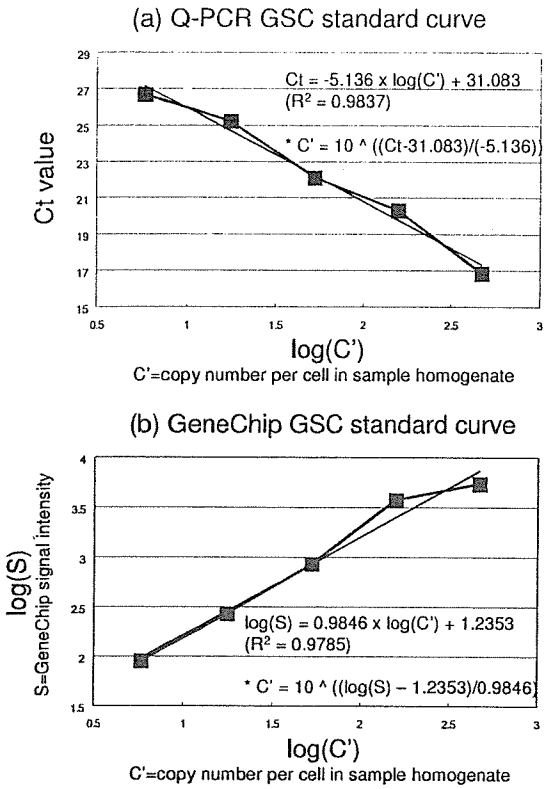

Figure 4
The dose-response linearity of the GSC spikes in Q-PCR and the Affymetrix GeneChip array system. Linear relationships are shown between (a) the Q-PCR Ct values and log of copy number (log (C')), and (b) the GeneChip log signal intensity (log(S)) and log of copy number (log (C')) of the GSC mRNAs. The regression functions were obtained by the least squares method. The inverse functions (*) were further used to generate the copy numbers of all other genes/probe sets for Percellome normalization.

were divided into 20 groups of 3 mice each. TCDD was administered once orally at doses of 0, 1, 3, 10 and 30 μg/kg, and the livers were sampled 2, 4, 8 and 24 h after administration. Nineteen primer pairs were prepared for Q-PCR and the Ct values of the liver transcriptome were measured. The same 60 liver samples were measured using the Affymetrix Mouse430-2 GeneChip [see Additional files 5 through 8 and 9 through 12]. Q-PCR and GeneChip data were normalized against cell number by functions {3} and {4}, respectively. The averages and standard deviations (sd) of each group (n = 3) were calculated and plotted as three layers of isoborograms on to 5 × 4 matrix three-dimensional graphs (Figure 5). Together with another sample set (data not shown), a total of thirty-six primer pairs were compared, and there was a correlation of up to 90% between the Q-PCR and GeneChip surfaces. It is notable that not only the average surfaces but also the +1sd and -1sd surfaces corresponded closely in shape and size. We infer that the differences resulted mainly from biological variations among the three animals in each experimental group rather than from measurement error (cf. Figure 7).

An important feature of Percellome normalization is its independence from the overall expression profile of the sample. When gene expression profiles differ among samples, Percellome normalization produces a robust transcriptome that is different from total-RNA dependent global normalization. As an example, Figure 6 shows the results of an experiment on the uterotrophic response of ovariectomized mice to estrogen treatment [12] [see Additional files 13 and 14]. The uteri of the vehicle control are atrophic because the ovaries, the source of intrinsic estrogens, are absent. The uteri of the treated groups are hypertrophic owing to estrogenic stimulus from the test compound administered. Global normalization (90 percentile) between the vehicle control group and the high-dose (1,000 mg/kg) group indicated that 4,600 of 12,000 probe sets showed 2-fold or greater increase, 470 were reduced by 0.5 or less, and 7,400 remained between these extremes. In contrast, analysis of Percellome-normalized data revealed that almost all the 12,000 probe sets showed a 2-fold or greater increase, including actin, GAPDH and other housekeeping genes. The hypertrophic tissues, consisting of cells with abundant cytoplasm, provide convincing evidence for the increases in various cellular components including housekeeping gene products.

Another important feature of Percellome normalization is the commonality of the expression scale across platforms. Batch conversion can be performed between results obtained from different platforms when the data are generated by the Percellome method. A practical strategy for such normalization is to prepare a set of samples from a target organ of interest with differences in gene expression, and measure them once by each platform. Data conversion functions with good linear dose-response relationships can be obtained individually for those genes/probe sets that are measured by both platforms (Figure 7).

Discussion

We have developed a novel method for normalizing mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration. For non-diploid or aneuploid samples, an average DNA content per cell should be determined beforehand for accurate adjustment. When there is significant DNA synthesis, a similar adjustment should be considered.

