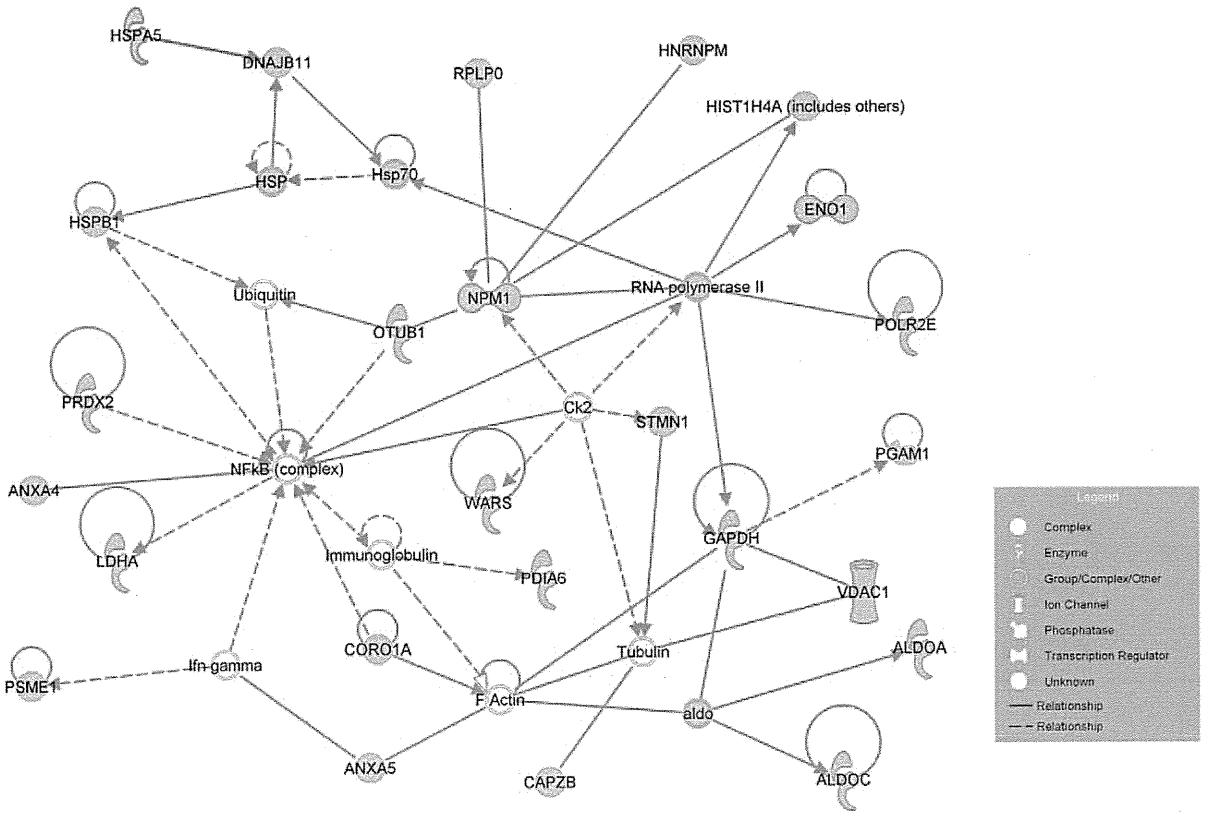
a



b

Category	*P*-value range	Molecules
Carbohydrate Metabolism	8.38E-10 - 3.27E-02	14
Cell Death	2.04E-05 - 4.72E-02	20

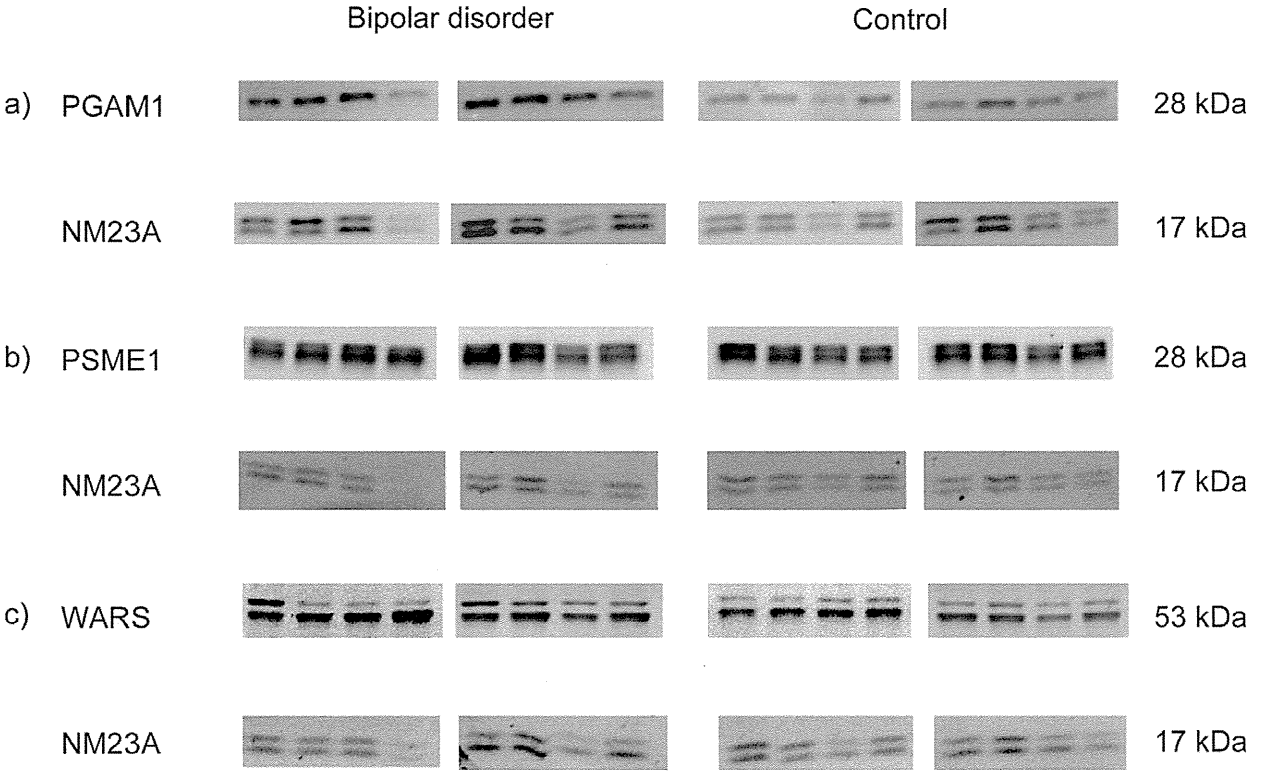
Figure 1. Gene networks showing interrelationships between differentially expressed genes in the twin with bipolar disorder.
doi:10.1371/journal.pone.0053855.g001

Functional grouping of altered proteins

The IPA software (Ingenuity Systems, Redwood City, CA) was used to identify the key biological relationships and functions of differentially expressed proteins and their interaction networks. For pathway analysis, Swiss-Prot and GenBank accession numbers were used to catalogue the identified protein into known interaction pathways based upon the Ingenuity Pathway Knowledge Base (IPKB). IPA classification data were derived from the published literature in a systematic way using a comprehensive ontology of functional annotations and protein–protein interaction data. The most significant interaction networks, biological functions and pathway associated with the differentially expressed proteins were identified. To confirm biological functions and gene ontology annotation between identified proteins, we used bioinformatics resources, PANTHER (http://www.pantherdb.org/) and DAVID (http://david.abcc.ncifcrf.gov/). DAVID program uses a modified Fisher's exact p value (EASE score) to rank gene clusters by statistical overrepresentation of individual genes, based on the co-occurrence/enrichment of the category within the gene list relative to all genes in the same category on the study.

Western blot analyses for validation

Lymphoblastoid cells in a case–control study were individually cultured. Total proteins were individually extracted using Q-proteome Mammalian Protein prep kit (Qiagen), and protein



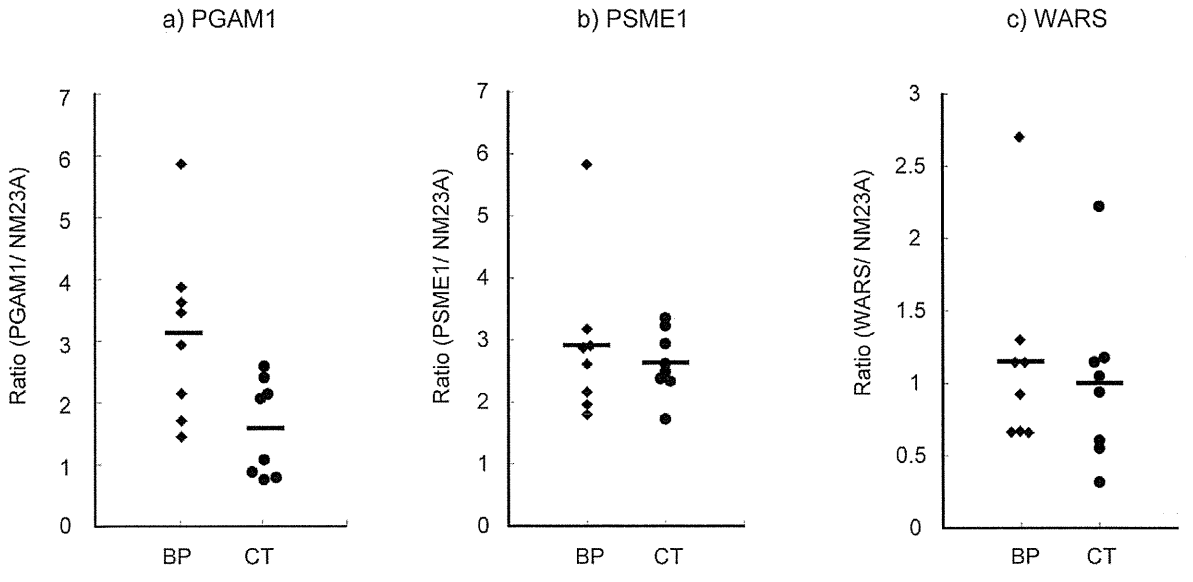


Figure 2. Protein expression validation by Western blot analysis with an internal standard, NM23A. Proteins from each of the eight pairs of samples were separated by SDS-PAGE and transferred to PVDF membranes. Proteins were immunodetected using the respective primary antibodies and fluorescent secondary antibodies. Signals were captured with FX and signal intensity and shown by the. a) PGAM1, b) PSME1, c) WARS. Scatter plots show the ratio of each protein to an internal standard protein, NM23A, measured by densitometric scanning of the band

intensities. The *p* values were calculated using a *t* test in all proteins. Number of the subjects is 8 for bipolar disorder and 8 for controls, respectively. The absolute band intensity for the PGAM1 was also significantly higher in patients with bipolar disorder (0.93+/−0.23 [mean +/− standard deviation] [arbitrary unit]) than control subjects (0.39+/−0.18, $p<0.0005$).
doi:10.1371/journal.pone.0053855.g002

concentrations were determined by the methods mentioned above. Equal concentration (5 μg per lane) of proteins from control and case samples were separated by 12% or 4–15% SDS-polyacrylamide gel electrophoresis and transferred onto Immun-Blot PVDF membranes (Bio-Rad) using a mini Trans-blot Cell (Bio-Rad). After transfer, the blotted membrane were blocked with 4% w/v ECL Advance Blocking Reagent (GE Healthcare Bio-Sciences) in phosphate-buffered saline containing 0.1% Tween20 (PBST) (MP Biomedicals Inc., Santa Ana, CA) at 4°C overnight and incubated with primary antibody in PBST with 4% w/v skim milk for 1 h at room temperature. PSME1, WARS, and PGAM1 were chosen for quantification by Western blot analysis. As an internal control, NM23A was used because there was no significant difference of protein levels of NM23A between patient and the co-twin in 2D-DIGE. Primary antibodies were as follows: rabbit antibody against human PSME1 (Calbiochem, La Jolla, CA), mouse antibody against human WARS (Abnova, Taipei City, Taiwan), goat antibody against human PGAM1 (Novus Biologicals, Littleton, CO), and rabbit monoclonal and polyclonal against human NM23A (Abcam, Cambridge, MA). The blotted membranes were washed in TBST and incubated at room temperature with each secondary antibodies, Alexa Fluor 488–labeled donkey anti-goat IgG antibody (Molecular Probes/Life Technologies Corporation), Cy5-conjugated affiniPure donkey anti rabbit IgG antibody and Cy3-conjugated affiniPure donkey anti mouse IgG antibody (Jackson Immuno Research, West Grove, PA). The membranes were directly scanned with the Molecular Imager FX (Bio-Rad). Protein bands were analyzed to give a quantitative estimation of intensity change using the Quantity One Software (Bio-Rad) adapted to the Molecular Imager FX. To estimate the relative molecular weight of each protein, molecular markers, Dual Color Precision Plus Protein Standards (Bio-Rad) and ECL Plex Fluorescent Rainbow Markers (GE Healthcare Bio-Sciences) were used. Preliminary experiments indicated that amounts of these proteins in the lysates of lymphoblastoid cells were within the linear range of detection.

Results

Detection of spots differentially expressed between monozygotic twins discordant for bipolar disorder by 2D-DIGE

First, we extracted total protein from cultured lymphoblastoid cells derived from a pair of monozygotic twins discordant for bipolar disorder. The total protein for each twin was separately labeled with different CyDyes (Cy3 or Cy5), and dyes were swapped between gels. A mixed sample composed of an equal amount of proteins from the patient and the co-twin was labeled with Cy2 and used as an internal standard. These processes minimized gel-to-gel variation and improved protein spot statistics at the analysis stage. These labeled proteins were mixed and analyzed by 2D-DIGE. To detect robust differences between the patient and the co-twin, we performed 2D-DIGE. To avoid artifacts, four gels of same condition were simultaneously run for each experiment. Three gels were used as analytical gels to detect differentially expressed spots between the patient and the co-twin, and the remaining gel was used as preparative gel for picking out differentially expressed spots. We performed 2D-DIGE and liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses in quadruplicate experiments using protein samples independently extracted from different aliquots of cell culture.

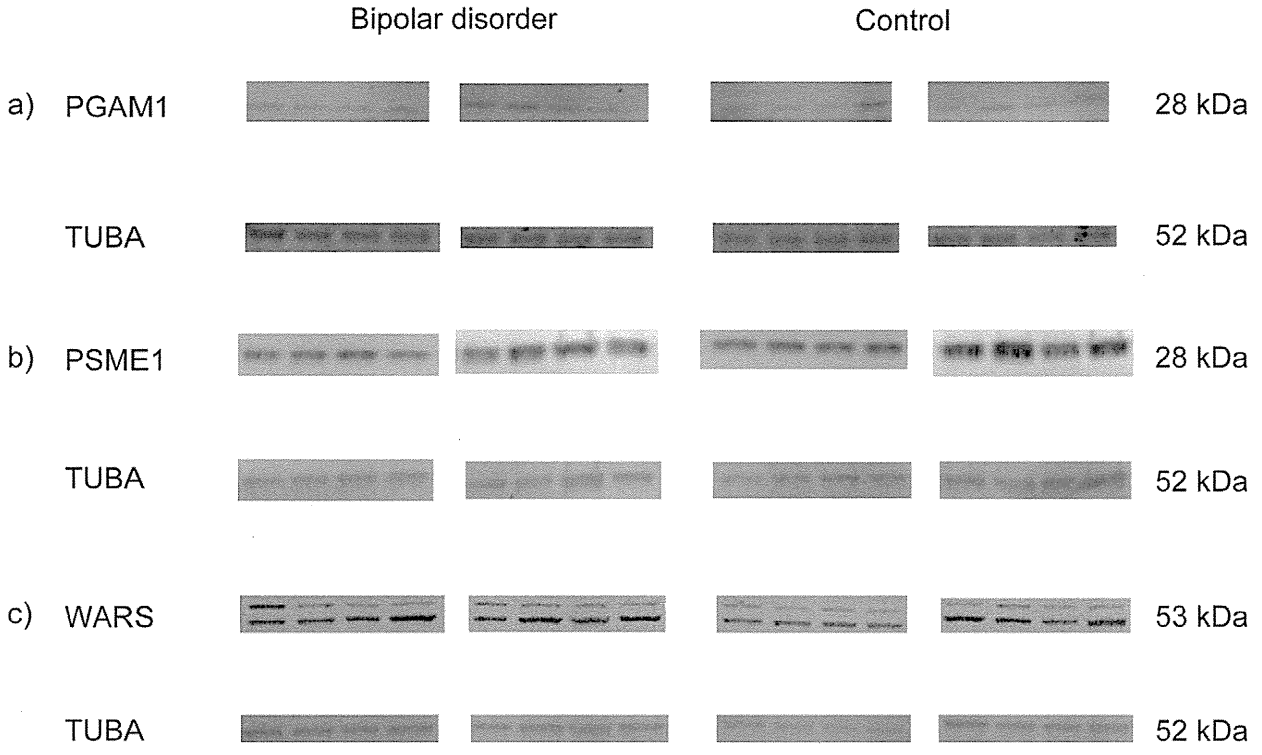
Using 2D-DIGE and DeCyder Ver.6.0 image analysis software, approximately 3200 protein spots were separated (3220±51 [mean ± SD]). The observed spot pattern images in each gel were very similar among quadruplicate experiments. The protein spots were selected if the intensity difference between the affected and nonaffected twin was larger than 1.25-fold (absolute value >1.25 or absolute value <-1.25). Approximately 200 spots (211±115, quadruplicate) were found to be significantly differentially expressed ($p<0.05$, Student's *t* test) between the twins per experiment.

