 Differentially expressed genes examined by quantitative RT-PCR in the lymphoblastoid cells. Expression value is normalized by dividing the mean of the value of control subjects (C, $N=11$), which is indicated by the dotted line in each plot. Bars indicate the mean of the values of patients (BP, bipolar disorder, $N=14$). In each analysis, expression values were determined by using three different control genes. All the differences shown here are significant (two-tailed Student's *t*-test, $P<0.05$). See Table 4 for the *P*-values.

Altered gene expressions in lymphoblastoid cells established from patients with bipolar disorder

Lymphoblastoid cell lines are the only available tissue that can be cultured semipermanently from patients. Although lymphoblastoid cells have clear limitations in that they are unrelated to neuronal cells, they have advantages in that they are free from the effects of medication, and their altered gene expression may have some pathophysiological importance such as the genetic abnormality. We examined the mRNA expression levels of the 10 genes, whose differential expressions were confirmed by RT-PCR, in the lymphoblastoid cells (Table 4). We could not detect the transcripts of the three genes, *GRIK1*, *AQP4*, and *HEPH*. Although no significant differences in the expression levels were found between bipolar disorder and control groups for most genes, *HSPF1* and *LIM* showed the differential expressions in the lymphoblastoid cells (Table 4; Figure 6).

In contrast to the upregulation in the brain, the expression of *LIM* was downregulated in the lymphoblastoid cells of patients with bipolar disorder (Figures 5b and 6). The cause of these changes in the opposite directions remains unknown, but may be explained by the difference of tissue type.

HSPF1 (HSP40) modulates the activity of HSP70 and direct unfolded proteins to HSP70, which leads to the translocation of proteins into the mitochondria and endoplasmic reticulum (ER).[40] Several recent studies delineated the altered function of mitochondria and ER in bipolar disorder. The altered levels of calcium ions, which were intracellularly stored in the mitochondria and ER, were reported in the lymphoblastoid cells in patients with bipolar disorder.[41,42] In addition, the altered functions of ER were suggested by the increased levels of molecular chaperones in ER by valproate, and by the altered expression of molecular chaperons in the lymphoblastoid cells of patients with bipolar disorder (CK *et al*, in preparation). Considering the above findings, the altered expression of *HSPF1* (Figures 5a and 6) could be involved in the aberration of protein translocation systems into the mitochondria and/or ER, which in turn affects the functions of these organelles.

Conclusion

In summary, we have performed microarray analysis of bipolar disorder using postmortem brains. By profiling the gene expression patterns of a large number of samples, we were able to remove possible effects of the confounding factors including aging, gender, and postmortem interval. By comparing the gene expression patterns of similar but different mental disorders, we identified the unique expression changes in bipolar disorder, and common expression changes between and among mental disorders. The genes identified in this study would be useful for the understanding of the pathophysiology of mental disorders, as well as the molecular genetic studies and drug development.

Since bipolar disorder as well as other psychiatric disorders are considered to be heterogeneous diseases, more detailed studies using a larger number of independent postmortem samples will be clearly needed to explore the gene expression differences among the possible subgroups of bipolar disorder. It is also not clear whether these gene expression changes in bipolar disorder are attributable to anatomical changes in brain such as glial loss or selective neuronal death. Histochemical analyses such as *in situ* hybridization, or single-cell transcript analysis [43] may be needed to address such question.

We successfully found the altered expression of two genes, *LIM* and *HSPF1*, in the lymphoblastoid cells established from patients with bipolar disorder. These findings will be needed to explore a larger number of samples and in the different ethnic groups. Even though it remains unclear whether the altered expressions of *LIM* and *HSPF1* are caused by the genetic variations within these genes in patients, or by the secondary effects induced by other unidentified causes, these two genes would be novel candidate genes for bipolar disorder.