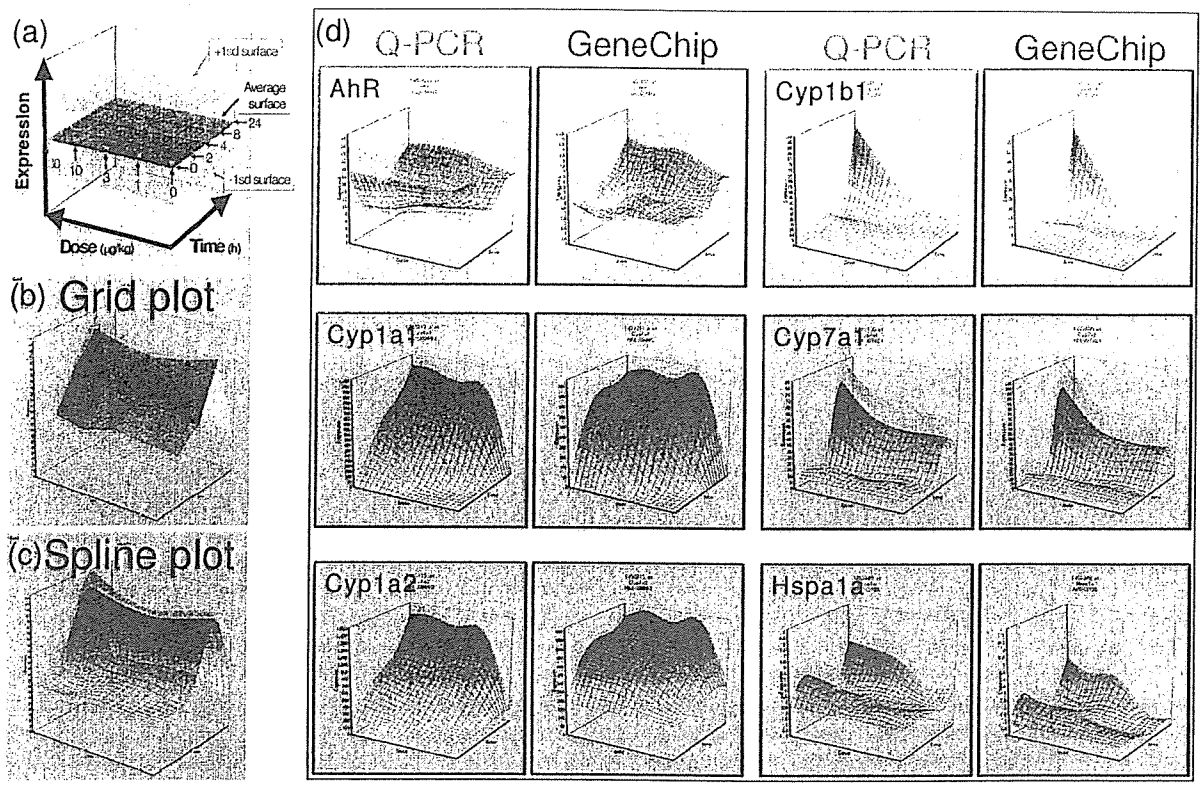

Figure 5

Correspondence between Q-PCR and GeneChip data. Sixty male C57BL/6 mice were divided into 20 groups of 3 mice each. 2,3,7,8-tetrachlorodibenzodioxin (TCDD) was administered once orally at doses of 0, 1, 3, 10 and 30μg/kg, and the liver was sampled 2, 4, 8 and 24 h after administration. The liver transcriptome was measured by the Affymetrix Mouse430-2 GeneChip. For Q-PCR, nineteen primary pairs were prepared and the Ct values of the same 60 liver samples were measured (19 genes and 5 spikes in duplicate, using a 96-well plate for 2 samples, total 30 plates). The Percellome data were plotted on to 3-dimensional graphs for average, +1sd, and – 1sd surfaces as shown in (a). The scale of expression (vertical axis) is the copy number per cell. The 0 h data (*) are copied from the 2 h/dose 0 point for better visualization of the changes after 2 h. The surfaces are demonstrated as a grid plot (b) where the grid points indicate one treatment group (n = 3), and a smoothened spline surface plot (c) for easier 3D recognition ((b), (c): Gys2 (glycogen synthase 2, 1424815_at) showing a typical circadian pattern. (d) the smoothened plots of 6 representative genes/ probe sets generated by Q-PCR (red) and GeneChip (blue). AhR (arylhydrocarbon receptor, 1450695_at) showed imperfect correspondence. Cyp1a1 (cytochrome P450, family 1, subfamily a, polypeptide 1, 1422217_a_at) and Cyp1a2 (1450715_at) showed good correlations between Q-PCR and GeneChip except for the saturation in GeneChips above c. 400 copies per cell. Cyp1b1 (1416612_at) and Cyp7a1 (1422100_at) showed good correspondence. Hspa1a (heat shock protein 1A, 1452888_at) showed fair correspondence despite low copy numbers, near the nominal detection limit of the Affymetrix GeneChip system.

The smallest sample to which we have successfully applied the direct DNA quantification method with sufficient reproducibility is the 6.75 dpc (days post coitus) mouse embryo which consists of approximately 5,000 cells. This sample size is also approximately the lower limit for double amplification protocol to obtain sufficient amount of RNA for Affymetrix GeneChip measurement (cf. http://www.affymetrix.com/Auth/support/downloads/manuals/expression_print_manual.zip.)

High-resolution technology such as laser-capture microdissection (LCM) has become popular and the average sample size analyzed is getting smaller. An alternative method for LCM samples is to count the cell number in the course of microdissection. Although we have not yet applied Percellome method to LCM samples, we have applied the alternative method to cell culture samples to gain Percellome data. Stereological and statistical calculations should become available to correct the number of partially sectioned cells in the LCM samples. Another issue for small samples is the yield of RNA. Approximately