Identification of spots by LC-MS/MS

The proteins in preparative gels were stained by SYPRO Ruby after electrophoresis, and the preparative gels were performed matching to analytical gels. The largest 100 spots out of the 200 differentially expressed protein spots were picked from each preparative gel and were considered suitable for subsequent analysis by LC-MS/MS. The spots were chosen sequentially from those with a large absolute value fold change. The 68 spots, averaged for quadruplicate values, were successfully identified as unique proteins through LC-MS/MS and are listed in Table 2. Since Epstein-Barr virus–transformed lymphoblastoid cells were used in this study, all immunoglobulin and B-cell–related proteins were removed from the analysis. Moreover, we also removed keratin and trypsin-related proteins because of the possibility of experimental contamination. Table 2 shows only proteins identified by LC-MS/MS in common with each experiment. Proteins identified twice or more with Mascot search results (ion scores of higher than 45) included PSME1, RPLP0, TPI1, ALDOC, ANXA4, PGAM1, and WARS. Fifty-three proteins had high ion scores and were identified at least twice in four experiments.

Functional grouping of altered proteins

To explore the biological function (protein-interaction network) of the 53 differentially expressed proteins, we performed protein classification using the Ingenuity Pathway Knowledge Base software. A data set containing the gene symbols by The HUGO Gene Nomenclature Committee was uploaded into the application. Each protein identifier was converted to its gene identification and mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base. These genes were overlaid onto a global molecular network developed from information contained in the Ingenuity pathways analysis (IPA) knowledge base, which is based entirely on findings reported in the literature. Networks of these focus genes were algorithmically generated based on their connectivity. One of the 53 proteins was omitted because it was not included in the database, and the remaining 52 proteins were mapped onto mainly three networks. The largest network, having the highest score, is associated with carbohydrate metabolism, neurological disease, and skeletal and muscular disorders (Fig. 1A). This network includes ALDOA, ALDOC, ANXA4, ANXA5, CAPZB, CORO1A, DNAJB11, ENO1, GAPDH, HIST1H4A (includes others), HNRNPM, HSPB1, LDHA, NPM1, OTUB1, PDIA6, PGAM1, POLR2E, PRDX2, PSME1, RNA polymerase II, RPLP0, STMN1, VDAC1, and WARS. Next, the 53 proteins were classified according to biological function and canonical



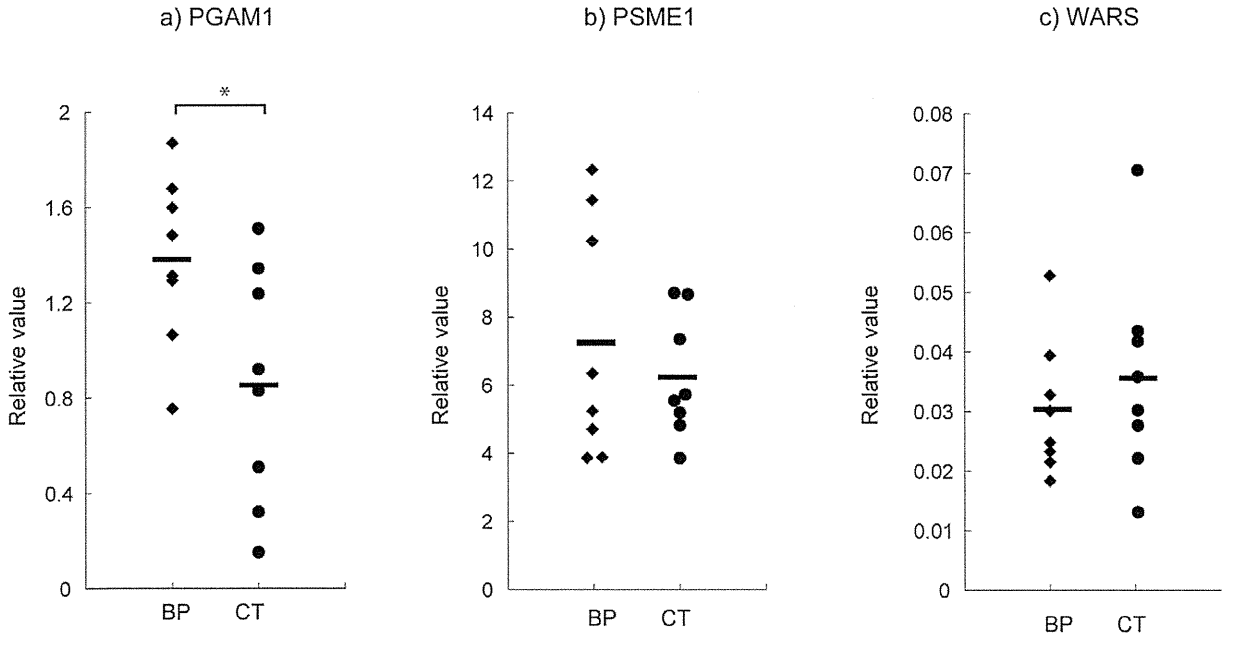


Figure 3. Verification of the alternation of PGAM1 using the other internal protein, tubulin alpha (TUBA). The methods are similar to the Figure 2, except that tubulin alpha (TUBA) was used for the internal standard. a) PGAM1, b) PSME1, c) WARS. Scatter plots show the ratio of each protein to an internal standard protein, TUBA, measured by densitometric scanning of the band intensities. The PGAM1/TUBA ratio was significantly

higher in patients with bipolar disorder compared with controls ($p<0.05$). Number of the subjects is 8 for bipolar disorder and 8 for controls, respectively.
doi:10.1371/journal.pone.0053855.g003

pathway. The categories pertained to carbohydrate metabolism (8.38E-10–3.27E-02, 14 molecules) and cell death (2.04E-05–4.72E-02, 20 molecules) (Fig. 1B). Pathway analysis and gene ontology classification using PANTHER and DAVID were conducted on the same protein IDs. These analyses also showed pathways and categories associated with glycolysis and anti-apoptosis. Taken together, most proteins identified in the present study were related to glycolysis and neurological diseases.

Case–control study by Western blot analysis

We postulated that the differentially expressed proteins might be candidate biomarkers for bipolar disorder. To validate the findings from the proteomic profiling study and to examine the possibility of biomarkers for bipolar disorder, Western blot analyses were performed using a case–control sample set consisting of eight subjects with bipolar disorder and eight healthy control subjects. To compare the protein levels across individuals, protein concentration was measured by the Bradford method, and equal amounts of proteins were loaded onto the gels. Commercially available antibodies for the candidate 7 proteins (PSME1, RPLP0, TPI1, ALDOC, ANXA4, PGAM1, and WARS) were searched for, and among available antibodies, those against PSME1, WARS, and PGAM1 showed good performance, and thus they were chosen for quantification by Western blot analysis. The levels of PGAM1, PSME1, and WARS were quantitatively investigated by Western blot analysis using NM23A as a standard (Fig. 2). Expression of PGAM1 was recognized by the presence of a single band at around 28 kDa and its protein expression was increased by 197% in bipolar disorder compared with controls ($p<0.05$). However, the levels of the other proteins were similar between bipolar disorder and controls in this case–control sample set (Fig. 2).

The absolute band intensity for the PGAM1 was also significantly higher in patients with bipolar disorder (0.93 ± 0.23 [mean ± standard deviation] [arbitrary unit]) than control subjects (0.39 ± 0.18, $p<0.0005$). In addition, we also performed an independent experiment using the other, more popular housekeeping protein, tubulin alpha (TUBA), as an internal standard. This analysis also showed higher PGAM1 levels in patients with bipolar disorder than controls ($p<0.05$) (Figure 3).

Discussion

In this study, we identified 53 proteins that were differentially expressed between a pair of monozygotic twins discordant for bipolar disorder; 34 were up-regulated and 19 were down-regulated. The differentially expressed proteins included those previously implicated in psychiatric disorders, such as ALDOC, ENO1, and PRDX2 [10,33]. Differences for ALDOC, ANXA4, PGAM1, PSME1, RPLP0, TPI1, and WARS between twins were regarded as robust because they were identified in three of four experiments with high scores.

To evaluate whether identified proteins might be biomarkers for bipolar disorder, we performed a case–control study for several proteins by Western blot analysis using available antibodies. An increased level of PGAM1 was observed in samples from patients with bipolar disorder. PGAM1 is an enzyme of the glycolytic pathway that catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate [34]. This enzyme also promotes glycolysis and ATP production via the TCA cycle and the electron transport system. Although previous studies using postmortem brains of patients with bipolar disorder and schizophrenia suggested altered protein expression of glycolysis enzymes, including PGAM1 [10,35], the results were controversial. The differentially expressed proteins between bipolar disorder and healthy control including PGAM1, might be a clue to understand the biological basis of bipolar disorder.

To examine whether the 53 identified proteins were related to each other and constituted a global molecular network, pathway, or category, we applied IPA to our data. The results showed that the networks having a high score belonged to cell death, energy production, and glucose metabolism categories. The cell death category included the following proteins: NPM1, P4HB, LGALS3, CASP3, PDIA3, ATP5A1, GAPDH, ANXA4, HSPA5, RPLP0, UCHL1, STMN1, ENO1, ANXA5, MZB1, PSMB1, ALDOA, VDAC1, LDHA, HSPB1, and PRDX2 (Fig. 1). These results are consistent with previous studies. Benes *et al.* [36] showed increased expression of pro-apoptotic gene transcripts in postmortem brains of bipolar disorder patients. Furthermore, Herbeth *et al.* [21] indicated altered cell death and inflammation-related proteins in peripheral blood mononuclear cells and serum from patients with euthymic bipolar disorder. Brain imaging studies demonstrated reductions in the mean gray matter volume of brains from patients with bipolar disorder [37]. Previous studies reported a decreased density of nonpyramidal neurons in layer II of the anterior cingulate and a lower number of glial cells in layer III with bipolar disorder [38]. Meta-analyses of volumetric magnetic resonance imaging studies showed reduced volume of gray matter in the anterior cingulate and bilateral insula [39,40]. Neuropathological studies of bipolar disorder showed decreases of each brain field and neuronal cells. Because mood stabilizers and antidepressants, which are used for treatment of bipolar disorder, have neuroprotective actions [5,41,42], it has been suggested that cells derived from patients with bipolar disorder are more vulnerable to factors related to cell death than those from controls. Patients with unipolar or bipolar depression exhibit decreased brain-derived neurotrophic factor levels [43]. Moreover, mood stabilizers have neuroprotective effects by increasing bcl-2 levels [42,44,45]. These findings suggest cellular vulnerability has a role in the pathology of bipolar disorder. Dysregulation of the apoptotic process found in the monozygotic twins discordant for bipolar disorder might be relevant to this hypothesis.

We examined the relationship of the identified proteins with canonical pathways and found that the proteins were related to the glycolysis pathway. The proteins included PKM2, ALDH2, ENO1, PGAM1, GAPDH, ALDOA, LDHA, and ALDOC. Glycolysis, or anaerobic respiration, is a fundamental metabolic process that produces energy for all cells. In order to maintain its functions, the brain needs an enormous amount of energy compared with other tissues. ALDOC is a brain-specific glycolysis enzyme that catalyzes the reversible aldol cleavage of fructose-1,6-biphosphate and fructose-1-phosphate to dihydroxyacetone phosphate and either glyceraldehyde-3-phosphate or glyceraldehyde [46]. In the present study, we found a decrease of the ALDOC protein level in the affected twin. However, previous reports showed that protein expression level of ALDOC was increased in the frontal cortex of patients, including those with mood disorder [35,47]. This discrepancy might reflect differences between tissues. Moreover, we found differential expression of many essential enzymes of glycolysis such as TPI1, ALDOA, and PGAM1. A

previous report using positron emission tomography showed that familial bipolar depressive patients had decreased blood flow in the cerebrum and a decreased rate of glucose metabolism in the ventral anterior cingulate cortex [37]. As indicated by an alteration in energy metabolism, compromised metabolic function has been reported in bipolar disorder [48,49]. In these studies, alteration of mitochondrial proteins was reported. Mitochondria are involved in processes including the TCA cycle, glycolysis and gluconeogenesis, lipogenesis, and malate-asparate shuttle [50]. Thus, changes in these proteins may lead to major alterations in the energy pathways, thus affecting ATP production. Recently, many reports have suggested that mitochondrial dysfunction is involved in bipolar disorder and other psychiatric disorders [51,52,53]. Mitochondria are also involved in other essential processes such as apoptosis, oxidative stress, and calcium regulation [50]. Thus, a decrease in energy production due to mitochondrial dysfunction in the brains of patients with bipolar disorder may be compensated for by an increase in energy production by glycolysis. It is possible that mitochondrial dysfunction affects neuronal cell death. Further study is needed to know whether these alterations in glycolysis-related proteins are a cause or consequence of the disease process.

This is the first study to our knowledge to apply proteomics for the analysis of monozygotic twins discordant for bipolar disorder, and it has major limitations. First of all, we analyzed only a single pair of monozygotic twins. Thus, results cannot be applied to bipolar disorder in general. Another limitation is the tissue examined; that is, lymphoblastoid cells. Although brain samples may be optimal to identify molecules directly related to bipolar disorder, brain samples of twins are difficult to access. In addition, accessible tissues such as body fluid and peripheral cells such as serum, plasma, cerebrospinal fluids, saliva, urine, and peripheral blood cells should be used for biomarkers. In this study, we used lymphoblastoid cells and avoided a possible effect of medication by culturing the cells in drug-free media. However, a possibility that the effect of medication at the collection of blood last even after culturing the cells in drug-free media for a month cannot be totally ruled out. The other major limitation is the small number of case–control samples.

In summary, we performed a proteomic analysis of lymphoblastoid cells in a pair of monozygotic twins discordant for bipolar disorder. The identified proteins were mainly categorized as those involved in cell death and glycolysis. In a case–control study, protein expression of PGAM1, which is related to glycolysis, was significantly higher in patients than in healthy controls. The present findings suggest future new targets that may be relevant to the pathology of bipolar disorder. The present results need to be tested in a larger, independent sample set to reach a valid conclusion.

Author Contributions

Conceived and designed the experiments: AK TK. Performed the experiments: AK K. Ohtawa K. Otsuki MU. Analyzed the data: AK TK. Contributed reagents/materials/analysis tools: HS YO. Wrote the paper: AK TK.