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Synaptic plasma membrane-bound acetylcholinesterase activity is not affected by docosahexaenoic acid-induced decrease in membrane order

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Abstract

We investigated the effect of administration of docosahexaenoic acid (C22:6, n-3; 300 mg/kg·day, for 12 weeks) on the degree of membrane order and membrane-bound acetylcholinesterase activity of the cerebral cortex synaptic plasma membrane in male Wistar rats. Docosahexaenoic acid levels in the synaptic plasma membrane increased significantly by 16% over levels in control rats concomitant with an increase in the molar ratio of docosahexaenoic acid to arachidonic acid. Synaptic plasma membrane order, assessed by 1,6-diphenyl-1,3,5-hexatriene, which measures order of the bulk internal hydrophobic lipid core, decreased significantly in the docosahexaenoic acid-fed rats. Lateral mobility of both global and annular lipids measured by pyrene also increased. Acetylcholinesterase activity of the synaptic plasma membrane was unaffected, and synaptic plasma membrane phospholipid contents increased in the docosahexaenoic acid-fed rats, with a concomitant decrease in the cholesterol/phospholipid molar ratio. Lipid peroxide and reactive oxygen species, indicators of tissue oxidative stress, decreased in both the cerebral cortex synaptosome and homogenate of the docosahexaenoic acid-fed rats. Arrhenius plot showed a break point in acetylcholinesterase activity at 22 °C and 24 °C in plasma membranes from docosahexaenoic acid-fed and control rats, respectively. The present experiment indicates that chronic administration of docosahexaenoic acid does not affect synaptic

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acetylcholinesterase activity and evoke oxidative stress, although it increases the disorder of the global and annular lipids of rat synaptic plasma membranes.
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Keywords: Docosahexaenoic acid; Acetylcholinesterase; Synaptic plasma membrane; Membrane disorder; Rat cortex

Introduction

Cholinesterase inhibition is targeted for the efficacious treatment approach of Alzheimer's disease (AD) (Giacobini et al., 2002), seen frequently associated with cholinergic dysfunctions including increased acetylcholinesterase (AChE) activity (Sáez-Valero et al., 2002), decreased choline acetyltransferase activity (Nitta et al., 1994, 1997; Nabeshima and Nitta, 1994) or impaired acetylcholine release (Itoh et al., 1996). Investigation of the effects of dietary docosahexaenoic acid (DHA, C22:6, n-3) on the activity of synaptic plasma membrane-bound AChE is of special significance, because (i) AChE is one of the key enzymes of cholinergic synapses in the CNS, (ii) the lipids of synaptic plasma membrane (SPM) contain large amounts of polyunsaturated fatty acids, especially DHA (Salem et al., 1986; Salem, 1989; Neuringer et al., 1994), and (iii) both cholinergic functions (Blockland, 1995) and DHA (Gamoh et al., 1999, 2001) play an important role in formation and/or retention of memory, which deteriorates in age-related pathologies such as Alzheimer's disease, which is characterized by a deficit in brain DHA concentration (Soderberg et al., 1991) and decrease in membrane fluidity (Scheuer et al., 1996; Muller et al., 1998). A decrease in DHA content in the neuronal membrane results in a decrease in membrane disorder or fluidity (Samuel et al., 1982) and synaptic plasma membrane-bound AChE enzyme activity is sensitive to a deficiency of n-3 fatty acids (Foot et al., 1983).