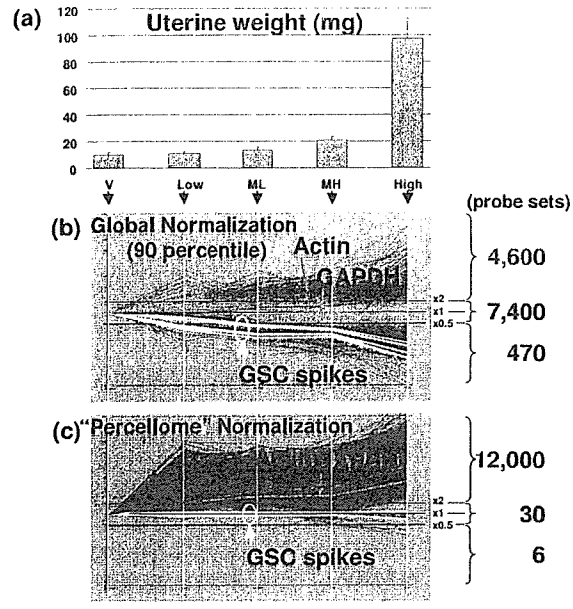

Figure 6
Uterotrophic response of ovariectomized female mice by an estrogenic test compound. (a) Shows the uterine weight, which increases in a dose-dependent manner; V, vehicle control; Low, low dose; ML, medium-low dose; MH, medium-high dose; High, high dose group. (b) Shows the line display of uterine gene expression (Affymetrix MG-U74v2 A GeneChips) normalized by global normalization (90 percentile), and (c) by the Percellome normalization. Averages of three samples per group were visualized (by K. A.). The five white lines are the GSC mRNAs. The green and blue lines are actin (AFFX-b-ActinMur/M12481_3_at) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, AFFX-GapdhMur/M32599_3_at), respectively. By global normalization, 7,400 probe sets remained unchanged and 4,600 probe sets increased more than two-fold in the H group compared to the V group, whereas almost all probe sets measured had increased. It is noted that housekeeping genes such as actin and GAPDH are significantly induced on a per cell basis.

30 ng of total RNA is retrieved from a single 6.75 dpc mouse embryo. This amount is sufficient for a double amplification protocol (DA) to prepare enough RNA for an Affymetrix GeneChip measurement. An inherent problem with the DA data is that the gene expression profile differs from that of the default single amplification protocol (SA). Consequently the DA percellome data differ from that of SA as if they were produced by a different platform. To bridge the difference, we applied the procedure that was used for data conversion between Q-PCR and GeneChip (cf. Figure 7). A set of spiked-in standard samples including the LBM sample set (of sufficient concentration) were measured by the SA protocol and diluted versions to the limit measured by the DA protocol. These data provided us with information about whether DA was successful as a whole (by comparing 5' signal to 3' signals of selected probe sets) and which probe sets were properly amplified by DA (by checking the linearity of the diluted LBM data). For those probe sets that proved to be linearly amplified, conversion functions between DA and SA were generated. These details, along with embryo expression data will be published elsewhere.

Figures 5 and 7 indicate a close correspondence between the data generated by Q-PCR and GeneChip analyses. Since each of the 60 samples was normalized individually against each GSC signal, the high similarity between the two platforms indicates the robustness and stability of this spike system (cf. Figure 7, Cyp7a1 data). Although more spikes could potentially increase the accuracy of normalization, our experience is that five spikes are practically sufficient for covering the detection range of GeneChip microarrays and Q-PCR, as long as they are used in combination with the "spike factor". The overall benefits of using a minimum number of external spikes include lower probability of cross-hybridization, a reduced number of wells and spots occupied by the spikes in the Q-PCR plates and small scale microarrays, and less effort in preparation, QC and supply.

The Percellome data can be truly absolute when all mRNA measurements including GSC spikes are strictly proportional to the original copy numbers in the sample homogenate. As noted earlier, this condition is not guaranteed by any platform despite linearity of response. Therefore, the Percellome-normalized values have some biases for each primer pair/probe set, depending on the steepness of the dose-response curves. An advantage of Percellome normalization is that, as long as such biases are consistently reproduced within a platform, the data can be compared directly among samples/studies on a common scale. Consequently, when a true value is obtained by any other measure, all the data obtained in the past can be simultaneously batch-converted to the true values.

This batch-conversion strategy can be extended to data conversion between different versions and different platforms, as long as the data are generated in copy numbers "per cell". We have shown an example between Affymetrix GeneChip and Q-PCR for limited numbers of probe sets (cf. Figure 7). Custom microarrays that accept our GSC for Percellome normalization are in preparation by Agilent Technologies (single color) and GE Healthcare (CodeLink Bioarray).