References

1. Gershon ES, Cloninger CR (1994) Genetic approaches to mental disorders. Washington, DC: American Psychiatric Press.
2. Goodwin FK, Jomison KR (1990) Manic-depressive illness. New York: Oxford University Press.
3. Bunney WE Jr, Davis JM (1965) Norepinephrine in depressive reactions. A review. Arch Gen Psychiatry 13: 483–494.
4. Meltzer H (1989) Serotonergic dysfunction in depression. Br J Psychiatry Suppl: 25–31.
5. Chuang DM, Chen RW, Chalecka-Franaszek E, Ren M, Hashimoto R, et al. (2002) Neuroprotective effects of lithium in cultured cells and animal models of diseases. Bipolar Disord 4: 129–136.
6. Sklar P, Smoller JW, Fan J, Ferreira MA, Perlis RH, et al. (2008) Whole-genome association study of bipolar disorder. Mol Psychiatry 13: 558–569.
7. Ferreira MA, O'Donovan MC, Meng YA, Jones IR, Ruderfer DM, et al. (2008) Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder. Nat Genet 40: 1056–1058.
8. Altamura AC, Dell'Osso B, Berlin HA, Buoli M, Bassetti R, et al. (2010) Duration of untreated illness and suicide in bipolar disorder: a naturalistic study. Eur Arch Psychiatry Clin Neurosci 260: 385–391.
9. Schwarz E, Bahn S (2008) The utility of biomarker discovery approaches for the detection of disease mechanisms in psychiatric disorders. Br J Pharmacol 153 Suppl 1: S133–136.
10. English JA, Pennington K, Dunn MJ, Cotter DR (2011) The neuroproteomics of schizophrenia. Biol Psychiatry 69: 163–172.
11. Iwamoto K, Bundo M, Kato T (2005) Altered expression of mitochondria-related genes in postmortem brains of patients with bipolar disorder or schizophrenia, as revealed by large-scale DNA microarray analysis. Hum Mol Genet 14: 241–253.
12. Fountoulakis M, Hardmeier R, Hoger H, Lubec G (2001) Postmortem changes in the level of brain proteins. Exp Neurol 167: 86–94.
13. Franzen B, Yang Y, Sunnemark D, Wickman M, Ottervald J, et al. (2003) Dihydropyrimidinase related protein-2 as a biomarker for temperature and time dependent post mortem changes in the mouse brain proteome. Proteomics 3: 1920–1929.
14. Li JZ, Vawter MP, Walsh DM, Tomita H, Evans SJ, et al. (2004) Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. Hum Mol Genet 13: 609–616.
15. Vawter MP, Tomita H, Meng F, Bolstad B, Li J, et al. (2006) Mitochondrial-related gene expression changes are sensitive to agonal-pH state: implications for brain disorders. Mol Psychiatry 11: 615, 663–679.
16. Li J, Gould TD, Yuan P, Manji HK, Chen G (2003) Post-mortem interval effects on the phosphorylation of signaling proteins. Neuropsychopharmacology 28: 1017–1025.
17. Hollander BA, Bennett GS (1991) Lithium chloride alters cytoskeletal organization in growing, but not mature, cultured chick sensory neurons. J Neurosci Res 28: 332–342.
18. Cannell GR, Bailey MJ, Dickinson RG (2002) Inhibition of tubulin assembly and covalent binding to microtubular protein by valproic acid glucuronide in vitro. Life Sci 71: 2633–2643.
19. Corena-McLeod Mdel P, Oliveros A, Charlesworth C, Madden B, Liang YQ, et al. (2008) Paliperidone as a mood stabilizer: a pre-frontal cortex synaptoneurosomal proteomics comparison with lithium and valproic acid after chronic treatment reveals similarities in protein expression. Brain Res 1233: 8–19.
20. Sussulini A, Dihazi H, Banzato CE, Arruda MA, Stuhmer W, et al. (2011) Apolipoprotein A-I as a candidate serum marker for the response to lithium treatment in bipolar disorder. Proteomics 11: 261–269.
21. Herberth M, Koethe D, Levin Y, Schwarz E, Krzyszton ND, et al. (2011) Peripheral profiling analysis for bipolar disorder reveals markers associated with reduced cell survival. Proteomics 11: 94–105.
22. Allen MG (1976) Twin studies of affective illness. Arch Gen Psychiatry 33: 1476–1478.
23. Bertelsen A, Harvald B, Hauge M (1977) A Danish twin study of manic-depressive disorders. Br J Psychiatry 130: 330–351.
24. Kato T, Iwamoto K, Kakiuchi C, Kuratomi G, Okazaki Y (2005) Genetic or epigenetic difference causing discordance between monozygotic twins as a clue to molecular basis of mental disorders. Mol Psychiatry 10: 622–630.
25. Kakiuchi C, Iwamoto K, Ishiwata M, Bundo M, Kasahara T, et al. (2003) Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder. Nat Genet 35: 171–175.
26. Hu VW, Frank BC, Heine S, Lee NH, Quackenbush J (2006) Gene expression profiling of lymphoblastoid cell lines from monozygotic twins discordant in severity of autism reveals differential regulation of neurologically relevant genes. BMC Genomics 7: 118.
27. Matigian N, Windus L, Smith H, Filippich C, Pantelis C, et al. (2007) Expression profiling in monozygotic twins discordant for bipolar disorder reveals dysregulation of the WNT signalling pathway. Mol Psychiatry 12: 815–825.
28. Kuratomi G, Iwamoto K, Bundo M, Kusumi I, Kato N, et al. (2008) Aberrant DNA methylation associated with bipolar disorder identified from discordant monozygotic twins. Mol Psychiatry 13: 429–441.
29. Sugawara H, Iwamoto K, Bundo M, Ueda J, Miyauchi T, et al. (2011) Hypermethylation of serotonin transporter gene in bipolar disorder detected by epigenome analysis of discordant monozygotic twins. Translational Psychiatry 1: e24.
30. Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720–1730.

31. Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 4: 117.
32. Kato T, Ishiwata M, Mori K, Washizuka S, Tajima O, et al. (2003) Mechanisms of altered Ca2+ signalling in transformed lymphoblastoid cells from patients with bipolar disorder. Int J Neuropsychopharmacol 6: 379–389.
33. Vercauteren FG, Bergeron JJ, Vandesande F, Arckens L, Quirion R (2004) Proteomic approaches in brain research and neuropharmacology. Eur J Pharmacol 500: 385–398.
34. Campbell JW, Watson HC, Hodgson GI (1974) Structure of yeast phosphoglycerate mutase. Nature 250: 301–303.
35. Novikova SI, He F, Cutrufello NJ, Lidow MS (2006) Identification of protein biomarkers for schizophrenia and bipolar disorder in the postmortem prefrontal cortex using SELDI-TOF-MS ProteinChip profiling combined with MALDI-TOF-PSD-MS analysis. Neurobiol Dis 23: 61–76.
36. Benes FM, Matzilevich D, Burke RE, Walsh J (2006) The expression of proapoptosis genes is increased in bipolar disorder, but not in schizophrenia. Mol Psychiatry 11: 241–251.
37. Drevets WC, Price JL, Simpson JR Jr, Todd RD, Reich T, et al. (1997) Subgenual prefrontal cortex abnormalities in mood disorders. Nature 386: 824–827.
38. Benes FM, Vincent SL, Todtenkopf M (2001) The density of pyramidal and nonpyramidal neurons in anterior cingulate cortex of schizophrenic and bipolar subjects. Biol Psychiatry 50: 395–406.
39. Ellison-Wright I, Bullmore E (2010) Anatomy of bipolar disorder and schizophrenia: a meta-analysis. Schizophr Res 117: 1–12.
40. Bora E, Fornito A, Yucel M, Pantelis C (2010) Voxelwise meta-analysis of gray matter abnormalities in bipolar disorder. Biol Psychiatry 67: 1097–1105.
41. Manji HK, Moore GJ, Chen G (2000) Clinical and preclinical evidence for the neurotrophic effects of mood stabilizers: implications for the pathophysiology and treatment of manic-depressive illness. Biol Psychiatry 48: 740–754.
42. Manji HK, Moore GJ, Rajkowska G, Chen G (2000) Neuroplasticity and cellular resilience in mood disorders. Mol Psychiatry 5: 578–593.
43. Kapczinski F, Frey BN, Kauer-Sant'Anna M, Grassi-Oliveira R (2008) Brain-derived neurotrophic factor and neuroplasticity in bipolar disorder. Expert Rev Neurother 8: 1101–1113.
44. Yuan PX, Huang LD, Jiang YM, Gutkind JS, Manji HK, et al. (2001) The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth. J Biol Chem 276: 31674–31683.
45. Manji HK, Chen G (2002) PKC, MAP kinases and the bcl-2 family of proteins as long-term targets for mood stabilizers. Mol Psychiatry 7 Suppl 1: S46–56.
46. Villar-Palasi C, Larner J (1970) Glycogen metabolism and glycolytic enzymes. Annu Rev Biochem 39: 639–672.
47. Johnston-Wilson NL, Sims CD, Hofmann JP, Anderson L, Shore AD, et al. (2000) Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium. Mol Psychiatry 5: 142–149.
48. Pennington K, Beasley CL, Dicker P, Fagan A, English J, et al. (2008) Prominent synaptic and metabolic abnormalities revealed by proteomic analysis of the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder. Mol Psychiatry 13: 1102–1117.
49. English JA, Dicker P, Focking M, Dunn MJ, Cotter DR (2009) 2-D DIGE analysis implicates cytoskeletal abnormalities in psychiatric disease. Proteomics 9: 3368–3382.
50. Scheffler IE (1999) Mitochondria: Wiley-Liss.
51. Kato T, Kato N (2000) Mitochondrial dysfunction in bipolar disorder. Bipolar Disord 2: 180–190.
52. Clay HB, Sillivan S, Konradi C (2011) Mitochondrial dysfunction and pathology in bipolar disorder and schizophrenia. Int J Dev Neurosci 29: 311–324.
53. Manji H, Kato T, Di Prospero NA, Ness S, Beal MF, et al. (2012) Impaired mitochondrial function in psychiatric disorders. Nat Rev Neurosci 13: 293–307.

International Journal of Psychophysiology 84 (2012) 26–32

Contents lists available at SciVerse ScienceDirect

International Journal of Psychophysiology

journal homepage: www.elsevier.com/locate/ijpsycho

P50 suppression in human discrimination fear conditioning paradigm using danger and safety signals

Taichi Kurayama [a], Daisuke Matsuzawa [a,b], Zen Komiya [b], Ken Nakazawa [b], Susumu Yoshida [c], Eiji Shimizu [a,b,*]

[a] Research Center for Child Mental Development, Graduate School of Medicine, Chiba University, Japan
[b] Department of Cognitive Behavioral Physiology, Chiba University Graduate School of Medicine, Japan
[c] Division of Physical Therapy, Department of Rehabilitation Sciences, Faculty of Health Care Sciences, Chiba Prefectural University of Health Sciences, Japan

ARTICLE INFO

Article history:
Received 15 March 2011
Received in revised form 22 December 2011
Accepted 4 January 2012
Available online 15 January 2012

Keywords:
Anxiety
Sensory gating
P50 suppression
Classical fear conditioning
Fear extinction

ABSTRACT

Auditory P50 suppression, which is assessed using a paired auditory stimuli (S1 and S2) paradigm to record the P50 mid-latency evoked potential, is assumed to reflect sensory gating. Recently, P50 suppression deficits were observed in patients with anxiety disorders, including panic disorder, post-traumatic stress disorder and obsessive–compulsive disorder, as we previously reported. The processes of fear conditioning are thought to play a role in the pathophysiology of anxiety disorders. In addition, we found that the P50 sensory gating mechanism might be physiologically associated with fear conditioning and extinction in a simple human fear-conditioning paradigm that involved a light signal as a conditioned stimulus (CS+). Our objective was to investigate the different patterns of P50 suppression in a discrimination fear-conditioning paradigm with both a CS+ (danger signal) and a CS− (safety signal). Twenty healthy volunteers were recruited. We measured the auditory P50 suppression in the control (baseline) phase, in the fear-acquisition phase, and in the fear-extinction phase using a discrimination fear-conditioning paradigm. Two-way (CSs vs. phase) Analysis of variance with repeated measures demonstrated a significant interaction between the two factors. Post-hoc LSD analysis indicated that the P50 S2/S1 ratio in the CS+ acquisition phase was significantly higher than that in the CS− acquisition phase. These results suggest that the auditory P50 sensory gating might differ according to the cognition of the properties (potentially dangerous or safe) of the perceived signal.

1. Introduction

Fear conditioning is a type of associative learning that involves the formation of linkages between a neutral stimulus and a stimulus with innate behavioral significance (Sanders et al., 2003). In a simple fear-conditioning paradigm, a conditioned stimulus (CS), such as a light signal or a tone, and an aversive unconditioned stimulus (US), such as an electric shock, are repeatedly and consistently paired. The CS alone begins to elicit a conditioned response (CR) in anticipation of presentation of the US (Cheng et al., 2006; Rosen et al., 1998; Wolpe, 1981). In humans, a CR is often assessed by measuring the skin-conductance responses (SCR). Fear extinction refers to the weakening of the CR through the repeated presentation of the CS in the absence of the US with which it was previously paired. It has been hypothesized that extinction does not erase the original memory (a previously established CS–US association) but rather forms a new memory of safety that inhibits fear expression (a newly established CS–no US association) (Myers and Davis, 2007). This behavioral model has been widely used for both basic (animal) and clinical (human) studies that investigate the pathophysiology of anxiety-related disorders, in which the core symptoms are excessive fear and anxiety that are hardly attenuated by extinction; in such disorders, deficits in the inhibitory control of the brain are considered to play a role (Sotres-Bayon et al., 2006).

In the discrimination fear-conditioning paradigm, the CSs that have been adopted are known as the CS+ (danger signal) and CS− (safety signal) (Hofmann, 2008). Discrimination learning in the respondent conditioning is indexed as the difference between the CRs to the CS+ and CS− (Lissek et al., 2005). Healthy adults should be able to suppress the fear response during CS− presentations and show higher rates of discrimination learning. If patients fail to inhibit fear in the presence of safety cues during fear conditioning, they might display the fear response to both the CS+ and the CS−, leading to low levels of discrimination learning even if these patients actually have the fear response to the CS+. In a meta-analytic review by Lissek et al., it was concluded that patients with anxiety disorders were more conditioned to danger cues (CS+) than were control subjects, and their inhibitory conditioning to safety signals (CS−) was impaired (Lissek et al., 2005). For example, Michael et al. reported that patients

* Corresponding author at: Department of Cognitive Behavioral Physiology, Chiba University Graduate School of Medicine, Inohana 1-8-1, Chiba 260-8670, Japan. Tel.: +81 43 226 2027; fax: +81 43 226 2028.
E-mail address: eiji@faculty.chiba-u.jp (E. Shimizu).

0167-8760/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpsycho.2012.01.004

with panic disorder (PD) could not reduce their SCRs to the CS- in the extinction phase as the healthy subjects could (Michael et al., 2007). Blechert et al. reported that patients with post-traumatic stress disorder (PTSD) showed delayed extinction of SCR to the CS+ and heightened SCR to the CS− during the acquisition phase compared to healthy participants who were not exposed to trauma (Blechert et al., 2007). Clinically, the above-mentioned finding in discrimination fear conditioning suggested that in anxiety disorders, the inability to distinguish the external stimulus seemed to be physiologically associated with a failure of the habituation to irrelevant sensory input.

Sensory gating is defined as the habituation to irrelevant sensory input, which might be an important function for the human brain as the central information-processing organ of the body. The failure of sensory gating might be associated with mental disturbances. A well-established method for sensory gating assessment is the suppression of an auditory evoked potential (AEP) of P50, which is a positive waveform of small amplitude that occurs 40–70 ms after an auditory stimulus. Using two paired auditory stimuli, P50 suppression is evaluated using the ratio of the amplitude of the second to the first stimulus response (S2/S1 ratio) or using the decrease from the 1st to the 2nd stimuli in terms of microvolts (Fuerst et al., 2007; Rentzsch et al., 2008b). Deficits in P50 suppression have been mainly demonstrated in clinical studies of schizophrenia, but these deficits have also been reported in patients with anxiety disorders, such as PTSD (Karl et al., 2006) and panic disorder (Ghisolfi et al., 2006a). In addition, we have found P50 suppression deficits in patients with obsessive–compulsive disorder (OCD) (Hashimoto et al., 2008). These data suggest that P50 suppression deficits may have considerable interest as a putative biomarker or endophenotype for the vulnerability to anxiety disorders.

In our previous studies, we hypothesized that there was a physiological association between P50 suppression and the mechanism of fear acquisition and extinction; this finding was based on the concept that if there was such an association, then the attenuated P50 suppression (i.e., loosened sensory gating) would manifest as a vulnerability to fear acquisition and a deficit in the inhibitory control of the brain, which could subsequently lead to a delay in the recovery from the fear. Therefore, we combined a P50-suppression measurement with a simple fear-conditioning paradigm, and we reported that the mean P50 S2/S1 ratio in the fear-acquisition phase was significantly more elevated than in the control phase but recovered to the basal level in the extinction phase in healthy participants (Kurayama et al., 2009). In contrast and in support of our hypothesis, the elevation of the P50 S2/S1 ratio, which represents attenuated P50 suppression, was sustained through the fear extinction phase in patients with OCD (Nanbu et al., 2010). These findings suggest a potential link between the processes of the acquisition/extinction of conditioned fear and P50 suppression.