We (Hossain et al., 1998, 1999c; Gamoh et al., 1999, 2001; Hashimoto et al., 2002) and others (Bourre et al., 1990; Green and Yavin, 1995; Makrides et al., 1996; Lim and Suzuki, 2001) showed that cerebral enrichment of DHA could be dependent on the supply in the diet. It is thus likely that chronic administration of DHA would affect the membrane fluidity and/or order of the synaptic area, where the AChE remains embedded and/or attached (Fernandez et al., 1996). DHA increases the fluidity of the plasma membranes of endothelial cells (Hashimoto et al., 1999b), liver cells (Hashimoto et al., 2001) and platelets (Hossain et al., 1999b) and is involved in modulation of the activity of several membrane-bound enzymes including Na^+-K^+-ATPase (Ruiz-Gutierrez et al., 1993), 5′-nucleotidase, adenylate cyclase (Brown and Subbaiah, 1994) and Mg^{2+}-ATPase (Hashimoto et al., 2001). If DHA exerts a similar influence on fluidity in the SPM, it could be expected that DHA would also affect the activity of SPM-bound AChE. Alterations of brain synaptic plasma membrane fluidity by ethanol (Tanii et al., 1995), benzene (Engelke et al., 1992), toluene (Edelfors and Ravn-Jonsen, 1992) and several anaesthetics (Ondrias et al., 1983; Sidek et al., 1984; Mazzanti et al., 1986) may influence the activity of SPM-bound enzyme such as AChE. Dietary cholesterol, which inversely relates to membrane fluidity (Hashimoto et al., 1999a,b; Hossain et al., 1999a,b), affects synaptosomal AChE activity (Sanchez-Yague et al., 1991). On the other hand, DHA may increase the susceptibility of the membrane to oxidative stress (Halliwell, 1992), which alters membrane fluidity (Hashimoto et al., 1999a). Oxidative damage to the cerebral cortex causes deficits in learning and memory (Akita et al., 1997). We therefore investigated the question of whether chronic administration of DHA does in fact modify membrane fluidity, oxidative status and SPM-bound AChE activity in the rat cerebral cortex.

Materials and methods

Materials

All the standard fatty acids, 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylamonium)-phenyl]-6-phenyl-1,3,5-hexatriene-DPH (TMA-DPH), pyrene, 2′,7′-dichlorofluorescein (DCF) and BW284c51 were purchased from Sigma (St. Louis, MO., USA), and dichlorofluorescin diacetate (DCFH-DA) from Molecular Probes (Eugene, OR., USA).

Animals and diets

All the rats were cared for and killed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane Medical University, compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Wistar rats (generation 1, G1) (Jcl: Wistar; Clea Japan, Osaka, Japan) were kept in an environmentally controlled room at 23 ± 2 °C and relative humidity of 50 ± 10%, with automatic lighting from 08:00 to 20:00, and fed an F1® diet containing no fish products (Funabashi Farm Co., Chiba, Japan), with water ad libitum. Second generation rats (G2) were bred from the G1 rats, and 10–15 litters of G2 rats were crossed to derive third generation (G3) rats. The siblings were not of the same group rather they were spread across the dietary group. The G3 rats were on the same F1® diets. Twenty G3 male rats at the age of 10 weeks (body weight 220–250 g) were randomly divided into two groups: the DHA group was orally fed DHA-95E (an ethyl-ester all-cis 4,7,10,13,16,19-docosahexaenoate with a purity of >95%; 300 mg/kg day; Harima Chemicals, Inc., Tokyo, Japan) gently emulsified in a 5% gum Arabic solution in ice-cold water before administration, and the control group was fed an equal volume of 5% gum Arabic solution. Both F1® pellet feeding and subsequent DHA administration were carried out for a period of 12 weeks (Table 1).

Preparation of brains

After sodium pentobarbital (65 mg/kg BW) intraperitoneal anesthesia, the rat cerebral cortex was separated from the whole brain on ice, blotted gently by filter paper to remove blood and extraneous tissue fragments, weighed and then homogenized in ice-cold 0.32 M sucrose buffer (pH 7.4) containing 5 mM HEPES, 0.5 mM EDTA and the following protease inhibitors: 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.2 mM phenylmethylsufonyl fluoride (PMSF), with a Polytron homogenizer (PCU 2-110, Kinematica GmbH, Steinhofhale, Switzerland). The homogenates were adjusted to a final concentration of 100 mg tissue/ml of buffer and immediately subjected to the assays described below and/or stored at − 80 °C after N_2 flash and bath until the assay could be performed.