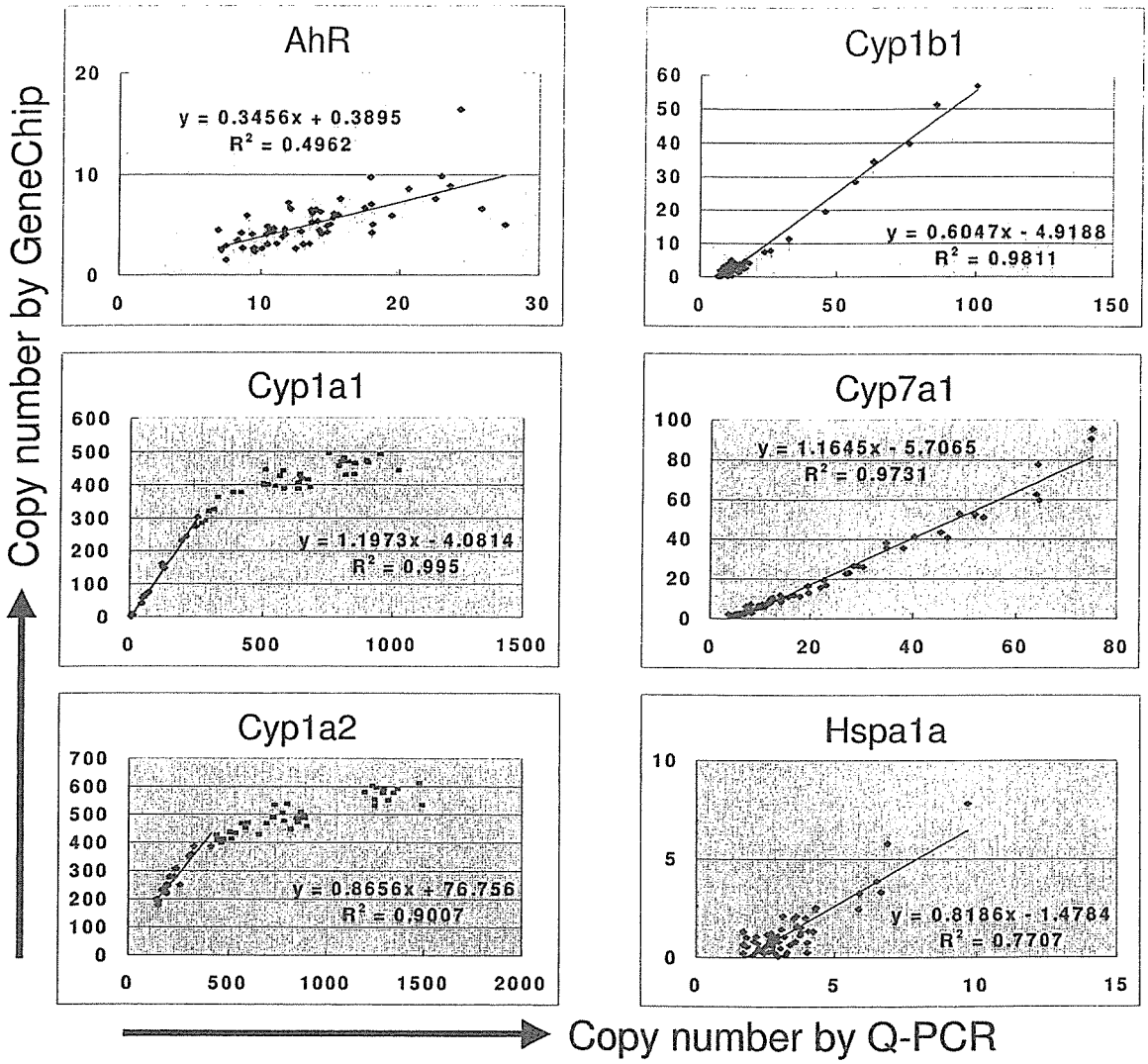

Figure 7
Conversion functions between Q-PCR and GeneChip. The data shown in Figure 5 as 3D surfaces are shown as a scatter plot (60 plots). The regression function can be used to convert Q-PCR to GeneChip and vice versa, with a level of certainty indicated by coefficient of correlation. It is noted that Cyp1a1 and Cyp1a2 became saturated above about 400 copies per cell in GeneChip system (indicated in pink plots). Cyp7a1 showed high linearity, indicating that the variation shown by the split +1sd and -1sd surfaces in Figure 5 reflected biological (animal) variation, not measurement errors.

Another important contribution of Percellome analysis is in the area of archived data in private and public domains. Firstly, Percellome data are the result of a simple linear transformation of the raw microarray data; preserving the distribution and order of the probe set data. Therefore, parametric or non-parametric methods should be able to align the data distribution and generate estimates of mRNA copy number of the non-spiked archival samples. Any archival samples that are re-measurable by Percellome method will greatly increase the accuracy of estimation. Secondly, percellome can provide appropriate bridging information between old and new versions of Affymetrix GeneChips, such as human HU-95 and HU-133, murine MU-74v2 and MOE430 series. This should also facilitate comparisons between newly generated and archived data.

The Percellome method was developed for a large-scale toxicogenomics project [13] using the Affymetrix GeneChip system. It was intended to compile a very large-scale database of comprehensive gene expression profiles in response to various chemicals from a series of experiments conducted over an extended time period. However, the method also proved to be useful for small-scale platforms such as 96 well plate-based Q-PCRs as shown above, and probably for small-scale targeted microarrays. In both cases, highly inducible or highly transcribed genes are likely to be selected. Therefore, the expression profiles may differ significantly among samples such that profile-dependent normalization (e.g. global normalization) may not be applicable. In such cases, the profile-independent nature of the Percellome method provides a robust normalization.

To demonstrate the profile-independence of the Percellome method, we chose an extreme case – the uterotrophic response assay (cf. Figure 6). The treated uteri were composed of hypertrophic cells with abundant cytoplasm whereas the untreated uteri were composed of hypoplastic cells with scant cytoplasm. This indicates that the uteri of untreated ovariectomized mice were quiescent, and that a majority of the inducible genes were probably transcriptionally inactive. Therefore, the identification of most genes as being induced by 2-fold or greater is reasonable and expected. In most *in vivo* experiments, the gene profiles of the samples are much more similar. However, there is always a set of genes that is found to be "increased" when analyzed on a "per one cell" basis that are declared to be "decreased" by global type normalization, or vice versa. Such increase/decrease calls made by the global type normalization can differ according to the normalization parameters. In both cases, the Percellome method can inform the researcher how much the expression profiles are distorted by the treatment, such as in the case of the uterotrophic assay. We also note that *in vitro* experiments such as cell-based studies tend to generate data similar to that of uterotrophic experiment.

Conclusion

Percellome data can be compared directly among samples and among different studies, and between different platforms, without further normalization. Therefore, "percellome" normalization can serve as a standard method for exchanging and comparing data across different platforms and among different laboratories. We hope that the Percellome method will contribute to transcriptome-based studies by facilitating data exchanges among laboratories.