These findings collectively suggest that the deficit in the sensory gating mechanism in the discrimination of safe signals or fear signals might be involved in the pathophysiology of anxiety disorders, but this possibility remained to be elucidated from our previous studies of the single fear-conditioning paradigm. In the current study, we aimed to measure a discriminable change in P50 suppression with the CSs in separate roles by first elucidating the physiological association between the sensory gating and discrimination learning of stimulus properties (potentially dangerous or safe) in healthy persons before proceeding to a study of patients with anxiety disorders.

2. Material and methods

2.1. Participants

This study was performed after approval by the ethics committee at Chiba University Graduate School of Medicine. Written informed consent was obtained from all the participants. Twenty-five healthy volunteers (11 men and 14 women), who ranged in age from 21 to 34 years (mean 27.5, standard deviation [SD] 4.0), were recruited to the study. None of the participants had a history of psychiatric, neurological, or hearing problems, as determined by a non-structured interview. All the participants were non-smokers, but because nicotine and caffeine can affect P50 suppression (Ghisolfi et al., 2006b; Knott et al., 2010), the participants were instructed to abstain from smoking and caffeine-containing products for 12 h prior to the experiment.

2.2. Experimental design and procedure

Though this study was conducted using methods similar to those described in our previous study (Kurayama et al., 2009), one conditioned stimulus (i.e., CS−) was added to the method. We used a red light signal (CS+) or a blue light signal (CS−) for the discrimination fear-conditioning paradigm. Participants were seated in a comfortable recliner and were instructed to relax with their eyes open and to keep viewing the light-emitting diode (LED) lamp that was used as a conditioned stimulus. The intensity of the electric shocks to the wrist (used as the US) was determined by each participant to be "aversive but not painful" (LaBar et al., 1998). The mean voltage of the electrical shock was 83.8 (SD = 19.7, range 41–195 V). Throughout the experiment, the participants were required to look at the LED lamp, which was used as the CS in the acquisition phase, and to listen to auditory clicks, which were used to measure the P50 suppressions. Fig. 1 summarizes the conditioning parameters that were used in the present study. The experiment consisted of the following three phases: phase 1 for control, phase 2 for fear acquisition, and phase 3 for fear extinction. Participants were exposed to 10 repetitive stimuli of each CS+ and CS−, alternating with a 30 ± 25 s intertribal interval throughout the three phases. In phase 2, only CS+ was paired with US, and in phase 3, the CSs were again individually presented. Skin conductance responses were also recorded throughout the three phases. To measure the P50 suppressions, the participants were given 60 pairs of click sounds (S1 as the first stimulus and S2 as the second stimulus) in each phase, independently of the presentation timing of the CSs. Control waveforms of P50 were recorded in phase 1.

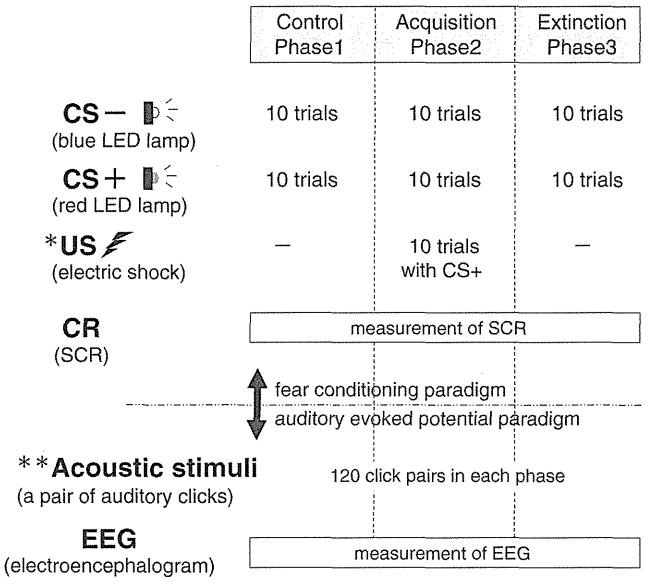


Fig. 1. The conditioning parameters used in the present study. Abbreviations: CS: conditional stimulus (CS−: safety signal, a blue LED lamp; CS+: danger signal, a red LED lamp); US: unconditioned stimulus; CR: conditioned responses; SCR: skin conductance responses; EEG: electroencephalogram. Note: *An acoustic stimulation was delivered as a click tone, the intensity of which was adjusted to 40 dB above the hearing threshold for each subject. **The US intensity was adjusted to the highest value that was aversive but not painful for each participant.

2.3. Stimulus presentation method

Fig. 2 illustrates the timeline of the stimulus parameters in the acquisition phase that was used in the present study. The CS and US were controlled and delivered using the RPvds software (Tucker-Davis Technologies, Alachua, Florida) and the RP2.1 Real-Time Processor (Tucker-Davis Technologies). The CS− and CS+ were a blue and red light, respectively, alternately delivered by an LED lamp that was placed 1.5 m in front of each participant. The US (200-ms duration, 50 Hz) was an electric shock that was delivered transcutaneously over the median-nerve area on the left wrist by a stimulating bar electrode. The electrode leads were secured using a Velcro strap and were attached to a stimulator (SEM-4201 Nihon Kohden, Tokyo, Japan). The US onset was a 200-ms timepoint before the CS+ offset, and the US offset was simultaneous with the CS+ offset. The total presentation time of one conditioning period (the CS+ and CS− added together) was fixed for 60 s. The CS+ presentation was initiated every 60 s during the conditioning phase and was presented for a duration that varied between 5 s and 55 s in a pseudorandom order. After the CS+ presentation period, the CS− was presented alternately; then, 60 s after the CS+ presentation start time, the CS− was terminated, and the CS+ of the next conditioning phase was presented alternately. An acoustic stimulus (a .1-ms pulse) to measure P50 was delivered through earphones. These stimuli (S1 and S2) were separated by a 500-ms interstimulus interval (ISI), and the intertrial interval (ITI) varied between 4.75 s and 5.25 s (Rentzsch et al., 2008a; Zouridakis and Boutros, 1992) for the removal of synchronization responses. The click-sound intensity was adjusted to 40 dB above each participant's hearing threshold (Kisley et al., 2001). As described above, the presentation timing of the paired click sounds and the CSs was not temporally related.

2.4. EEG and SCR recording methods

An electroencephalogram (EEG) was recorded from three midline-scalp electrode sites, Fz, Cz, and Pz (according to the 10/20 system), and was referenced to electrodes that were placed on the left ear lobe. An electrooculogram (EOG) was recorded by placing electrodes on the outer canthus of the left eye and below the right eye. EOGs were recorded from a pair of Ag/AgCl cup electrodes, and all impedances for recording electrodes were below 5 kΩ. The EEG and EOG signals were amplified and analog-filtered at .1 and 250 Hz. The output signals from the amplifiers were digitized using a 16-bit AD converter at 1000 Hz (Power1401; Cambridge Electronic Design, United Kingdom) and were recorded on a computer. The skin conductance was sampled through Ag/AgCl electrodes, using a UFI Bioderm model 2701 constant voltage device (1081 FG, UFI, Morro Bay, California) that was set at .5 V. This system was used to output a tonic DC-coupled SCR, measured in micro-Siemens (μS). Electrodes were attached by Velcro straps to the middle phalanges of the second and third digits of the right hand. A UFI 1090 BioGel contact medium was used as an electrolyte. The skin conductance was sampled at 250 Hz during the course of each trial, amplified, and stored on a computer.

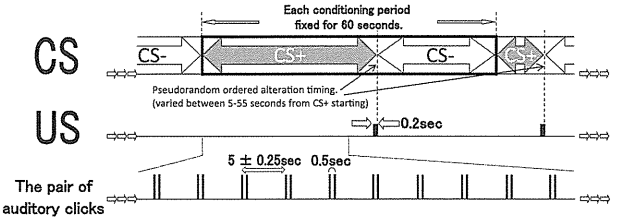


Fig. 2. Timeline illustrating stimulus parameters in the acquisition phase. Note: The conditioned stimulus (CS+) co-terminated with the delivery of a brief electric shock to the wrist (unconditioned stimulus [US]). The parameters for extinction were identical, except that no US was delivered.

2.5. EEG and SCR data analysis

We analyzed all the EEG data offline using the Spike2 software program (Spike2, Cambridge Electronic Design Ltd., Cambridge, UK). The EEG data were manually inspected and were rejected if they were contaminated by substantial movement (segments with deviations greater than ±100 mV on any of the EEG channels) (Metzger et al., 2002) or eye-blink artifacts (segments with deviations greater than ±85 mV on the bipolar EOG channels) (Smith et al., 1994). The rejected EEG data reached 20 at maximum in a single phase. Each trial was digitally filtered at 10–50 Hz for the measurement of P50. All P50 components were scored at the Cz site (Patterson et al., 2008). The P50 component was identified by a computer program according to published guidelines with a base-to-peak measure (Picton et al., 2000). The P50 amplitude was identified as the maximum positivity between 40 and 70 ms post-stimulus, and these were scored relative to a 200-ms pre-stimulus control (White and Yee, 2006). The P50 suppression was assessed using the S2/S1 amplitude ratios. To remove the influence of the visually evoked potential by the CSs (CS+ and CS−) and the somatosensory evoked potential by the US, the waveforms for 1 s from the CS and US onsets were rejected. The SCR, except in the CS− phase, was scored according to the methods described in previous studies (LaBar et al., 1998; Prokasy and Kumpser, 1973). During the CS+ presentation period, we used the first SCR (First Interval Responses: FIR) as the conditioned response (Prokasy and Kumpser, 1973). In the CS− presentation period, the first positive peak after the unconditioned stimulus responses but not the FIR was used to measure the canceling effect of the US responses.

To examine the alterations of each P50 component in each pair of signals (CS− and CS+) throughout the three phases, we analyzed the data as follows: First, we constructed each grand average waveform of the AEP from each participant. Second, we measured each P50 component in each pair of signals throughout the three phases for each participant, using a base-to-peak measure (identified by a computer program). Third, we input these parameters of all 25 participants together into a datasheet for statistical analyses and compared the P50 component parameters among the three phases for each pair of signals. Fourth, the EEG data were screened for outliers, which were defined as those cases falling more than two standard deviations from the mean amplitudes of S1 and S2 and of the S2/S1 ratio throughout the three phases (Csomor et al., 2008; Edgar et al., 2003; Nagamoto et al., 1991). Even if only one piece of data from the 6 phases met the exclusion criteria, we excluded all the data from the analyses so that the final number of subjects analyzed was 20, which was a reduction from the 25 subjects who were initially recruited.

2.6. Statistical analysis

Comparisons of each AEP parameter at the Cz site (S2/S1 ratios, S1 and S2 amplitude, and latencies) and the SCR were performed using the two-way (CS+/− and the three phases as factors) analysis of variance (ANOVA) with repeated measures. The Greenhouse–Geisser correction was used for violations of the sphericity assumption. Post-hoc LSD (least significant difference) analyses were applied when a significant ANOVA result was obtained. The level of statistical significance was set to $p<.05$. All analyses were performed using the Windows software SPSS 19.0 (SPSS, Chicago, Illinois).

3. Results

All subjects were aware of the CS–US contingencies and felt the US (electrical shock) as aversive, as assessed by post-experimental interviews. The mean ± SD of the P50 S2/S1 ratio, amplitude of the P50 components and SCR amplitude for each phase are shown in Table 1. For the P50 S2/S1 ratio, a significant interaction between

Table 1
Mean ± SD of the P50 S2/S1 ratios, amplitudes, and SCR amplitudes in each phase.

	CS-			CS+		
P50	control	acquisition	extinction	control	acquisition	extinction
S2/S1ratio*	0.59 ± 0.38	0.46 ± 0.29	0.71 ± 0.58	0.52 ± 0.34	0.79 ± 0.50	0.44 ± 0.28
S1amplitude [μV]	1.50 ± 0.78	1.53 ± 0.76	1.43 ± 0.89	1.55 ± 0.94	1.44 ± 0.86	1.49 ± 0.64
S2amplitude [μV]	0.94 ± 0.77	0.76 ± 0.64	0.99 ± 0.89	0.77 ± 0.72	1.01 ± 0.62	0.65 ± 0.45
SCR**	control	acquisition	extinction	control	acquisition	extinction
amplitude [μS]	0.07 ± 0.15	0.10 ± 0.13	0.04 ± 0.08	0.08 ± 0.15	0.20 ± 0.21	0.03 ± 0.06

Data are shown as the mean ± standard deviation.
*p<.05; **p<.01; p values were determined using 2-way ANOVA with repeated measures and post-hoc LSD analysis.

the CS type and the three phases ($F(2, 38) = 5.044$, $p = 0.011$) was observed using two-way ANOVA with repeated measures. A post-hoc LSD test revealed that the ratio in the acquisition phase was significantly higher for the CS+ than for the CS− ($p = 0.021$), and in the CS+ signal, the mean P50 ratio in the acquisition phase was greater than that in the control phase ($p = 0.049$) and the extinction phase ($p = 0.014$) (Figs. 3, 4 and Table 1). No significant difference was observed in the amplitudes and latencies of S1 and S2 between the CS+ and the CS−. Two-way ANOVA with repeated measures revealed a significant interaction between the mean SCR amplitude of the CS− and that of the CS+ ($F(1.544, 21.809) = 8.453$, $p = 0.001$). Furthermore, post-hoc LSD analysis revealed that the amplitude of the SCR in the acquisition phase was significantly higher in the CS+ than in the CS− ($p = 0.011$), and in the CS+ signal, the mean SCR amplitude in the acquisition phase was greater than that in the control phase ($p = 0.003$) and extinction phase ($p = 0.002$) (Fig. 5 and Table 1). No significant correlations were observed between the P50 components and SCR.

4. Discussion

Our study's major results revealed that the P50 S2/S1 ratios of the CS+ were elevated from the control level in the acquisition phase and recovered to the control level in the extinction phase. In contrast, the P50 S2/S1 ratio of the CS− did not significantly change in any of the three phases. To the best of our knowledge, this is the first report that demonstrates different patterns of auditory P50 suppression between the CS+ and CS− in the discrimination fear-conditioning paradigm. Our findings suggested that the P50 sensory gating might differ according to the presented signal properties (i.e., potentially dangerous or safe).

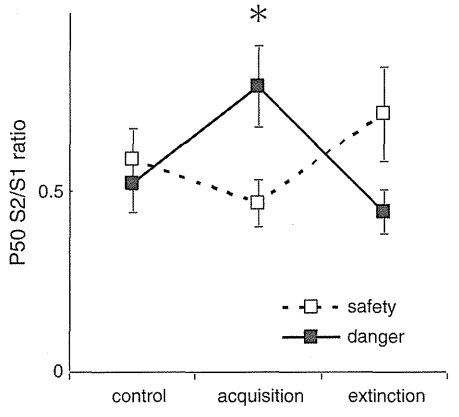


Fig. 3. P50 S2/S1 ratios of the CS− and CS+ in each phase. Note: Data are shown as the mean ± SEM (standard error of the mean).

Regarding these results, it should be noted that the P50 S1 amplitude in the acquisition phase revealed no change between that of the CS+ and CS−, but the P50 S2 amplitude of the CS+ in the acquisition phase demonstrated a 1.3-fold increase compared to that of the CS−, though this increase was not statistically significant. These observations suggest that the increase of the P50 S2/S1 ratio in the fear-acquisition phase in the CS+, but not in the CS−, occurred mainly because of a failure to suppress the second P50 response and not due to changes of the first response after the initial auditory stimulus.

The term "gating" is a hypothetical psychophysiological construct in the brain and refers to a central inhibitory function that occurs at the neural level (Brenner et al., 2004). We propose that the gating response is discriminatory because the P50 sensory gating temporarily opens in the fear-acquisition phase for danger signals but not for safety signals, and the opened "gate" that responds to the danger signal closes again to shut out irrelevant sensory input in the fear-extinction phase. We believe that our current study offers evidence to support this idea. In our previous study (Nanbu et al., 2010), the patients with OCD presented P50 suppression deficits in the fear-extinction phase during simple fear conditioning compared to healthy controls. Some studies reported that patients with anxiety disorders exhibited hypersensitive SCR in the fear-acquisition phase and that such responses were sustained even in the extinction phase and involved the CS− safety signal in particular (Blechert et al., 2007; Lissek et al., 2005; Michael et al., 2007). If our results apply to future studies of patients with anxiety disorders, then the discriminate role of P50 suppression that is observed in healthy controls would differ from that of patients. Because the scalp-recorded evoked potential is a summation of all the simultaneously active neuronal generators that are possible in a certain period (Korzyukov et al., 2007), it is difficult to localize the generators of the components of P50. However, several lines of evidence have suggested that the limbic, temporal and parietal regions were the major sources of P50 and that the frontal lobe was involved in P50 suppression that is, the gating function as an inhibitory role of the brain (Huang et al., 2003; Knott et al., 2009; Weisser et al., 2001). Prefrontal cortex (PFC) and limbic regions, such as the hippocampus and amygdala, form a part of the widespread network of structures that are involved in fear conditioning/extinction (Ehrlich et al., 2009; Sotres-Bayon et al., 2006), and the medial PFC (mPFC) is particularly considered to

contribute to the extinction process with the mPFC's inhibitory regulation on the limbic structures. Taken together, we can hypothesize that the P50 suppression deficits in the fear-extinction phases in patients with anxiety disorders might appear not only in response to danger cues (CS+) but also to safety signals (CS−). In addition, an interesting question is whether treatment, such as medication or exposure therapy, could normalize the enhanced resistance to the normalization of sensory gating.