Preparation of synaptosomal plasma membrane

Synaptosomal plasma membrane (SMP) was prepared according to procedures described previously (Mason et al., 1999) with slight modification. The cortex homogenate was centrifuged at 578 g for 10 min, the supernatant was again centrifuged at 17,300 g for 10 min, and the resulting pellet was suspended in 0.32 M sucrose buffer and then layered over 7.5 and 13% Ficoll solutions (wt/vol; Ficoll/sucrose buffer) containing 0.5 mM EDTA and the above protease inhibitors. The gradients were

Table 1
Composition and fatty acid composition of F1 diet[1]

	[2]F_1 (g/100 g)	Fatty acid	[2]F_1 (g/100 g)
Corn grain	19.1	Myristic acid ($C_{14:0}$)	0.09 ± 0.09
Wheat bran	21.8	Palmitic acid ($C_{16:0}$)	15.6 ± 0.53
Wheat flour	35.8	Palmitoleic acid ($C_{17:1,\ n-7}$)	ND
Soybean meal	8.0	Stearic acid ($C_{18:0}$)	5.41 ± 0.09
Casein	4.0	Oleic acid ($C_{18:1,\ n-9}$)	21.1 ± 0.17
Dry skim milk	3.8	Linoleic acid ($C_{18:2,\ n-6}$)	52.4 ± 0.80
Soybean oil	1.5	Linolenic acid ($C_{18:2,\ n-6}$)	4.49 ± 0.13
[3]Mineral mixture	1.0	Arachidic acid ($C_{20:0}$)	0.14 ± 0.09
[4]Vitamin mixture	1.0	Ecosenoic acid ($C_{20:1,\ n-9}$)	0.33 ± 0.13
[5]Amino acid mixture	1.0	Arachidonic acid ($C_{20:4,\ n-6}$)	ND
[5]DL-methionine	0.1	Eicosapentaenoic acid ($C_{20:5,\ n-3}$)	0.06 ± 0.06
[6]Calcium carbonate	0.9	Docosapentaenoic acid ($C_{22:5,\ n-3}$)	ND
		Docosahexaenoic acid ($C_{22:6,\ n-3}$)	ND
		Lignoceric acid ($C_{24:0}$)	0.11 ± 0.07

[1] Values are mean ± SEM, n = 4; ND = not detected.

[2] The F1 standard diet containing no fish products contains [(in g/100 g : protein, 21.3; fat 5.1; fiber 3.1; carbohydrate, 5; nonnitrigen, 57.5; and total energy, 17.7 J/g] and was purchased from Funabashi Farm, Chiba, Japan.

[3] Mineral mixture (g/Kg) (as formulated by Takeda Kagaku Shiryo, Tokyo, Japan): $MnSO_4$, 15.7; $FeSO_4$, 23.8; $CoSO_4$, 0.7; $CuSO_4$, 1.0; Ca $(IO_3)_2$, 0.5; $MgCO_3$, 3.0; Na Cl, 300.0;$CaCO_3$, 655.3.

[4] Vitamin mixture (g/kg) as formulated by Takeda Kagaku Shiryo: retinal, 1,000,000 IU/kg; vitamin C oil, 200,000 IU/kg; *dl*-α-tocopherol acetate, 5.0; menadione, 1.0; thiamine nitrate, 0.7; riboflavin, 0.8; pyridoxine hydrchloride, 1.0; nicotinamide, 4.0; calcium pantothenate, 1.7; choline chloride, 65.0; cyanocobalamine, 0.5; biotin, 0.015; saccharine sodium, 8.5; mil S-Na_2 [natural spice (in g/100 g: carbohydrate 8; protein 16; lactate 52; fat, 18.5)], 100.0; glucose 90.0.

[5] Amino acid mixture (g/kg) as formulated by Takeda Kagaku Shiryo; dl-methionine, 300.0; l-lysine hydrochloride, 300.0; defatted rice bran, 400.0.