Methods

Animal experiments

C57BL/6 Cr Slc (SLC, Hamamatsu, Japan) mice maintained in a barrier system with a 12 h photoperiod were used in this study. For the liver transcriptome experiments, twelve week-old male mice were given a single dose of the test compound by oral gavage, and the liver was sampled at 2, 4, 8 and 24 h post-gavage. For the uterotrophic experiment, 6 week old female mice were ovariectomized 14 days prior to the 7 day repeated subcutaneous injection of a test compound [12]. Animals were euthanized by exsanguination under ether anesthesia and the target organs were excised into ice-cooled plastic dishes. Tissue blocks weighing 30 to 60 mg were placed in an RNase-free 2 ml plastic tube (Eppendorf GmbH., Germany) and soaked in RNAlater (Ambion Inc., TX) within 3 min of the beginning of anesthesia. Three animals per treatment group were used and individually subjected to transcriptome measurement.

Sample homogenate preparation

The tissue blocks soaked in RNAlater were kept overnight at 4°C or until use. RNAlater was replaced in the 2 ml plastic tube with 1.0 ml of RLT buffer (Qiagen GmbH., Germany), and the tissue was homogenized by adding a 5 mm diameter Zirconium bead (Funakoshi, Japan) and shaking with a MixerMill 300 (Qiagen GmbH., Germany) at a speed of 20 Hz for 5 min (only the outermost row of the shaker box was used).

Direct DNA quantitation

Three separate 10 μl aliquots were taken from each sample homogenate to another tube and mixed thoroughly. A final 10 μl aliquot therefrom was treated with DNAse-free RNase A (Nippon Gene Inc., Japan) for 30 min at 37°C, followed by Proteinase K (Roche Diagnostics GmbH., Germany) for 3 h at 55°C in 1.5 ml capped tubes. The aliquot was transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes Inc., USA) was added to each well, shaken for 10 seconds four times and then incubated for 2 min at 30°C. The DNA concentration was measured using a 96 well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. λ phage DNA (PicoGreen Kit, Molecular Probes Inc., USA) was used as standard. Measurement by this PicoGreen method and the standard phenol extraction method correlated well (coefficient of correlation = 0.97, data not shown). The smallest sample size for reproducible and reliable DNA quantitation is about 5,000 cells that corresponds to a 6.75 dpc mouse embryo.

The grade-dosed spike cocktail (GSC)

The following five *Bacillus subtilis* RNA sequences were selected from the gene list of Affymetrix GeneChip arrays (AFFX-ThrX-3_at, AFFX-LysX-3_at, AFFX-PheX-3_at, AFFX-DapX-3_at, and AFFX-TrpnX-3_at) present in the MG-U74v2, RG-U34, HG-U95, HG-U133, RAE230 and MOE430 arrays: thrC, thrB genes corresponding to nucleotides 248–2229 of X04603; lys gene for diami-

nopimelate decarboxylase corresponding to nucleotides 350–1345 of X17013; pheB, pheA genes corresponding to nucleotides 2017–3334 of M24537, dapB, jojF, jojG genes corresponding to nucleotides 1358–3197 of L38424; TrpE protein, TrpD protein, TrpC protein corresponding to nucleotides 1883–4400 of K01391. The corresponding cDNAs were purchased from ATCC, incorporated into expression vectors, amplified in *E. coli* and transcribed using the MEGAscript kit (Ambion Inc., TX). The mRNA was purified using a MACS mRNA isolation kit (Miltenyi Biotec GmbH., Germany). The concentrations of spike RNAs in the GSC were in threefold steps, from 777.6 pM for AFFX-ThrX-3_at, 259.4 pM for AFFX-LysX-3_at, 86.4 pM for AFFX-PheX-3_at, 28.8 pM for AFFX-DapX-3_at, to 9.6 pM for AFFX-TrpnX-3_at. In general, the ratio depends on the linear range of the measurement system and the available number of spikes.

Setting of the "spike factor" and addition of GSC to a sample homogenate according to its DNA concentration

The GSC was added to the sample homogenates in proportion to their DNA concentrations, assuming that all cells contain a fixed amount of genomic DNA (g/cell) across samples. The amount of GSC added to each sample G (l) was given as

$$G = C * v * f \quad (l),$$

where C is the DNA concentration (g/l), v(l) is the volume of homogenate further used for RNA extraction and f (l/g) is the "spike factor", which is an adjustment factor to ensure that the sample is properly spiked by the GSC (cf. Figure 3). Spike factors have been pre-determined for various organs/tissues to reflect differences in their total RNA/genomic DNA ratios (cf. Table 1). In this way, five spike mRNA signals can properly cover the linear dose-response range of the platform. In practice, for the Affymetrix GeneChips, the spike factor is set so that the five GSC spikes cover the range of "Present" calls given by the Affymetrix system, which corresponds to approximately 80 to 7000 in raw readouts given by the Affymetrix MAS5.0 software. A raw readout of 10 by the current Affymetrix GeneChip system corresponds to approximately one copy per cell in mouse liver (spike factor = 0.2), whereas in mouse thymus (spike factor = 0.01) it corresponds to approximately 0.05 copy per cell. For Q-PCR, the same spike factor corresponds to Ct values ranging approximately from 17 to 27, which is well within the linear range of Q-PCR (data not shown).

"Per cell" normalization (Percellome normalization)

Since murine haploid genomic DNA is made of 2.5×10^9 base pairs and one base pair is approximately 600 Daltons (Da), the haploid genomic DNA weighs 1.5×10^{12} Da, corresponding to

$d = 5 \times 10^{-12}$ (g DNA per diploid cell).

Therefore, the cell number per liter of the sample homogenate (N) is given as

$$N = C/d \text{ (cells/l)}$$

where C is the DNA concentration (g/l).