As for the discriminative role of P50, its associations with cognitive discrimination have been suggested by previous studies. Yadon et al. reported that P50 suppression was significantly correlated with the interference score on the Stroop test and the error rate of the go/no-go task (Yadon et al., 2009). Lijffijt et al. reported a significant relationship between stronger P50 suppression and the commission of fewer errors in the delayed memory task (Lijffijt et al., 2009).

The SCR to the CS+ in the acquisition phase was significantly higher than the baseline level, and it recovered again in the extinction phase. In contrast, SCR to the CS− did not change throughout the three phases (Fig. 5). These SCR results were consistent with the previous findings in healthy subjects (Blechert et al., 2007; Lissek et al., 2005; Michael et al., 2007). In contrast, there was no significant correlation between SCR and the P50 components in the current study, which is consistent with our previous data (Kurayama et al., 2009; Nanbu et al., 2010). However, the limitation should be noted that we cannot fully deny the possibility of a correlation between SCR and the P50 components, considering that our EEG data were recorded only from the Cz site. Additional investigations, using multiple EEG recordings and source localization techniques or magnetoencephalography, are required to clarify the possible relationships between the conditioned SCR and localized auditory sensory gating in fear conditioning and extinction.

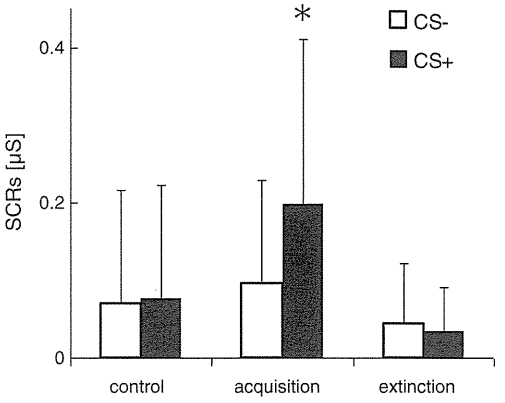


Fig. 5. Amplitudes of the SCR of the CS− and CS+ in each phase. Note: Data are shown as the mean ± SD.

In further studies, several additional factors from our conditioning paradigm should be addressed. The first point relates to the CS–US pairing rate. In the current study, the US was presented in 100% of CS+ trials, whereas in other conditioning designs, the CS and US are paired intermittently. As discussed in previous studies (Klucken et al., 2009; Weike et al., 2007), the pairing of CS and US could generate two independent processes in fear conditioning, i.e., certain automatic and possibly unconscious conditioning responses and the contingency awareness of these two stimuli. Because we aimed to investigate the

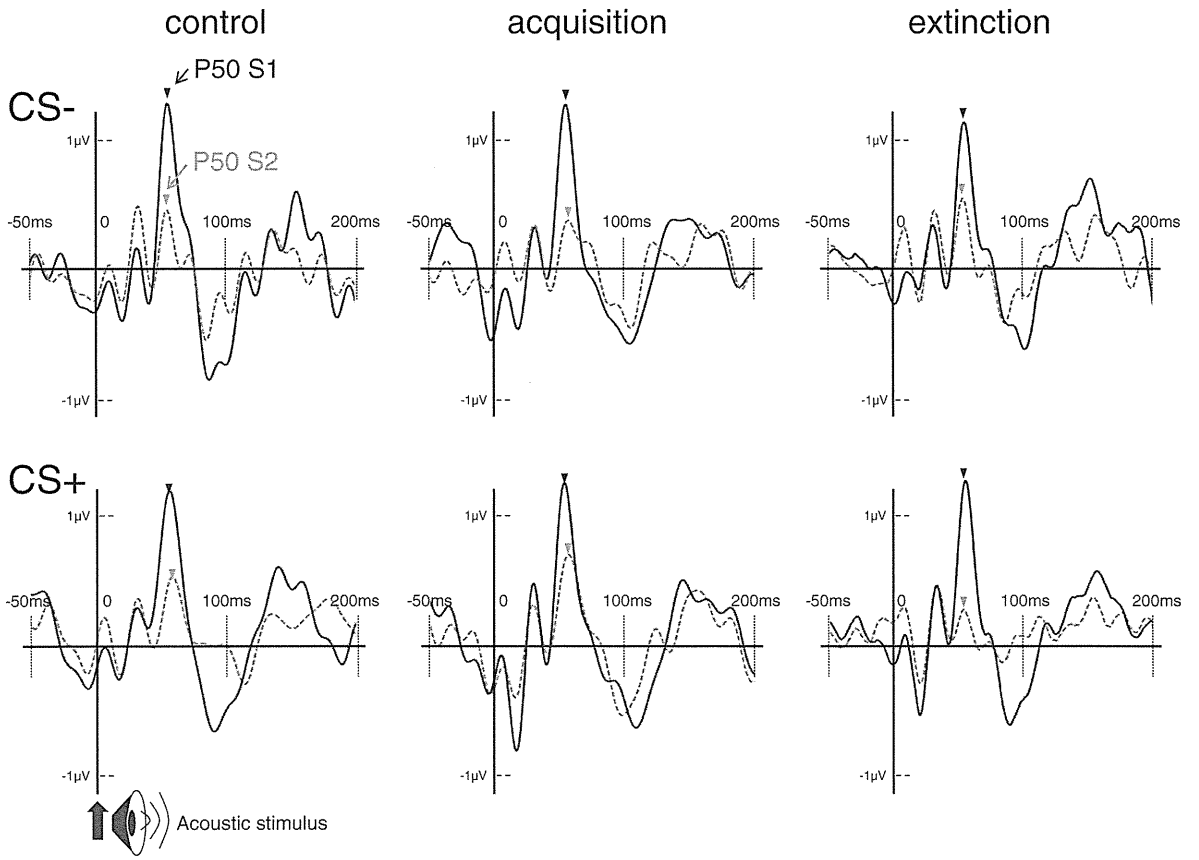


Fig. 4. Grand average waveforms of the P50 auditory evoked potentials. Note: This figure depicts the P50 auditory evoked potentials' first stimulus (S1, solid line) and second stimulus (S2, dotted line) in the control (Phase 1), acquisition (Phase 2), and extinction (Phase 3) phases of each CS+ and CS− signal from all participants. Arrowheads show the top of each wave. Each trial was digitally filtered at 10–50 Hz for the measurement of P50. All components were scored at the Cz site. This grand wave demonstrates the quality of the overall recording but not the quality of the data analysis.

change of the sensory gating (measured by P50 suppression) in the process of fear conditioning and extinction, we did not want to obtain large individual differences in the levels of the fear response measured by SCR. Thus, to equalize the participants' individual contingency awareness as much as possible, we adopted a paradigm using the UCS paired 100% with CS+; as a result, all the subjects were aware of the CS–US contingencies. A second point relates to the design of the CS–US pairing. Two types of conditioning design, i.e., delayed and trace conditioning, should be distinguished because they differ in the temporal relationship (or temporal contiguity) between the CS+ and US (Sehlmeyer et al., 2009; Shors, 2004). In delayed conditioning, the CS+ overlaps or is immediately followed by the US, which allows the participants to learn rapidly with a shorter acquisition time, and fewer trials are needed than in trace conditioning. In contrast, in trace conditioning, the presentation of the CS+ is separated from the US; thus, the working memory processes are more strongly involved in trace conditioning because the gap between the CS+ and the delayed US has to be bridged explicitly (Sehlmeyer et al., 2009; Weike et al., 2007). Therefore, to enable the participants to rapidly learn the CS+–US association, and (as mentioned above) to equalize the participants' individual contingency awareness, we employed the delayed conditioning design in this study. However, because the sensory gating process itself is implicit, the use of another conditioning design would expand the idea of the role of P50 suppression in the fear acquisition and extinction process.

Several limitations in the present study should be noted. First, nearly 100 trials are generally required to analyze a high-quality P50 auditory evoked potential (Light et al., 2010). However, in the present study, the total experiment time was rather long; thus, we deemed it necessary to limit the number of trials to prevent fatigue and drowsiness in the subjects. A total of 60 presentations of paired clicks in each phase were set to measure P50. Although, based on our experience, we considered this number sufficient for obtaining P50 waveforms with an adequate S/N ratio, a greater number of trials would increase our confidence in these results (Fig. 4). Second, we measured the SCR as an acquired conditioned fear response. However, other interpretations are possible, including the fact that a participant's response does not necessarily have a fearful (affective) property but may indicate an arousal level of stimuli; thus, the subject has simply learned the CS–US contingencies (Hamm and Vaitl, 1996; Weike et al., 2007). Other physiological measures, such as the startle-blink response, could be better indices of genuine fear learning (Light and Braff, 2001). Adding such a measure would clarify the relation of the sensory gating mechanism to fear conditioning/extinction. Third, several studies have reported that stress affects P50 suppression. For example, stressors such as cold stimulation (Ermutlu et al., 2005; Johnson and Adler, 1993) or mental arithmetic (White and Yee, 2006) resulted in the transient impairment of P50 suppression. Although we confirmed the fear acquisition and extinction phases by the measurement of SCR, we did not directly evaluate the stress itself, which the subjects might have felt during the course of the experiments. Although it might be difficult to remove stress factors from fear-conditioning experiments, further studies are necessary to investigate the different effects of fear and stress on sensory gating systems. As one approach to solve the problem in the near future, we will plan our next study to measure P50 after a sufficiently long interval for stress recovery from fear conditioning. Lastly, our final sample size was rather small (20 after 5 samples were rejected); thus, further study with a larger sample size would improve the quality of the EEG evaluation.

5. Conclusions

This is the first trial that measured P50 suppression in the discrimination paradigm of human classical fear conditioning and extinction. Consequently, in the acquisition phase but not in the extinction phase, the P50 S2/S1 ratio in the CS+ was significantly greater than that in the CS−. Our findings support the idea that the auditory P50 sensory gating might exhibit different patterns between danger and safety in the human fear-conditioning paradigm. Thus, the gating of the P50 components might differ according to the presented signal properties (potentially dangerous or safe).

References

Blechert, J., Michael, T., Vriends, N., Margraf, J., Wilhelm, F.H., 2007. Fear conditioning in posttraumatic stress disorder: evidence for delayed extinction of autonomic, experiential, and behavioural responses. Behaviour Research and Therapy 45, 2019–2033.

Brenner, C.A., Edwards, C.R., Carroll, C.A., Kieffaber, P.D., Hetrick, W.P., 2004. P50 and acoustic startle gating are not related in healthy participants. Psychophysiology 41, 702–708.

Cheng, D.T., Knight, D.C., Smith, C.N., Helmstetter, F.J., 2006. Human amygdala activity during the expression of fear responses. Behavioral Neuroscience 120, 1187–1195.

Csomor, P.A., Stadler, R.R., Feldon, J., Yee, B.K., Geyer, M.A., Vollenweider, F.X., 2008. Haloperidol differentially modulates prepulse inhibition and p50 suppression in healthy humans stratified for low and high gating levels. Neuropsychopharmacology 33, 497–512.

Edgar, J.C., Huang, M.X., Weisend, M.P., Sherwood, A., Miller, G.A., Adler, L.E., Canive, J.M., 2003. Interpreting abnormality: an EEG and MEG study of P50 and the auditory paired-stimulus paradigm. Biological Psychology 65, 1–20.

Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., Luthi, A., 2009. Amygdala inhibitory circuits and the control of fear memory. Neuron 62, 757–771.

Ermutlu, M.N., Karamursel, S., Ugur, E.H., Senturk, L., Gokhan, N., 2005. Effects of cold stress on early and late stimulus gating. Psychiatry Research 136, 201–209.

Fuerst, D.R., Gallinat, J., Boutros, N.N., 2007. Range of sensory gating values and test–retest reliability in normal subjects. Psychophysiology 44, 620–626.

Ghisolfi, E.S., Heldt, E., Zanardo, A.P., Strimitzer Jr., I.M., Prokopiuk, A.S., Becker, J., Cordioli, A.V., Manfro, G.G., Lara, D.R., 2006a. P50 sensory gating in panic disorder. Journal of Psychiatric Research 40, 535–540.

Ghisolfi, E.S., Schuch, A., Strimitzer Jr., I.M., Luersen, G., Martins, F.F., Ramos, F.L., Becker, J., Lara, D.R., 2006b. Caffeine modulates P50 auditory sensory gating in healthy subjects. European Neuropsychopharmacology 16, 204–210.

Hamm, A.O., Vaitl, D., 1996. Affective learning: awareness and aversion. Psychophysiology 33, 698–710.

Hashimoto, T., Shimizu, E., Koike, K., Orita, Y., Suzuki, T., Kanahara, N., Matsuzawa, D., Fukami, G., Miyatake, R., Shinoda, N., Fujisaki, M., Shirayama, Y., Hashimoto, K., Iyo, M., 2008. Deficits in auditory P50 inhibition in obsessive–compulsive disorder. Progress in Neuro-Psychopharmacology & Biological Psychiatry 32, 288–296.

Hofmann, S.G., 2008. Cognitive processes during fear acquisition and extinction in animals and humans: implications for exposure therapy of anxiety disorders. Clinical Psychology Review 28, 199–210.

Huang, M.X., Edgar, J.C., Thoma, R.J., Hanlon, F.M., Moses, S.N., Lee, R.R., Paulson, K.M., Weisend, M.P., Irwin, J.G., Bustillo, J.R., Adler, L.E., Miller, G.A., Canive, J.M., 2003. Predicting EEG responses using MEG sources in superior temporal gyrus reveals source asynchrony in patients with schizophrenia. Clinical Neurophysiology 114, 835–850.

Johnson, M.R., Adler, L.E., 1993. Transient impairment in P50 auditory sensory gating induced by a cold-pressor test. Biological Psychiatry 33, 380–387.

Karl, A., Malta, L.S., Maercker, A., 2006. Meta-analytic review of event-related potential studies in post-traumatic stress disorder. Biological Psychology 71, 123–147.

Kisley, M.A., Olincy, A., Freedman, R., 2001. The effect of state on sensory gating: comparison of waking, REM and non-REM sleep. Clinical Neurophysiology 112, 1154–1165.

Klucken, T., Tabbert, K., Schweckendiek, J., Merz, C.J., Kagerer, S., Vaitl, D., Stark, R., 2009. Contingency learning in human fear conditioning involves the ventral striatum. Human Brain Mapping 30, 3636–3644.

Knott, V., Millar, A., Fisher, D., 2009. Sensory gating and source analysis of the auditory P50 in low and high suppressors. NeuroImage 44, 992–1000.

Knott, V., Millar, A., Fisher, D., Albert, P., 2010. Effects of nicotine on the amplitude and gating of the auditory P50 and its influence by dopamine D2 receptor gene polymorphism. Neuroscience 166, 145–156.

Korzyukov, O., Pflieger, M.E., Wagner, M., Bowyer, S.M., Rosburg, T., Sundaresan, K., Elger, C.E., Boutros, N.N., 2007. Generators of the intracranial P50 response in auditory sensory gating. NeuroImage 35, 814–826.

Kurayama, T., Nakazawa, K., Matsuzawa, D., Yoshida, S., Nanbu, M., Suto, C., Shimizu, E., 2009. Alterations of auditory p50 suppression in human fear conditioning and extinction. Biological Psychiatry 65, 495–502.

LaBar, K.S., Gatenby, J.C., Gore, J.C., LeDoux, J.E., Phelps, E.A., 1998. Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. Neuron 20, 937–945.

Light, G.A., Braff, D.L., 2001. Measuring P50 suppression and prepulse inhibition in a single recording session. The American Journal of Psychiatry 158, 2066–2068.

Light, G.A., Williams, L.E., Minow, F., Sprock, J., Rissling, A., Sharp, R., Swerdlow, N.R., Braff, D.L., 2010. Electroencephalography (EEG) and event-related potentials (ERPs) with human participants. Current Protocols in Neuroscience 25, 21–24 (Chapter 6, Unit 6).