[6] Wako Pure Chemicals (Osaka, Japan).

centrifuged at 80,000 g for 30 min. The interface between 7.5 and 13% Ficoll enriched in synaptosome was carefully removed, placed in the same sucrose buffer and again centrifuged at 17,300 g for 15 min. The synaptosomal pellet was resuspended in the sucrose buffer and centrifuged again by the above procedure to remove the remaining Ficoll. The pellets suspended in an appropriate volume of cold Tris-HCl buffer (pH. 7.4) containing 137 mM NaCl, 5.4 mM KCl and 11 mM glucose were lysed by freeze-thaw cycles using liquid N2 to isolate SPM. The lysed suspensions were centrifuged at 41,000 g for 20 min. The synaptosomal pellet was resuspended in cold double distilled water and centrifuged at 41,000 g for 30 min. Finally, the SPM pellet was resuspended in Tris-HCl buffer (pH. 7.4) with 137 mM NaCl, 5.4 mM KCl and 11 mM glucose.

Measurements of annular and average/global membrane order

When excited at its own wavelength of 334 nm, a pyrene molecule in close proximity to another pyrene molecule (monomer, M) forms a monomer-monomer dimer (excimer, E). The ratio of E/M fluorescence intensity can therefore be used as an index of lateral diffusion (lateral mobility of the membrane environment); thus, the higher the ratio, the higher the membrane lateral mobility. In principle, pyrene diffuses randomly at all nonpolar regions into the bilayer membrane, irrespective of the

presence of any microdomains. Accordingly, the resulting E/M ratio gives an average or global membrane lateral mobility of the membrane. When a fluorescence emission is created through energy transfer from the tryptophan of the membrane protein, only the pyrene molecules localized in the annular lipid are excited and the molar E/M molar ratio gives the lateral mobility of the annular lipids (Mason et al., 1999; Avdulov et al., 1997).

Briefly, 100 μL of SPM suspension containing100 μg of protein was suspended in 2.0 ml of 25 mM Tris-HCl buffer (pH. 7.4) containing 137 mM NaCl, 5.4 mM KCl and 11 mM glucose, incubated in the dark at 37 °C for 30 min, transferred to a cuvette and injected with 2 μl of 10 mM pyrene. After incubation for 5 min, the pyrene was excited at a wavelength of 286 nm, and the fluorescence emission spectrum was recorded from 320 to 530 nm. After tracing, the pyrene was excited at 334 nm and the intensity profile was again recorded from 320 to 530 nm. The E/M ratio obtained at 286 nm wavelength was taken as the annular fluidity, and the ratio at 334 nm as the global (bulk) fluidity, where E is the fluorescence intensity of pyrene excimer at 480 nm and M the fluorescence intensity of pyrene monomer at 373 nm.

The global membrane order of BCM was also determined by measuring the polarization of the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and its 1-[4-trimethylamonium]-phenyl]-6-phenyl-1,3,5-hexatriene-DPH (TMA-DPH) as described previously (Hashimoto et al., 1999a,b).

Acetylcholinesterase assay

SPM-bound AChE was determined by measuring the hydrolysis of acetylthiocholine. Total cholinesterase (ChE) activity was measured by the method of Ellman et al. (1961) at 25 °C. The standard 1.0 ml reaction mixture for the assay contained 100 mM phosphate buffer (pH 8.0), 1 mM $MgCl_2$, 0.50 mM acetylthiocholine, 0.125 mM 5,5′-dithiobis-2-nitrobenzoic acid and 100 ~ 150 μg SPM protein. The blank consisted of solutions without the SPM protein. The reaction was recorded at 412 nm by using a Hitachi U-2000 spectrophotometer and the rate was calculated as μmoles of substrate hydrolyzed per min per mg protein. AChE activity was defined as the difference of total ChE activity between in the absence and presence of 0.1 mM BW284c51 (specific AChE inhibitor) as described (Lassiter et al., 1998).