On the other hand, the copy numbers of GSC RNAs in the homogenate are given as follows:

if Sj (mole/l) (j = 1,2,3,4,5) is the mole concentration of one of the five spike RNAs in the GSC solution and G(l) is the amount of GSC added to each homogenate, the mole concentrations of the spike RNAs in the homogenate (CSj) are given as,

$$CSj = Sj*C*f \text{ (mole/l)}.$$

The GSC RNAs in moles per cell (MSj) are given as

$$MSj = CSj/N$$

$$= Sj*C*f/(C/d)$$

$$= Sj*f*d \text{ (mole/cell)}$$

The copy numbers of the GSC RNAs per cell (NSj) are given as

$$NSj = MSj*A$$

$$= Sj*f*d*A \text{ (copies per diploid cell)}$$

where A is Avogadro's number.

As a result, the GSC spikes AFFX-TrpnX-3_at, AFFX-DapX-3_at, AFFX-PheX-3_at, AFFX-LysX-3_at and AFFX-ThrX-3_at correspond approximately to 5.8, 17.3, 52.0, 156.0 and 468.1 copies per cell (per diploid DNA template) for mouse liver sample homogenates, where the spike factor = 0.2. It is our observation that the RNA/DNA ratios are virtually constant across polyploid hepatocytes (data not shown).

For each Q-PCR plate or GeneChip, the coefficients, α, β, γ and δ of functions {1} or {2} are determined from the GSC values using the least-square method. The signal values or Ct values of all the other mRNAs measured are then converted to copy numbers per cell by {3} or {4}, i.e. the inverses of functions {1} or {2}.

Table 2: Primers for Q-PCR

Gene	Forward	Reverse
AFFX-TrpnX-3_at	TTCTCAGCGTAAAGCAATCCA	GCAAATCCTTTAGTGACCGAATACC
AFFX-DapX-3_at	TCAGCTAACGCTTCCAGACC	GGCCGACAGATTCTGATGACA
AFFX-PheX-3_at	GCCAATGATATGGCAGCTTCTAC	TGCGGCAGCATGACCATTA
AFFX-LysX-3_at	CCGCTTCATGCCACTGAATAC	CCGGTTCGATCCAAATTTCC
AFFX-ThrX-3_at	CCTGCATGAGGATGACGAGA	GGCATCGGCATATGGAAAC
Ahr_1450695_at	CAGAGACCACTGACGGATGAA	AGCCTCTCCGGTAGCAAACA
Cyp1a1_1422217_a_at	TGCTCTTGCCACCTGCTGA	GGAGCACCCTGTTTGTTTCTATG
Cyp1a2_1450715_at	CCTCACTGAATGGCTTCCAC	CGATGGCCGAGTTGTTATTG
Cyp1b1_1416612_at	GCCTCAGGTGTGTTTGATGGA	AGTACAGCCCTGGTGGGAATG
Cyp7a1_1422100_at	TTCTACATGCCCTTTGGATCAG	GGACACTTGGTGTGGCTCTC
Hspa1a_1452388_at	ACCATCGAGGAGGTGGATTAGA	AGGACTTGATTGCAGGACAAAC

The "LBM" ("liver-brain mix") standard sample

A pair of samples having dissimilar gene expression profiles was chosen to evaluate the linearity of the platform. The pairs chosen were brain and liver for mouse and rat, two distinct cancer cell lines for humans, and adult liver and embryo for *Xenopus laevis*. The sample pairs were processed as described above including addition of the GSC. Two final homogenates were then blended at ratios of 100:0, 75:25, 50:50, 25:75 and 0:100 (based on cell numbers) to make five samples. These five samples were measured by Q-PCR and/or GeneChips (MG-U74v2A, MEA430A, MEA430B, MG430 2.0 (shown in Figure 1), RAE230A, HG-U95A, HG-U133, and Xenopus array).

Quantitative-PCR

Duplicate homogenate samples were treated with DNaseI (amplification grade, Invitrogen Corp., Carlsbad, CA, USA) for 15 min at room temperature, followed by Super-Script II (Invitrogen) for 50 min at 42°C for reverse transcription. Quantitative real time PCR was performed with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (TAKARA BIO Inc., Japan), with initial denaturation at 95°C for 10 s followed by 45 cycles of 5 s at 95°C and 60 s at 60°C, and Ct values were obtained. Primers for the genes explored in this study were selected from sequences close to the areas of Affymetrix GeneChip probe sets as shown in Table 2.

Affymetrix GeneChip measurement

The sample homogenates with GSC added were processed by the Affymetrix Standard protocol. The GeneChips used were MG-U74v2A for the uterotrophic study and Mouse 430-2 for the TCDD study (singlet measurement). The efficiency of *in vitro* transcription (IVT) was monitored by comparing the values of 5' probe sets and 3' probe sets of the control RNAs (AFFX- probe sets) including the GSC (see Quality Control below). The dose-response linearity of the five GSC spikes was checked and samples showing saturation and/or high background were re-measured from either backup tissue samples, an aliquot of homogenate, or a hybridization solution, depending on the nature of the anomaly.

Quality control

Any external spiking method, including our Percellome method, is valid for high-quality RNA samples. Therefore, the quality of the sample RNA should be carefully monitored. In addition to a common checkup by RNA electrophoresis (including capillary electrophoresis if necessary), OD ratio, and cRNA yield, we monitor the performance of IVT (*in vitro* translation) or amplification. The 3' and 5' probe set data of the spiked-in RNAs and sample RNAs (actin, GAPD and other AFFX- probe sets) that are prepared in Affymetrix GeneChip are compared to monitor the extension of RNA by the IVT process. When both the spiked-in RNAs and the sample RNAs have similar levels of 5' and 3' signals respectively, it is judged that the IVT extension was normally performed. When both spiked-in and sample RNAs have significantly lower 5' signal than 3' signal, it is judged that the IVT extension was abnormal. When only the sample RNAs showed significantly lower 5' signal than 3' signal, it is judged that the IVT extension was normal but the sample RNAs were degraded. When only the spiked-in RNAs showed significantly lower 5' signal than 3' signal, it is judged that the IVT extension was normal but the spiked-in RNAs were degraded (although we have not encountered this situation). In addition, if the degraded sample was spiked-in by the non-degraded spike RNAs and measured by GeneChip, the position of spiked-in RNAs will be offset toward abnormally higher intensity. Together, this battery of checkups considerably increases the ability to detect abnormal events that will affect the reliability of the Percellome method. When any abnormality was found, each step of sample preparation was reevaluated to regain normal data for Percellome normalization.