Lijffijt, M., Lane, S.D., Meier, S.L., Boutros, N.N., Burroughs, S., Steinberg, J.L., Moeller, F.G., Swann, A.C., 2009. P50, N100, and P200 sensory gating: relationships with behavioral inhibition, attention, and working memory. Psychophysiology 46, 1059–1068.

Lissek, S., Powers, A.S., McClure, E.B., Phelps, E.A., Woldehawariat, G., Grillon, C., Pine, D.S., 2005. Classical fear conditioning in the anxiety disorders: a meta-analysis. Behaviour Research and Therapy 43, 1391–1424.

Metzger, L.J., Carson, M.A., Paulus, L.A., Lasko, N.B., Paige, S.R., Pitman, R.K., Orr, S.P., 2002. Event-related potentials to auditory stimuli in female Vietnam nurse veterans with posttraumatic stress disorder. Psychophysiology 39, 49–63.

Michael, T., Blechert, J., Vriends, N., Margraf, J., Wilhelm, F.H., 2007. Fear conditioning in panic disorder: enhanced resistance to extinction. Journal of Abnormal Psychology 116, 612–617.

Myers, K.M., Davis, M., 2007. Mechanisms of fear extinction. Molecular Psychiatry 12, 120–150.

Nagamoto, H.T., Adler, L.E., Waldo, M.C., Griffith, J., Freedman, R., 1991. Gating of auditory response in schizophrenics and normal controls. Effects of recording site and stimulation interval on the P50 wave. Schizophrenia Research 4, 31–40.

Nanbu, M., Kurayama, T., Nakazawa, K., Matsuzawa, D., Komiya, Z., Haraguchi, T., Ogura, H., Hashimoto, T., Yoshida, S., Iyo, M., Shimizu, E., 2010. Impaired P50 suppression in fear extinction in obsessive–compulsive disorder. Progress in Neuro-Psychopharmacology & Biological Psychiatry 34, 317–322.

Patterson, J.V., Hetrick, W.P., Boutros, N.N., Jin, Y., Sandman, C., Stern, H., Potkin, S., Bunney Jr., W.E., 2008. P50 sensory gating ratios in schizophrenics and controls: a review and data analysis. Psychiatry Research 158, 226–247.

Picton, T.W., Bentin, S., Berg, P., Donchin, E., Hillyard, S.A., Johnson Jr., R., Miller, G.A., Ritter, W., Ruchkin, D.S., Rugg, M.D., Taylor, M.J., 2000. Guidelines for using human event-related potentials to study cognition: recording standards and publication criteria. Psychophysiology 37, 127–152.

Prokasy, W.F., Kumpser, K.L., 1973. Classical conditioning. In: Prokasy, W.F., Raskin, D.C. (Eds.), Electrodermal Activity in Psychological Research. Academic Press Inc.

Rentzsch, J., de Castro, A.G., Neuhaus, A., Jockers-Scherubl, M.C., Gallinat, J., 2008a. Comparison of midlatency auditory sensory gating at short and long interstimulus intervals. Neuropsychobiology 58, 11–18.

Rentzsch, J., Jockers-Scherubl, M.C., Boutros, N.N., Gallinat, J., 2008b. Test–retest reliability of P50, N100 and P200 auditory sensory gating in healthy subjects. International Journal of Psychophysiology 67, 81–90.

Rosen, J.B., Fanselow, M.S., Young, S.L., Sitcoske, M., Maren, S., 1998. Immediate-early gene expression in the amygdala following footshock stress and contextual fear conditioning. Brain Research 796, 132–142.

Sanders, M.J., Wiltgen, B.J., Fanselow, M.S., 2003. The place of the hippocampus in fear conditioning. European Journal of Pharmacology 463, 217–223.

Sehlmeyer, C., Schoning, S., Zwitserlood, P., Pfleiderer, B., Kircher, T., Arolt, V., Konrad, C., 2009. Human fear conditioning and extinction in neuroimaging: a systematic review. PLoS One 4, e5865.

Shors, T.J., 2004. Memory traces of trace memories: neurogenesis, synaptogenesis and awareness. Trends in Neurosciences 27, 250–256.

Smith, D.A., Boutros, N.N., Schwarzkopf, S.B., 1994. Reliability of P50 auditory event-related potential indices of sensory gating. Psychophysiology 31, 495–502.

Sotres-Bayon, F., Cain, C.K., LeDoux, J.E., 2006. Brain mechanisms of fear extinction: historical perspectives on the contribution of prefrontal cortex. Biological Psychiatry 60, 329–336.

Weike, A.I., Schupp, H.T., Hamm, A.O., 2007. Fear acquisition requires awareness in trace but not delay conditioning. Psychophysiology 44, 170–180.

Weisser, R., Weisbrod, M., Roehrig, M., Rupp, A., Schroeder, J., Scherg, M., 2001. Is frontal lobe involved in the generation of auditory evoked P50? Neuroreport 12, 3303–3307.

White, P.M., Yee, C.M., 2006. P50 sensitivity to physical and psychological state influences. Psychophysiology 43, 320–328.

Wolpe, J., 1981. The dichotomy between classical conditioned and cognitively learned anxiety. Journal of Behavior Therapy and Experimental Psychiatry 12, 35–42.

Yadon, C.A., Bugg, J.M., Kisley, M.A., Davalos, D.B., 2009. P50 sensory gating is related to performance on select tasks of cognitive inhibition. Cognitive, Affective, & Behavioral Neuroscience 9, 448–458.

Zouridakis, G., Boutros, N.N., 1992. Stimulus parameter effects on the P50 evoked response. Biological Psychiatry 32, 839–841.

Progress in Neuro-Psychopharmacology & Biological Psychiatry 39 (2012) 273–279

Contents lists available at SciVerse ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Effects of perinatal exposure to low dose of bisphenol A on anxiety like behavior and dopamine metabolites in brain

Shingo Matsuda [a,*], Daisuke Matsuzawa [a], Daisuke Ishii [a], Haruna Tomizawa [a], Chihiro Sutoh [a], Ken Nakazawa [a], Keiko Amano [b], Junko Sajiki [a], Eiji Shimizu [a]

[a] Department of Cognitive Behavioral Physiology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chiba 260-8670, Japan
[b] Seifuso Hospital, 1-9-28 Horinouchi, Saitama 352-0023, Japan

ARTICLE INFO

Article history:
Received 5 March 2012
Received in revised form 1 June 2012
Accepted 20 June 2012
Available online 1 July 2012

Keywords:
Anxiety
BPA
Dopamine
MAO
Sex differences

ABSTRACT

Bisphenol A (BPA), an endocrine-disrupting chemical, is widely present in the environment. It has been reported that perinatal exposure to low doses of BPA that are less than the tolerable daily intake level (50 μg/kg/day) affects anxiety-like behavior and dopamine levels in the brain. Although the dopaminergic system in the brain is considered to be related to anxiety, no study has reported the effects of low-dose BPA exposure on the dopaminergic system in the brain and on anxiety-like behavior using the same methods of BPA exposure.

To investigate the relationship between alterations in anxiety-like behavior and changes in the dopaminergic system in the brain induced by BPA, we examined the effects of BPA on anxiety-like behavior using an open field test in juvenile and adult mice and measured DA and DOPAC levels and the DOPAC/DA ratio in the dorsal hippocampus (HIP), amygdala (AMY), and medulla oblongata (MED) using high-performance liquid chromatography (HPLC) in adult mice.

In males, BPA decreased the time spent in the center area of the open field in both juveniles and adults. In addition, BPA increased DA levels in the dorsal HIP and MED and decreased the DOPAC/DA ratio in the dorsal HIP, AMY, and MED in adults. The activity of monoamine oxidase (MAO)-B, the enzyme that metabolizes DA into DOPAC, was reduced in the MED. In females, those changes were not observed.

These results suggest that an increase in anxiety-like behavior induced by perinatal exposure to BPA may be related to decreases in DA metabolites in the brain, and there are sex differences in those BPA effects.

1. Introduction

Bisphenol A (BPA), an endocrine-disrupting chemical, affects estrogen, androgen, and thyroid hormone systems (Wetherill et al., 2007) BPA is used primarily in the production of polycarbonate plastics and epoxy resins that are used in most food containers and beverage cans (Kawamura et al., 2001). BPA can be widely detected in the environment, including food (Sajiki et al., 2007) and human biological samples (Sajiki et al., 1999; Vandenberg et al., 2010). Many studies have reported that BPA is transferred from the maternal body to the newborn via maternal blood and breast milk (Schonfelder et al., 2002; Sun et al., 2004; Vandenberg et al., 2007) and that perinatal exposure to BPA affects the brain development of offspring, which leads to alterations in the central nervous system that affect behaviors (Cox et al., 2010; Gioiosa et al., 2007; Patisaul and Bateman, 2008; Rubin et al., 2006; Zhou et al., 2011) and neurotransmitter levels (Honma et al., 2006; Matsuda et al., 2010b; Nakamura et al., 2010).

Cox et al. (2010) reported that perinatal exposure to BPA increased anxiety-like behaviors in both juvenile and adult mice. These authors suggest that an alteration of the dopaminergic system is one of the mechanisms of the anxiogenic effect. Dopamine (DA) is metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) in the terminal of synapses and mitochondria by monoamine oxidase-A/B (Thorpe et al., 1987). DA levels and DA metabolites, including the DOPAC/DA ratio (DA turnover) and MAO-A/B activity, are related to anxiety behavior (Chen et al., 2004; Chiavegatto et al., 2009; Pandaranandaka et al., 2006; Thiemann et al., 2009).

The medulla oblongata (MED) is an important brain region for DA metabolism (Kitahama et al., 2000; Phillips et al., 2001). DA neurons in the MED project to the limbic regions (Reyes and Van Bockstaele, 2006; Zagon et al., 1994) including the amygdala (AMY) and the hippocampus (HIP), which play important roles in emotional behavior (de la Mora et al., 2005; Matsuda et al., 2010a; Zarrindast et al., 2010). BPA has been reported to induce abnormal development of these regions. BPA

Abbreviations: AMY, Amygdala; BPA, Bisphenol A; CON, Control; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, Dopamine; EPM, Elevated plus maze; E_2, Estradiol; ER, Estrogen receptor; GD, Gestational day; HPLC, high-performance liquid chromatography; HIP, Hippocampus; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MAO, Monoamine oxidase; MED, Medulla oblongata; OFT, Open field test; PBS, Phosphate buffered saline; PND, postnatal day.

* Corresponding author. Tel.: +81 226 2027; fax: +81 43 226 2028.
E-mail address: matsutsuma@gmail.com (S. Matsuda).

0278-5846/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.pnpbp.2012.06.016

suppresses the expression of estrogen beta protein and the density of NeuN-positive neurons in the HIP (Kunz et al., 2011; Xu et al., 2010). In addition, BPA causes GABAergic disinhibition and DAergic enhancement in the AMY (Zhou et al., 2011). An alteration of the DAergic system in the limbic regions of BPA-exposed mice may be associated with a change in anxiety-like behavior. To our knowledge, there has been no study investigating the effects of BPA on DA levels and DA turnover in the AMY other than 2 studies of the HIP in which the effects of BPA on behavior were not examined (Honma et al., 2006; Matsuda et al., 2010b). Different methods of BPA exposure may cause different results (Kubo et al., 2003; Matsuda et al., 2010b; Tian et al., 2010; Xu et al., 2010).

To investigate the relationship between the alteration of anxiety-like behavior and BPA-induced changes in the dopaminergic system in the brain, we examined the effect of BPA on anxiety-like behavior and DA and DOPAC levels, the DOPAC/DA ratio, and MAO-A and MAO-B activity levels in the brain (dorsal HIP, AMY, and the whole MED) in both sexes using the same methods of BPA exposure.

2. Materials and methods

2.1. Reagents

Standard DA hydrochloride and DOPAC were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Wako Pure Chemicals (Tokyo, Japan), respectively. DL-Isoproterenol hydrochloride was purchased from Sigma Chemical Co. as an internal standard. Authentic BPA, LC-grade ethanol and acetonitrile were purchased from Wako Pure Chemicals. All other chemicals were of special grade (Wako Pure Chemicals, Osaka, Japan). DA and DOPAC standards were freshly prepared from stock solutions of 1 mg/mL of the same 0.1 M perchloric acid (PCA) solution. The BPA stock solution (10 μg/mL) contained BPA, phosphate buffered saline (PBS; pH 7.4), and 0.01% methanol. The BPA stock solution was diluted with PBS to 0.075 μg/mL for the BPA group. The vehicle contained 0.01% methanol in PBS (pH 7.4). From the preliminary studies, we had confirmed that the vehicle did not have any effect compared to non-treatment. Thereafter, all solutions were sterilized at 120 °C for 20 min. Pure MAO-A and MAO-B were purchased from Sigma-Aldrich Japan, Inc. (Tokyo, Japan).

2.2. Animals and treatments

C57BL/6J female mice at gestational day (GD) 8 were purchased from SLC (Shizuoka, Japan). After the mice were housed in the laboratory for two days, the female mice were injected with the vehicle for the control group (offspring, CON) or with BPA (250 ng/kg, calculated using 30 g of expected female mouse body weight) for the BPA group (offspring, BPA) from GD 10 to postnatal day (PND) 20. The injection volume was 100 μL. The vehicle or BPA was administered daily by subcutaneous injection between 10 a.m. and 12 noon. The dose and injection method were obtained from a previous study (Rubin et al., 2006). The female mice were weighed from GD 11 to PND 21 every three days. The litters were culled to six pups (male:female = 3:3) at PND 2. We determined gender by measuring the distance between the anus and external genitalia. The pups were weaned and housed in same-sex groups (3–5/cage) at PND 21. To eliminate litter effects, sibling pups were not used in the same experiments.

One week after the open field test described below (conducted at 9 weeks of age), the mice were anesthetized with CO_2, and brain samples were collected. The whole MED was dissected out with scissors, and the AMY and dorsal HIP were dissected out from the slices with a sharp scalpel under a stereoscopic microscope according to Matsuda et al. (2010a). The samples were immediately frozen in dry-ice/ethanol and randomly divided into two groups for the measurements of dopamine levels and MAO activity. The samples were stored at −85 °C until use.

The mice were maintained under a controlled temperature (23 ± 1 °C) with a 12/12 h light/dark photoperiod. They had free access to laboratory chow (CE-2, CLEA Japan, Inc., Tokyo) and tap water.

The animal use procedures were approved in advance by the Guide for Animal Experimentation of the Chiba University Graduate School of Medicine.

2.3. The open field test for juvenile and adult mice

The open field apparatus was a square field (50 × 50 × 30 cm) made of white acrylic material. Open field tests were performed on juvenile and adult mice. At 4 and 8 weeks of age, each mouse was placed in the corner of the apparatus at the beginning of the test and allowed to move freely for 10 min. The total distance and total center time were recorded. The center area was 16 × 16 cm. The total distance was evaluated as an index of locomotor activity, and the total center time was evaluated as an index of anxiety. The data analysis was performed using Image J OF4 (O'Hara & Co., Ltd.), a modified software based on the public domain Image J program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij).

2.4. The measurement of dopamine levels in adult mice

Dopamine and DOPAC were extracted from each part of the brain according to Matsuda et al. (2010b). An LC/ECD system (LCSS905 series; JASCO International, Tokyo, Japan) equipped with an electrochemical detector (CoulochemII 5200A; ESA) was used for monoamine determinations. The detector conditions were as follows: guard cell (5020) potential, E, 450 mV; analytical cell (5011) potentials, E_1, −50 mV and E_2, 400 mV; and sensitivity, 1 μA. Separation was performed using a 5-μm SunFire C18 (4.6 × 150 mm; Waters) column at 35 °C under isocratic conditions with a mobile phase of acetonitrile, methanol and 0.04 M phosphate buffer adjusted to pH 3.0 with phosphoric acid containing 0.04 M citrate, 7.5 mM sodium 1-heptasulfonate and 0.08 mM EDTA·2Na (2.4:5.8:91.8, v/v/v). The flow rate and injection volume were 1.0 mL/min and 10 μL, respectively.