The temperature dependence of AChE also was measured over a temperature range 5 to 45 °C. The samples of individual groups were mixed (to have a large volume) and assayed at 2.5 °C intervals, keeping the samples at a constant temperature by a thermostatted cell holder coupled to a circulating water bath. Lines were fitted to the data points in Arrhenius plots by regression analysis.

Reactive oxygen species assay

The fluorescent probe 2′,7′-dichlorofluorescin diacetate (2′,7′-DCFH-DA) was used for the assessment of reactive oxygen species (ROS) as described previously (Hashimoto et al., 2001). Briefly, 200 μg of the protein of the homogenate was diluted in 1.5 ml of 25 mM Tris-HCl buffer (pH 7.4) containing 137 mM NaCl, 5.4 mM KCl and 11 mM glucose, and then incubated with DCFH-DA (at a final concentration of 100 μM) in methanol for 1 h at 37 °C. The dye-loaded samples were centrifuged at 12,500 g for 10 min at 4 °C. The pellet was vortexed in the same Tris-HCl buffer (pH 7.4) at ice-cold temperature and again incubated for 30 min at 37 °C. Fluorescence was measured with a Hitachi 850 spectrofluorometer at wavelengths of 488 nm for excitation and 525 nm for emission. The temperature

of the cuvette holder overlying a magnetic stirrer was maintained at 37 °C. ROS were determined from the standard curve for DCF.

Lipid peroxide and reduced glutathione assay

Lipid peroxide content (LPO) was estimated by the thiobarbituric acid reactive substances (TBARS) test of Ohkawa et al. (1979), as previously described (Hashimoto et al., 1999a,b). Malonaldehyde levels were calculated relative to a standard preparation of 1,1,3,3-tetraethoxypropane. Reduced glutathione (GSH) levels were determined by the method of Hissin and Hilf (1976). GSH levels were determined from the standard curve for GSH and expressed as nanomoles per mg protein.

Lipid analyses

Liver fatty acid profile was measured from liver homogenate. Briefly, 10% homogenate of the liver was prepared in chilled buffer containing 300 mM mannitol, 5 mM EGTA, 18 mM Trsi-HCl, 1 mM PMSF, (pH 7.4) with a polytron homogenizer (PCU 2-110, Kinematica GmbH, Steinhofhale, Switzerland).

SPM total lipids were extracted by the method of Folch et al. (1957) using a chloroform/methanol mixture (2:1, v/v). The solvent was evaporated by blowing N_2 gas at 25 °C. An appropriate volume of Tris-HCl buffer was added to the dried lipid and it was resuspended with a bath sonicator at ice-cold temperature. The suspension was used to estimate phospholipid content by measuring inorganic phosphate (Pi) using an inorganic phosphorus kit (Sigma, St. Luis, USA). Phospholipid content of the SPM was calculated as 25 times the Pi in the Folch extract (Shattil. et al., 1975).

SPM cholesterol was measured using gas chromatography on a Model 5890 plus gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and an automatic sampler (Model 7673), as previously described (Naeemi et al., 1995) with a few modifications. Briefly, 50 μl of SPM suspension (100–150 μg protein) containing 50 μl of α-cholestane (1.0 μg/μl) in ethanol as an internal standard was dissolved in saturated methanolic KOH and incubated at 80 °C for 30 min. After cooling, 1.0 ml of saturated NaCl solution and 200 μl of cyclohexane were added to the mixture. The mixture was vigorously shaken and centrifuged at 2000 g, and the upper organic layer was directly supplied to gas chromatography using a 30 m × 0.25 mm inner diameter fused silica column coated with methyl siloxane to a film thickness of 0.25 μM (HP-1, Hewlett-Packard), helium with flow rate of 1.5 ml/min as the carrier gas, the oven temperature programmed from 180 °C to 280 °C at 20 °C/min and at 280 °C for 10 min, and the injector splitter at 20 min/min and temperature 290 °C.