The web site for GeneChip data

The GeneChip data are accessible at http://www.nihs.go.jp/tox/TTG_Archive.htm.

Authors' contributions

JK drafted the concept of the Percellome method, led the project at a practical level, and drafted the manuscript. KA developed the algorithm for the Percellome calculation and wrote the calculation/visualization programs. KI developed the laboratory protocols for the Percellome procedures to the level of SOP for technicians. NN developed the Percellome Q-PCR protocol and performed the measurements, and helped in analyzing the Percellome data. AO helped develop the algorithm. YK led the animal studies. TN provided advice and led the toxicogenomics project using the Percellome method, to be approved by the Ministry of Health, Labour and Welfare of Japan.

Additional material

Additional File 1

Excel spreadsheet file containing 15 Affymetrix Mouse 430–2 GeneChip raw data of five LBM samples in triplicate (cf. Figure 1). The column name LBM-100-0-X_Signal indicates the component percentages, i.e. 100% liver 0% brain, and X = 1,2,3 indicates the triplicates. The LBM-100-0-X_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S1.zip]

Additional File 2

Excel spreadsheet file containing Percellome data of the same LBM samples, of which raw data is listed in Additional file 1 (cf. Figure 1).

Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S2.zip]

Additional File 3

Excel spreadsheet file containing 2 Affymetrix MG-U74v2 raw data of a blank sample with the GSC (horizontal axis of Figure 2a) and blank with the five spike RNAs at a high dosage (vertical axis of Figure 2a).

Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S3.zip]

Additional File 4

Excel spreadsheet file containing 2 Affymetrix MG-U74v2 raw data of a liver sample with GSC (horizontal axis of Figure 2b) and without GSC (vertical axis of Figure 2b).

Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S4.zip]

Additional File 5

(first quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 2 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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[http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S5.zip]

Additional File 6

(second quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 4 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 7

(third quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 8 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 8

(last quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 24 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 9

(first quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 2 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 5.
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Additional File 10

(second quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 4 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 6.
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Additional File 11

(third quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 8 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 7.
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Additional File 12

(last quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation)]: an Excel spreadsheet file containing 24 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 8.
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Additional File 13

Excel spreadsheet file containing 15 Affymetrix MG-U74v2 A GeneChip raw data of the uterotrophic response study (cf. Figure 6). The column name X-Y_Signal indicates the treatment (V = vehicle, Low = low dose, etc) and animal triplicate (Y = 1,2,3). The X-Y_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.
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Additional File 14

Excel spreadsheet file containing Percellome data of the same 15 samples of the uterotrophic response study (cf. Figure 6), of which raw data is listed in Additional file 13.
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Acknowledgements

The authors thank Tomoko Ando, Noriko Moriyama, Yuko Kondo, Yuko Nakamura, Maki Abe, Nae Matsuda, Kenta Yoshiki, Ayako Imai, Koichi Morita, Hisako Aihara and Chiyuri Aoyagi for technical support, and Dr. Bruce Blumberg and Dr. Thomas Knudson for critical reading of the manuscript. This study was supported by Health Sciences Research Grants H13-Seikatsu-012, H13-Seikatsu-013, H14-Toxico-001 and H15-Kagaku-002 from the Ministry of Health, Labour and Welfare, Japan.

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Research Report

Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats

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ARTICLE INFO

Article history:
Accepted 2 November 2005
Available online 27 December 2005

Theme:
Development and regeneration
Topic:
Hormones and development

Keywords:
Bisphenol A
Prenatal exposure
Sexual differentiation
Exploratory behavior
Depressive behavior

ABSTRACT

Perinatal exposure to bisphenol A (BPA, 0.1 and 1 ppm in drinking water applied to mother rats for 6 weeks) has been shown to impair the sexual differentiation in exploratory behavior, but the exact critical period of this disrupting effect is still unknown. In this study, we examined the effects of prenatal exposure to BPA (0.1 ppm in drinking water applied to dams during the final week of pregnant) on emotional and learning behaviors in addition to exploratory behavior. Estimated daily intake was 15 μg/kg/day, below the reference dose (RfD) in the United States and the daily tolerable intake (TDI) in Japan (50 μg/kg/day). The rats were successively tested in open-field test, elevated plus maze test, passive avoidance test and forced swimming test during development from 6 to 9 weeks of juvenile period. Prenatal exposure to BPA mainly affected male rats and abolished sex differences in rearing behavior in the open-field test and struggling behavior in the forced swimming test. BPA increased the immobility of male rats in the forced swimming test. The avoidance learning and behaviors in the elevated plus maze were not affected. The present study demonstrates that male rats at the final week of prenatal period are sensitive to BPA, which impairs sexual differentiation in rearing and struggling behavior and facilitate depression-like behavior.