2.5. The measurement of MAO activity in adult male mice

We measured MAO-A/B activity in males, because DA turnover was altered only in males as mentioned in the Results section. MAO-A and MAO-B were extracted from the dorsal HIP, AMY, and whole MED of the adult mice. Each brain sample was added to 0.5 mL of buffer containing 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl, and 1 mM EDTA (pH 7.4). After homogenization, the samples were centrifuged at 700 × *g* for 10 min at 0 °C. The supernatants were centrifuged at 5000 × *g* for 10 min at 0 °C. The pellets were suspended with 0.5 mL buffer and centrifuged at 24,000 × *g* for 10 min at 0 °C. The pellets were also suspended with 200 μL of either MAO reaction buffer consisting of 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7.5) and 5% glycerol (for measuring MAO-A activity) or MAO-B reaction buffer consisting of 100 mM HEPES (pH 7.5), 5% glycerol, and 10% dimethyl sulfoxide (for measuring MAO-B activity). Then, we measured MAO activity by the MAO-Glo Assay that was purchased from Promega KK (Tokyo, Japan). The luminescent signal was measured and recorded using Gene Light GL-200 (Microtec Co., Ltd., Tokyo, Japan). The signal integration time was three seconds.

2.6. Statistical analyses

For behavioral tests, DA levels, DOPAC levels, and the DOPAC/DA ratio, a two-way (sex × group) ANOVA and multiple analyses according to Bonferroni were used to test the significance of differences. For MAO activity, a one-way (group) ANOVA was used to test the significance of differences. Correlations between anxiety-like

behavior and DA levels or DA turnover were analyzed using Pearson's product-moment correlation test. All statistical analyses were performed using SPSS 12.0 J for Windows (SPSS Japan Inc., Tokyo, Japan). The data are presented as the mean ± SEM.

3. Results

3.1. Open field test of juvenile and adult mice

For total distance (at 4 and 8 weeks), a two-way ANOVA showed no significant main effect of group ($F_{(1,38)}=2.46$ and $F_{(1,37)}=0.90$, respectively), sex ($F_{(1,38)}=0.04$ and $F_{(1,37)}=3.23$, respectively) or the interaction of group × sex ($F_{(1,38)}=2.24$ and $F_{(1,37)}=0.02$, respectively; Figs. 1A and 2A).

For the time spent in the center area (at 4 weeks), a two-way ANOVA showed a significant main effect of group ($F_{(1,38)}=9.58$, $p<0.01$) but no significant main effect of sex ($F_{(1,38)}=1.13$) or the interaction of group × sex ($F_{(1,38)}=0.23$). *Post hoc* analysis showed that the male BPA-exposed mice spent less time in the center than the controls ($p<0.05$), but a parallel difference was not found in the females (Fig. 1B).

For the time spent in the center area (at 8 weeks), a two-way ANOVA showed a significant main effect of group ($F_{(1,37)}=4.44$, $p<0.05$) but no significant main effect of sex ($F_{(1,37)}=3.32$) or the interaction of group × sex ($F_{(1,37)}=2.679$). *Post hoc* analysis showed that the male BPA-exposed mice spent less time in the center than the controls ($p<0.05$), but a parallel difference was not found in the females (Fig. 2B).

3.2. DA and DOPAC levels in adult mice

In the dorsal HIP (Fig. 3A), a two-way ANOVA showed no significant main effect of group, sex or the interaction of group × sex on DOPAC levels ($F_{(1,17)}=1.50$, $F_{(1,17)}=3.70$ and $F_{(1,17)}=1.18$, respectively). A two-way ANOVA showed no significant main effect of group or sex on DA levels ($F_{(1,17)}=1.69$ and $F_{(1,17)}=1.22$, respectively), but it did show a significant interaction of group × sex on DA levels ($F_{(1,17)}=4.65$, $p<0.05$). *Post hoc* analysis showed that BPA increased the DA levels in the males ($p<0.05$) but not in the females.

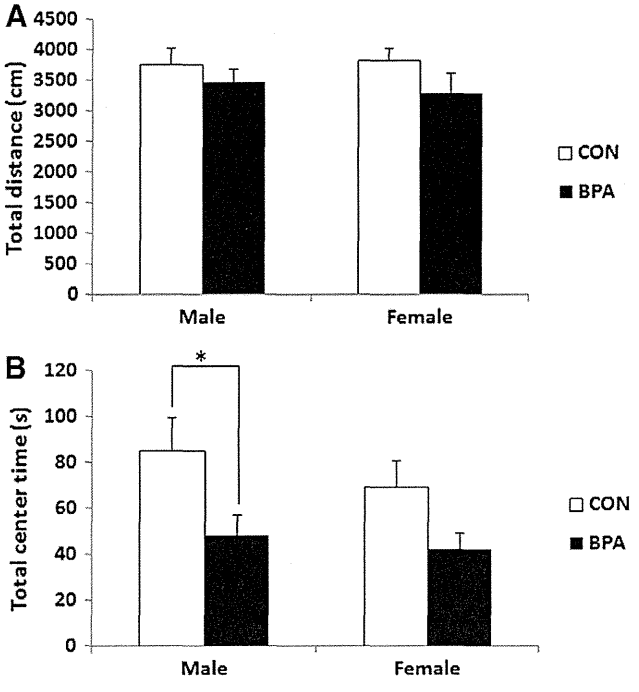


Fig. 1. The effects of BPA on locomotor activity (A) and anxiety-like behavior (B) in juvenile mice. The data represent the mean ± SEM (CON, n = 9; BPA, n = 12). * indicates $p<0.05$, CON vs. BPA.

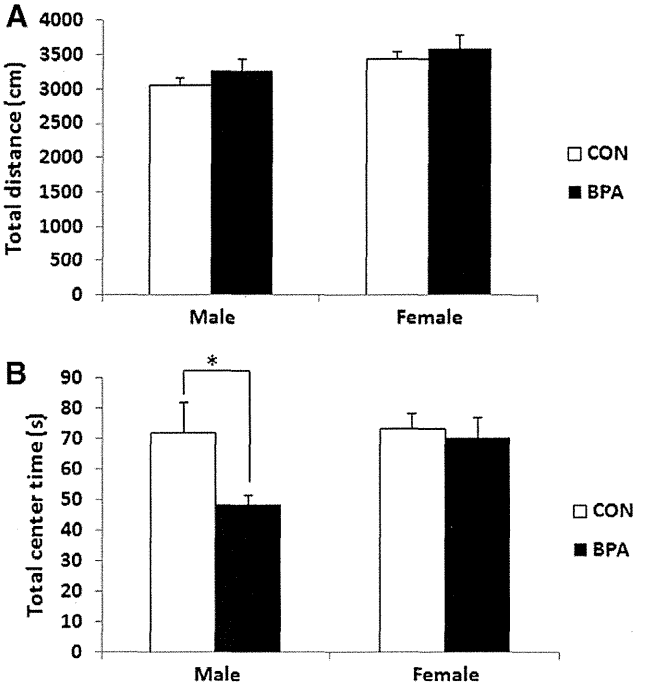


Fig. 2. The effects of BPA on locomotor activity (A) and anxiety-like behavior (B) in adult mice. The data represent the mean ± SEM (CON, n = 8; BPA, n = 12). * indicates $p<0.05$, CON vs. BPA.

In the AMY (Fig. 3B), a two-way ANOVA showed no significant main effect of group, sex or the interaction of group × sex on DOPAC levels ($F_{(1,18)}=1.58$, $F_{(1,18)}=0.07$ and $F_{(1,18)}=1.42$, respectively) or DA levels ($F_{(1,18)}=0.43$, $F_{(1,18)}=0.03$ and $F_{(1,18)}=0.00$, respectively).

In the whole MED (Fig. 3C), a two-way ANOVA showed no significant main effect of group, sex or the interaction of group × sex on DOPAC levels ($F_{(1,16)}=0.70$, $F_{(1,16)}=1.50$ and $F_{(1,16)}=2.58$, respectively). However, a two-way ANOVA showed a significant main effect of group and the interaction of group × sex ($F_{(1,16)}=12.41$, $p<0.01$, and $F_{(1,16)}=5.70$, $p<0.05$, respectively), but no main effect of sex ($F_{(1,16)}=1.57$), on DA levels. *Post hoc* analysis showed that BPA increased the DA levels in males ($p<0.01$) but not in females and that DA levels were higher in females than in males among the vehicle-exposed mice ($p<0.05$); however, there was no difference between the sexes among the BPA-exposed mice.

3.3. DA turnover in adult mice

In the dorsal HIP (Fig. 4A), a two-way ANOVA showed no significant main effect of group or sex ($F_{(1,17)}=2.26$ and $F_{(1,17)}=0.49$, respectively) but did show a significant interaction of group × sex ($F_{(1,17)}=6.84$, $p<0.05$) on the DOPAC/DA ratio. *Post hoc* analysis showed that BPA decreased the DOPAC/DA ratio in the males ($p<0.05$) but not in the females and that the DOPAC/DA ratio was higher in males than in females among the vehicle-exposed mice ($p<0.05$); however, there was no difference between the sexes among the BPA-exposed mice.

In the AMY (Fig. 4B), a two-way ANOVA showed a significant main effect of group ($F_{(1,18)}=11.81$, $p<0.01$) but did not show a main effect of sex or the interaction of group × sex ($F_{(1,18)}=0.03$ and $F_{(1,18)}=3.28$, respectively) on the DOPAC/DA ratio. *Post hoc* analysis showed that BPA decreased the DOPAC/DA ratio in the males ($p<0.01$) but not in the females.

In the whole MED (Fig. 4C), a two-way ANOVA showed a significant main effect of group and the interaction of group × sex ($F_{(1,16)}=28.34$,

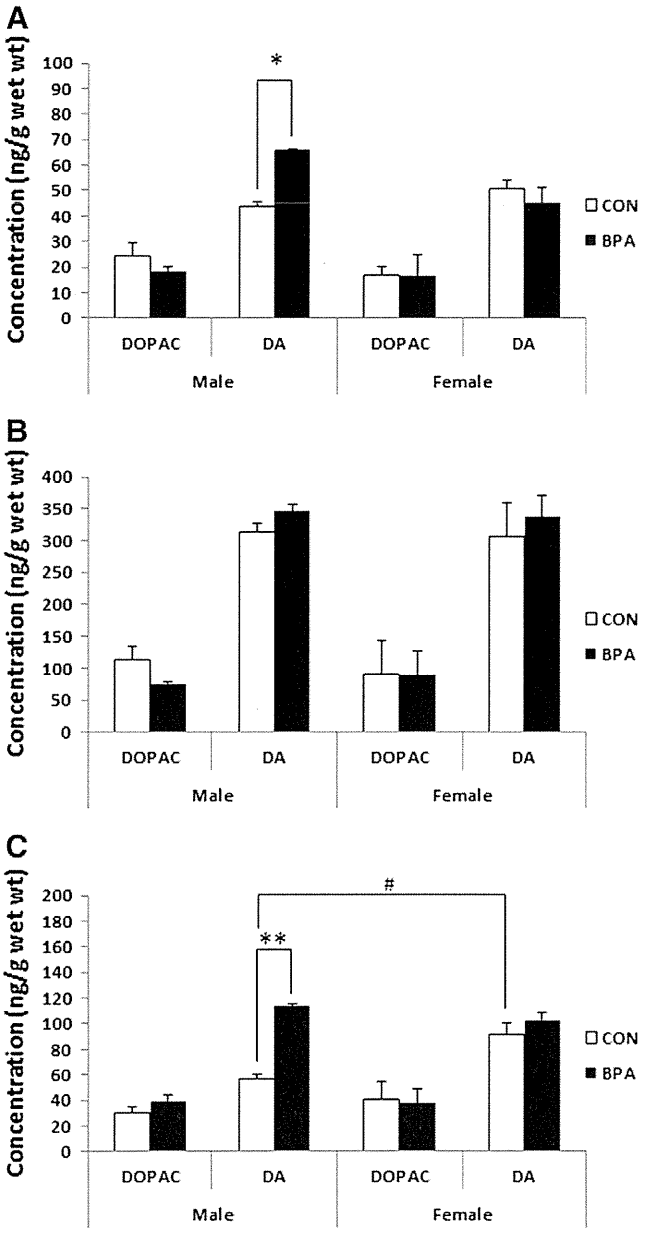


Fig. 3. The effects of BPA on DOPAC and DA levels in the dorsal HIP (A), AMY (B), and whole MED (C) in adult mice. The data represent the mean ± SEM (CON, n = 4–6; BPA, n = 5–6). * and ** indicate $p<0.05$ and $p<0.01$, respectively, CON vs. BPA. # indicates $p<0.05$, male vs. female.

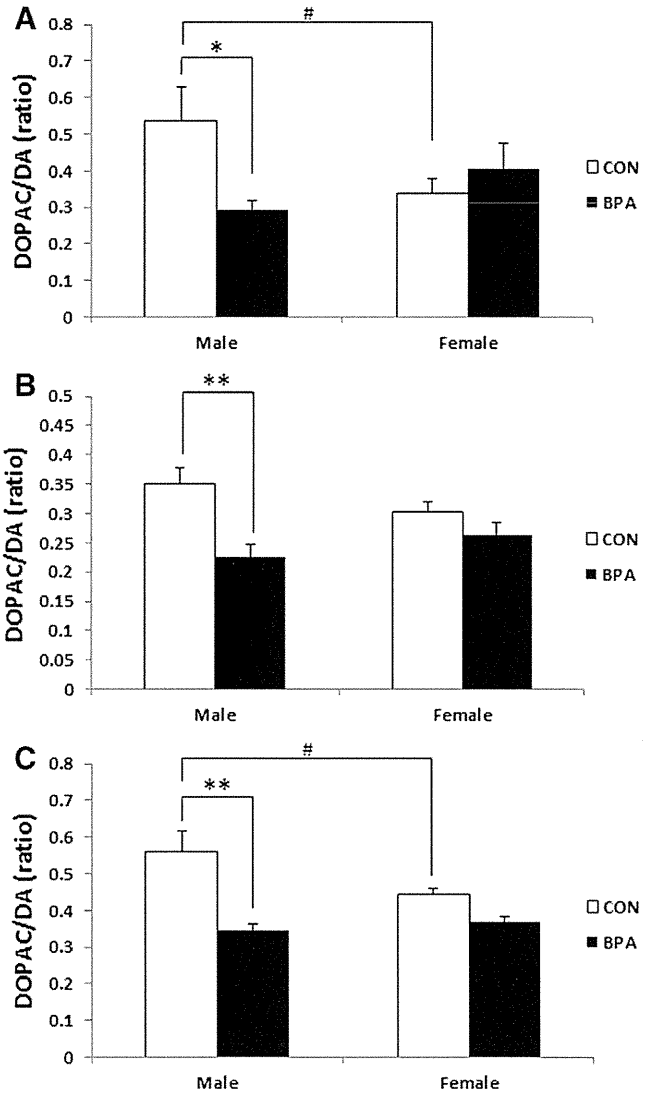


Fig. 4. The effects of BPA on the DOPAC/DA ratio in the dorsal HIP (A), AMY (B), and whole MED (C) in adult mice. The data represent the mean ± SEM (CON, n = 4–6; BPA, n = 5–6). * and ** indicate $p<0.05$ and $p<0.01$, respectively, CON vs. BPA. # indicates $p<0.05$, male vs. female.

$p<0.01$ and $F_{(1,16)}=6.73$, $p<0.05$, respectively) but did not show a main effect of sex ($F_{(1,16)}=2.65$) on the DOPAC/DA ratio. *Post hoc* analysis showed that BPA decreased the DOPAC/DA ratio in males ($p<0.01$) but not in females and that the DOPAC/DA ratio was higher in males than in females among the vehicle-exposed mice ($p<0.05$); however, there was no difference between the sexes among the BPA-exposed mice.

3.4. MAO-A and MAO-B activity levels in adult male mice

In the dorsal HIP (Fig. 5A), Student's *t*-test showed no significant differences in group on MAO-A activity ($t(7)=-1.18$) and MAO-B activity ($t(7)=-1.01$).

In the AMY (Fig. 5B), Student's *t*-test showed no significant differences in group on MAO-A activity ($t(8)=-0.24$) and MAO-B activity ($t(8)=-0.38$).

In the whole MED (Fig. 5C), Student's *t*-test showed significant differences in group on MAO-B activity ($t(7)=3.38$, $p<0.05$) but not on MAO-A activity ($t(7)=-0.24$).

4. Discussion

The major finding of the present study was that perinatal exposure to a low dose of BPA increased anxiety-like behavior and DA levels and decreased the DOPAC/DA ratio and MAO-B activity levels in the limbic and MED of male mice, but those changes were not observed in female mice. Table 1 summarizes the data from the open field test and indicates the DA and DOPAC levels and the DOPAC/DA ratio in the brain of adult mice.