The liver homgenate and SPM fatty acid profile were determined by one step analysis of Lepage and Roy (1986) using gas chromatography as described previously (Hashimoto et al., 1999a,b).

Protein concentration was estimated by the method of Lowry et al. (1951).

Statistical analysis

Results were expressed as the mean ± SE. Statistical analysis was performed by Student's *t*-test. Correlation coefficients were determined by simple regression analyses. All data were analyzed using

MindVision software, StatView® 4.01 (Abacus Concepts, Inc., Berkeley, CA, USA). A level of $p < 0.05$ was considered statistically significant.

Results

SPM lateral mobility

Lateral mobility of both the annular and global regions (which include both annular and nonannular regions) of the cerebral cortex SPM were measured by pyrene excimer fluorescence spectroscopy (Fig. 1A and 1B). Dietary DHA administration significantly increased the lateral mobility of both the annular and global regions of the SPM.

DPH-polarization value reflects the average membrane order of the mid-acyl chain region of the phospholipid bilayer (Hashimoto et al., 1999a,b). Membrane disorder is inversely related to fluorescence polarization. DPH-determined average membrane disorder of the SPM of DHA-fed rats increased to a significantly greater extent than that of the SPM of control rats (Fig. 1C). Differences in SPM membrane acyl chain order between the DHA-fed and control rats were not revealed to the same extent by TMA-DPH, a cationic derivative of DPH that measures the fluidity at the lipid-water interfacial region of the bilayer membrane and provides information on the fatty acyl carbonyls just below the polar head group region (Prendergast et al., 1981; Kuhry et al., 1983) (Fig. 1D).

Effect of DHA administration on SPM-bound acetylcholinesterase

AChE activity in the SPM of DHA-fed rats remained at the same level as that in the SPM of the control rats (Fig. 2).

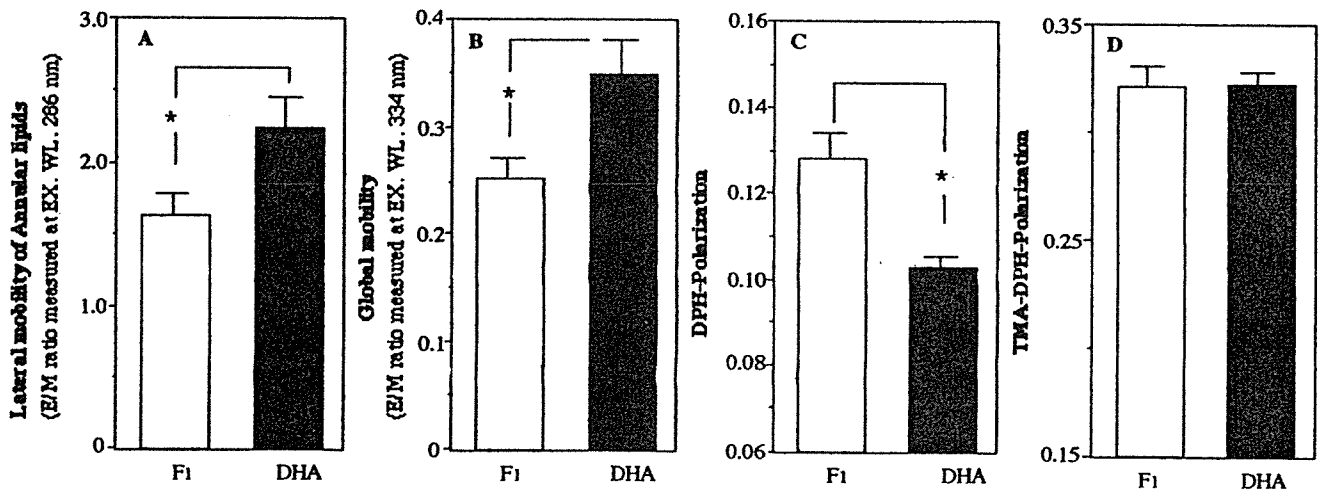

Fig. 1. Effects of DHA administration on membrane order of rat synaptic plasma membrane (SPM). Pyrene-determined annular lipid lateral mobility is shown in (A), average or global lateral mobility in (B), and average membrane order as determined by the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) in (C) and of 1-[4-(trimethylamonium)-phenyl]-6-phenyl-1,3,5-hexatriene-DPH (TMA-DPH) in (D). Results are mean ± SE for 10 to 12 rats each with duplicate determinations. See Materials and methods section for details. *$p < 0.05$, unpaired student's t test; □ = F1, control rats; ■ = F1 + DHA, DHA-administered rats.

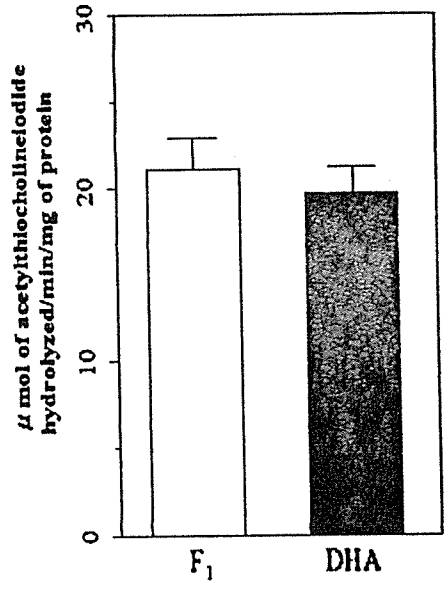

Fig. 2. Effects of DHA administration on synaptic plasma membrane (SPM)-bound acetylcholinesterase (AChE) activities. AChE activity is defined as the difference between total choline esterase activity occurring in the absence and presence of 0.1 mM BW284c51 (specific AChE inhibitor). Results are mean ± SE for 10 to 12 rats each with duplicate determinations. See Materials and methods section for details. □ = F1, control rats; ■ = F1+ DHA, DHA-administered rats. No significant difference was found between the groups.

Effect of DHA on oxidative stress

Lipid peroxidation alters membrane fluidity (Aksentsev et al., 1995; Urano et al., 1997). We therefore measured the LPO (TBARS) level in the cerebral cortex SPM fraction and the ROS level in the cortex whole homogenate of the DHA-fed and control rats to determine whether dietary administration of DHA either ameliorates or exacerbates the oxidative status of the nerve terminals (Fig. 3). In the DHA-fed rats, dietary DHA administration reduced both the LPO level in the SPM (Fig. 3A) and the ROS level in the cortex homogenate to a significantly greater extent than in the control rats (Fig. 3B).

The GSH levels in the cortex homogenate of DHA rats significantly increased over that of the control rats (DHA-fed rats: 4.0 ± 0.20 nmol/mg protein; control rats: 3.2 ± 0.15 nmol/mg protein).

Effect of dietary DHA administration on fatty acid profiles of liver, and fatty acid profiles, cholesterol and phospholipid composition of the SPM

Table 2 shows the fatty acid profiles of liver homogenate. After DHA feeding, n-6 arachidonic acid significantly decreased with a concomitant increase of n-3 eicosapentaenoic, docosapentaenoic and docosahexaenoic acid in the liver tissues. Among the n-3 PUFAs, DHA was doubled. This was consistent with the report of Bourre et al. (1990). Palmitic, steraric, oleic, linoleic and linolenic acids were not altered. Finally, the changes in the fatty acid profiles brought about a significant change in the total unsaturation index (USI), n-3 USI and the ratio of n-3 USI/n-6 USI of the liver of DHA-fed rats.

The DHA content of the SPM increased concomitantly with a decrease in arachidonic acid (AA) content in the DHA-fed rats, leading to an increase in the DHA/AA molar ratio (Table 3). Oleic acid