BPA did not affect the locomotor activity of either sex or age group (juvenile or adult mice) (Figs. 1A and 2A). BPA decreased the time spent in the center area of the open field for both juvenile and adult males, while BPA did not affect the females in either age group (Figs. 1B and 2B). These results suggest that perinatal exposure to

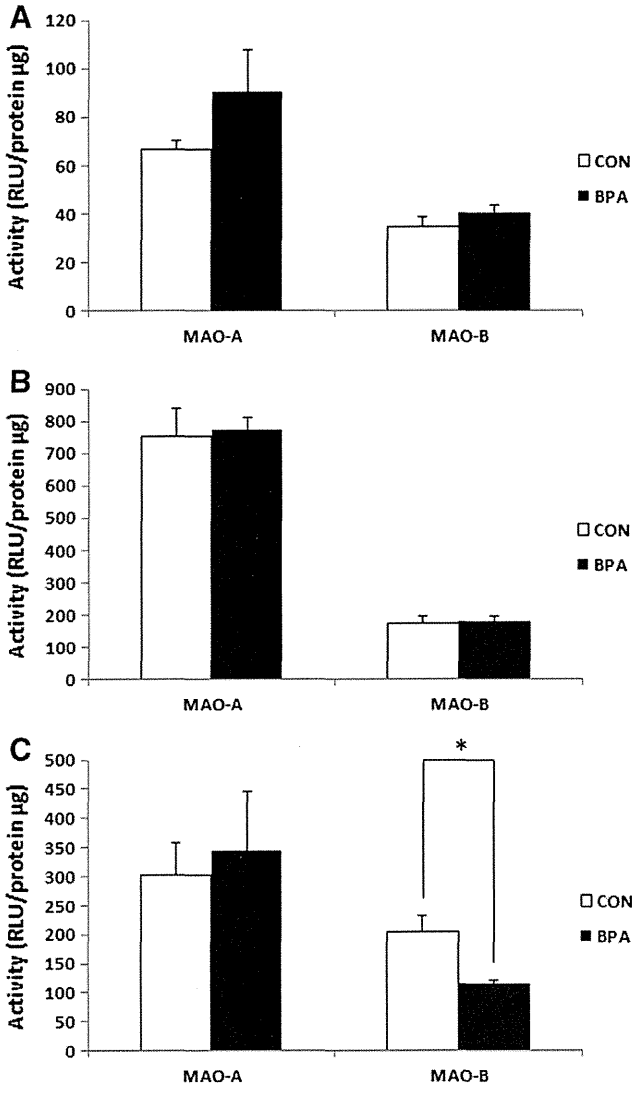


Fig. 5. The effects of BPA on MAO-A and MAO-B activity levels in the whole HIP (A), AMY (B), and whole MED (C) in adult male mice. The data represent the mean ± SEM (CON, n = 3–6; BPA, n = 4–6). * indicates $p<0.05$, CON vs. BPA.

Table 1
The effects of BPA on behavior and dopamine metabolites in adult mice.

Sex	Anxiety-like behavior	Locomotor activity	Brain regions	DOPAC	DA	DOPAC/DA
Male			Dorsal HIP	→	⇧	⇩
	⇧	→	AMY	→	→	⇩⇩
			Whole MED	→	⇧⇧	⇩⇩
Female			Dorsal HIP	→	→	→
	→	→	AMY	→	→	→
			Whole MED	→	→	→

⇧ and ⇩ indicate significant increase and decrease by BPA, respectively ($p<0.05$, compared to CON).

⇧⇧ and ⇩⇩ indicate significant increase and decrease by BPA, respectively ($p<0.01$, compared to CON).

→ indicates no significant change by BPA.

BPA increases anxiety-like behavior and that the change of behavior persists from the juvenile stage to adulthood in males. This result is consistent with previous studies using an elevated plus maze, another anxiety-like behavioral test (Cox et al., 2010; Xu et al., 2011). Although it should be noted that some studies have shown anxiolytic effects (Farabollini et al., 1999; Tian et al., 2010; Yu et al., 2011). The inconsistency in results may be due to differences in BPA exposure methods and the species studied; for example, mice were exposed to BPA during perinatal periods in our study and the study by Cox et al. (2010), while BPA was administered after weaning in the studies by Tian et al. (2010) and Yu et al. (2011).

Similar to the effect of BPA on anxiety-like behavior, BPA increased DA levels in the dorsal HIP and MED and decreased DA turnover in the dorsal HIP, AMY, and MED in males but not in females (Figs. 3 and 4). As to the correlation between the data of DA metabolism and the behavior in male, no statistically significant correlation was observed perhaps due to the small sample size, but the DA levels in MED showed a strong negative trend with the time spent in the center area ($r=-0.59$, $p=0.094$ by Pearson's correlation coefficient). These results suggest that the alteration of DA levels and DA turnover in the brain may be related to a change in anxiety-like behavior. A strain of rats showing higher anxiety-like behavior had a lower DA turnover in the HIP compared to other rat strains (Chiavegatto et al., 2009). Cannabinoid receptor antagonist-administered mice showed increases in anxiety-like behavior and DA levels in the HIP and a decrease in DA turnover in the septum (Thiemann et al., 2009). The AMY is mostly involved in anxiety-like behavior. Although it is not clear whether DA turnover in the AMY is related to anxiety-like behavior, the administration of a dopamine D1 receptor agonist and antagonist in the basolateral amygdala showed anxiogenic and anxiolytic effects, respectively (Bananej et al., 2012). BPA enhanced dopamine D1 receptor function in the AMY (Zhou et al., 2011). To our knowledge, there has been no study reporting that the DAergic system in the MED is related to anxiety-like behavior. However, the changes in DA levels and DA turnover cannot be ignored. The DAergic system in the MED regulates cardiovascular responses such as blood pressure and heart rate. An increase in DA levels causes hypertension and an increase in heart rate (Granata and Woodruff, 1982). An alteration in cardiovascular responses may be associated with anxiety (Fiedorowicz et al., 2011; Williams et al., 2009). Thus, the alteration of DA levels and DA turnover in the MED might be indirectly related to anxiety.

We could not measure DA levels in the juvenile mice because the same mice were used for behavioral tests throughout the experimental period. However, DA levels may be expected to increase in juveniles. In a previous study, intracranial injections of BPA into neonatal male rats showed a significant increase in DA levels in the brainstem one week after the injection (Matsuda et al., 2010b). Moreover, DA levels tended to increase and DA turnover tended to decrease in the HIP, although the changes were not significant (Matsuda et al., 2010b). The timing of DA level measurements in our previous study and the behavioral test of juveniles in the present study were similar: seven days and eight days from the last day of BPA exposure, respectively.

BPA-exposed males showed decreases in DA turnover in the limbic region and the whole MED as adults, as mentioned above (Fig. 4A–C). We hypothesized that the decrease in DA turnover resulted from a reduction in MAO-A and MAO-B activity levels, the enzymes that metabolize DA into DOPAC. To test this hypothesis, we measured MAO-A and MAO-B activity levels in three brain regions in males. BPA-exposed males showed lower MAO-B activity in the whole MED compared with vehicle-exposed males; however, there was no change in MAO-A activity (Fig. 5A and B). It has been shown that MAO-B contributes to DA metabolism at higher concentrations of DA in mice, although DA is largely metabolized by MAO-A under normal physiological conditions (Fornai et al., 1999). These results suggest that the decline in DA turnover may be attributed to an increase in DA levels through the reduction of MAO-B activity in the whole MED.

In contrast to the whole MED, BPA did not affect either MAO-A or MAO-B activity level in the dorsal HIP and AMY. This inconsistency might result from the different impacts of BPA among the brain regions. BPA strongly increased DA levels ($p<0.01$) and decreased the DOPAC/DA ratio ($p<0.01$) in the whole MED, while significant ($p<0.05$) increases in DA and decreases in the DOPAC/DA ratio were found in the dorsal HIP. Moreover, the observed change was only DOPAC/DA ratio ($p<0.01$) in the AMY. These results suggest that there may be different BPA-related mechanisms causing the decreases in DA turnover among the brain regions. Tyrosine hydroxylase (TH), a time-limiting enzyme of DA synthesis, is also influenced by BPA (Ishido et al., 2007; Tando et al., 2007; Tanida et al., 2009). TH and MAO regulate DA levels and are considered to be related to anxiety-like behavior (Chen et al., 2004; Picazo et al., 2009). The effects of BPA on the expression or activity of TH, especially in the limbic regions, should be further investigated.

One possible explanation for the decrease in MAO-B activity is that BPA inhibited the expression of MAO-B. The promoter of MAO-B (but not MAO-A) contains 22 CpG sites (Shih and Chen, 2004). In general, the methylation status at CpG sites can regulate gene transcription (Hsieh, 2000). Methylation at the 22 CpG sites is inversely correlated with MAO-B gene expression in vitro (Shih and Chen, 2004). Low doses of BPA change the methylation pattern in the developing mouse brain (Yaoi et al., 2008). In addition, estrogen receptors and estrogen-related receptors, which are putative main target receptors of BPA (Takayanagi et al., 2006; Wetherill et al., 2007), regulate the expression of the MAO-B gene (Zhang et al., 2006).

BPA abolished the sex differences observed in the vehicle-exposed mice in DA levels in the whole MED and the DOPAC/DA ratio in the dorsal HIP and whole MED (Figs. 3C, 4A, and C). The DA levels and DA turnover of the BPA-exposed males appeared to be near those of the vehicle-exposed females. Nakamura et al. (2010) also reported that the DOPAC/DA ratio of BPA-exposed males is near that of vehicle-exposed females in the lateral hypothalamus/preoptic area. These results suggest that BPA-exposed males might be feminized. Estrogen masculinizes the brain during early periods of brain development in male rodents, although the default brain is the female type. Estrogen is converted from testosterone by aromatase in male offspring and binds to estrogen receptors (ERs), resulting in the masculinization of the brain (MacLusky and Naftolin, 1981; Wu et al., 2009). Akingbemi et al. (2004) reported that BPA decreased the expression of aromatase mRNA and E_2 levels during BPA exposure periods in males. In addition, BPA inhibited the activity of aromatase in vitro (Benachour et al., 2007).

Based on embryology, GD 10 is approximately the time when the amygdala begins to develop in rodents (McConnell and Angevine, 1983), while the amygdala begins to develop between the 2nd and 3rd fetal months in humans (Tuchmann-Duplessis et al., 1974). On the other hand, PND 21 was the weaning time in the present study. The timeframe of BPA exposure in the present study most likely aligns with the time frame from 2nd–3rd fetal month to weaning in humans. As prenatal and early-life exposure to BPA concentrations may be associated with poorer emotional control in human children (Braun et al., 2011; Perera et al., 2012), our results may contribute to understanding the effects of BPA exposure on the brain of children from the viewpoint of environmental hygiene.

5. Conclusions

BPA-exposed males showed an increase in anxiety-like behavior and DA levels and a decrease in DA turnover in the brain. Furthermore, BPA inhibited MAO-B activity in the MED. In contrast, females did not exhibit those changes. These results indicate that an alteration in anxiety-like behavior and the DAergic system occur in parallel and support the possibility that alteration of the DAergic system is a mechanism of the anxiogenic effects of BPA.

Acknowledgments

This research was supported by a Grant-in-Aid for Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology in Japan and by the Chiba Serum Institute Memorial Fund for Health Medical Welfare.

References

Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 2004;145:592–603.
Bananej M, Karimi-Sori A, Zarrindast MR, Ahmadi S. D1 and D2 dopaminergic systems in the rat basolateral amygdala are involved in anxiogenic-like effects induced by histamine. J Psychopharmacol 2012;26:564–74.
Benachour N, Moslemi S, Sipahutar H, Seralini GE. Cytotoxic effects and aromatase inhibition by xenobiotic endocrine disrupters alone and in combination. Toxicol Appl Pharmacol 2007;222:129–40.
Braun JM, Kalkbrenner AE, Calafat AM, Yolton K, Ye X, Dietrich KN, et al. Impact of early-life bisphenol a exposure on behavior and executive function in children. Pediatrics 2011;128:873–82.
Chen K, Holschneider DP, Wu W, Rebrin I, Shih JC. A spontaneous point mutation produces monoamine oxidase A/B knock-out mice with greatly elevated monoamines and anxiety-like behavior. J Biol Chem 2004;279:39645–52.
Chiavegatto S, Izidio GS, Mendes-Lana A, Aneas I, Freitas TA, Torrao AS, et al. Expression of alpha-synuclein is increased in the hippocampus of rats with high levels of innate anxiety. Mol Psychiatry 2009;14:894–905.
Cox KH, Gatewood JD, Howeth C, Rissman EF. Gestational exposure to bisphenol A and cross-fostering affect behaviors in juvenile mice. Horm Behav 2010;58:754–61.
de la Mora MP, Cardenas-Cachon L, Vazquez-Garcia M, Crespo-Ramirez M, Jacobsen K, Hoistad M, et al. Anxiolytic effects of intra-amygdaloid injection of the D1 antagonist SCH23390 in the rat. Neurosci Lett 2005;377:101–5.
Farabollini F, Porrini S, Dessi-Fulgherit F. Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol Biochem Behav 1999;64:687–94.
Fiedorowicz JG, He J, Merikangas KR. The association between mood and anxiety disorders with vascular diseases and risk factors in a nationally representative sample. J Psychosom Res 2011;70:145–54.
Fornai F, Chen K, Giorgi FS, Gesi M, Alessandri MG, Shih JC. Striatal dopamine metabolism in monoamine oxidase B-deficient mice: a brain dialysis study. J Neurochem 1999;73:2434–40.
Gioiosa L, Fissore E, Ghirardelli G, Parmigiani S, Palanza P. Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Horm Behav 2007;52:307–16.
Granata AR, Woodruff GN. Dopaminergic mechanisms in the nucleus tractus solitarius and effects on blood pressure. Brain Res Bull 1982;8:483–8.
Honma T, Miyagawa M, Suda M, Wang RS, Kobayashi K, Sekiguchi S. Effects of perinatal exposure to bisphenol A on brain neurotransmitters in female rat offspring. Ind Health 2006;44:510–24.
Hsieh CL. Dynamics of DNA methylation pattern. Curr Opin Genet Dev 2000;10:224–8.
Ishido M, Yonemoto J, Morita M. Mesencephalic neurodegeneration in the orally administered bisphenol A-caused hyperactive rats. Toxicol Lett 2007;173:66–72.
Kawamura Y, Inoue K, Nakazawa H, Yamada T, Maitani T. (Cause of bisphenol A migration from cans for drinks and assessment of improved cans)Shokuhin Eiseigaku Zasshi 2001;42:13–7.
Kitahama K, Nagatsu I, Geffard M, Maeda T. Distribution of dopamine-immunoreactive fibers in the rat brainstem. J Chem Neuroanat 2000;18:1–9.
Kubo K, Arai O, Omura M, Watanabe R, Ogata R, Aou S. Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci Res 2003;45:345–56.
Kunz N, Camm EJ, Somm E, Lodygensky G, Darbre S, Aubert ML, et al. Developmental and metabolic brain alterations in rats exposed to bisphenol A during gestation and lactation. Int J Dev Neurosci 2011;29:37–43.
MacLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981;211:1294–302.
Matsuda S, Matsuzawa D, Nakazawa K, Sutoh C, Ohtsuka H, Ishii D, et al. d-Serine enhances extinction of auditory cued fear conditioning via ERK1/2 phosphorylation in mice. Prog Neuropsychopharmacol Biol Psychiatry 2010a;34:895–902.
Matsuda S, Saika S, Amano K, Shimizu E, Sajiki J. Changes in brain monoamine levels in neonatal rats exposed to bisphenol A at low doses. Chemosphere 2010b;78:894–906.
McConnell J, Angevine JBJ. Time of neuron origin in the amygdaloid complex of the mouse. Brain Res 1983;272:150–6.
Nakamura K, Itoh K, Yoshimoto K, Sugimoto T, Fushiki S. Prenatal and lactational exposure to low-doses of bisphenol A alters brain monoamine concentration in adult mice. Neurosci Lett 2010;484:66–70.
Pandaranandaka J, Poonyachoti S, Kalandakanond-Thongsong S. Anxiolytic property of estrogen related to the changes of the monoamine levels in various brain regions of ovariectomized rats. Physiol Behav 2006;87:828–35.
Patisaul HB, Bateman HL. Neonatal exposure to endocrine active compounds or an ERbeta agonist increases adult anxiety and aggression in gonadally intact male rats. Horm Behav 2008;53:580–8.
Perera F, Vishnevetsky J, Herbstman JB, Calafat AM, Xiong W, Rauh V, et al. Prenatal bisphenol A exposure and child behavior in an inner city cohort. Environ Health Perspect 2012. [Epub ahead of print].