1. Introduction

Gonadal hormones play an important role in the perinatal sexual differentiation of neural tissues and patterns of behavior (Davidson and Levine, 1972; Gorski, 1973). Testicular androgen is thought to masculinize and defeminize the male rats central nervous system (CNS) during the critical perinatal period that was presumed between days 18 and 27 after conception (MacLusky and Naftolin, 1981). Androgen action is thought to be exerted through its conversion by brain aromatase into estrogen (Lephart, 1996; MacLusky and Naftolin, 1981). In addition to converted estrogen, Sato et al. reported that androgen receptors (ARs) were also important in the induction of male-typical behaviors (Sato et al., 2004).

Many kinds of industrial chemical substances are being released into our surrounding environment, and some of them may influence the CNS using similar mechanisms in gonadal hormones during perinatal period. These chemicals are called 'environmental endocrine disrupters' (EEDs). Bisphenol A (BPA) is one of the well-known EEDs that is known to have both estrogenic and anti-androgenic action

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doi:10.1016/j.brainres.2005.11.028

in vitro (Krishnan et al., 1993; Sohoni and Sumpter, 1998). BPA has been released from polycarbonate plastics used in dental prostheses and sealants (Olea, 2000; Suzuki et al., 2000), in the linings of metal cans for preserving foods (Brotons et al., 1995; Kang et al., 2003), and in such items as baby bottles (Brede et al., 2003). According to U.S. Environmental Protection Agency (EPA), the reference dose (RfD) of BPA is calculated as 50 μg/kg/day by dividing the Lowest-Observed-Adverse-Effect-Level (LOAEL, 50 mg/kg/day) (from an earlier chronic toxicity study) by an uncertainty factor of 1000. Applying the same factor to the reproductive toxicity No-Observed-Adverse-Effect-Level (NOAEL, 50 mg/kg/day) from the Tyl et al. (2002) study confirms the safety of the RfD (available on the web site at http://www.bisphenol-a.org/human/polyplastics.html#exposure). Recently, the European Commission's Scientific Committee on Food (SCF) set up the independent safety standards in which tolerable daily intake level (TDI, 10μg/kg/day) was calculated from overall NOAEL (5 mg/kg/day) by applying 500-fold uncertainty factor (available on the web site at http://europa.eu.int/comm/food/fs/sc/scf/out128_en.pdf).

Previously, many studies in rodents have reported the effects of BPA on the reproductive system, however, recent studies focus mainly on its effects on the CNS and nonreproductive behaviors (Facciolo et al., 2002; Funabashi et al., 2004; Ishido et al., 2004; Kawai et al., 2003; MacLusky et al., 2005; Mizuo et al., 2004; Negishi et al., 2004; Ramos et al., 2003; Shikimi et al., 2004; Suzuki et al., 2003). The effects of perinatal exposure to BPA have been shown to affect various aspects of nonsocial behaviors such as exploratory behavior (Farabollini et al., 1999), anxiety level (Farabollini et al., 1999), nociception (Aloisi et al., 2002), sociosexual behaviors (Farabollini et al., 2002), play behaviors (Dessi-Fulgheri et al., 2002) and the response to D-amphetamine (Adriani et al., 2003). Neonatal effects of BPA on spatial learning were demonstrated in gender-dependent manner (Carr et al., 2003).

We have investigated the behavior and brain structure in Wistar rat offspring when mothers were administered BPA during pregnancy and lactation periods (for 6 weeks) (Kubo et al., 2001, 2003). In these studies, 5 parts per million (ppm) of BPA (1.5 mg/kg/day) treatment disrupted the sexual differentiation of the avoidance behavior (Kubo et al., 2001). Administration of 0.1 ppm or 1 ppm of BPA (30, 300 μg/kg/day, respectively) impaired the sexual differentiation of the exploratory behavior and the size of the locus coeruleus (LC). BPA, however, did not affect the reproductive function or the size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Kubo et al., 2003).

Previous studies have found that sex differences in open-field behavior were abolished in rats when they were exposed to androgen or estrogen in the neonatal period (Blizard et al., 1975; Stevens and Goldstein, 1981; Stewart et al., 1975), but these studies did not examine the prenatal effects of these hormones. However, prenatal hormones are necessary for sexual differentiation of LC (Guillamon et al., 1988; Pinos et al., 2001; Rodriguez-Zafra et al., 1993) and the catecholamine systems (Siddiqui and Gilmore, 1988; Stewart and Rajabi, 1994) which are deeply involved in the regulation of the open-field behavior (Britton et al., 1984; Kubo et al., 2001, 2003). In Vessey's report, administration of diethylstilbestrol (DES, a synthetic estrogenic compound) in pregnant women increased the incidence of psychiatric diseases such as depression and anxiety in their offspring (Vessey et al., 1983). These findings raised a hypothesis that BPA induce emotional disturbances especially in the case of prenatal exposure. In the present study, we demonstrated that exposure to BPA at the final week of prenatal period results in disrupting of sexual differentiation in behavior and enhanced depression-like behavior in male rats without causing major effects on female rats.

2. Results

2.1. *Open-field test*

The frequency and duration of rearing and the time spent in center area were evaluated as the exploratory behavior. The total distance moved and the numbers of rapid movement were evaluated as the locomotor activity. A significant main effect of sex was found in the frequency ($F_{1,88} = 5.25$, $P < 0.05$) and duration ($F_{1,88} = 4.50$, $P < 0.05$) of rearing by two-way ANOVA. The duration of rearing had a significant sex × treatment interaction ($F_{1,88} = 4.09$, $P < 0.05$) and BPA increased the duration of rearing in males ($P < 0.05$). Sex differences found in controls (female > male, $P < 0.01$) were abolished by BPA (Fig. 1).

The time spent in the center area (control: 50.7 ± 10.4 (male), 78.8 ± 13.7 (female); BPA: 48.8 ± 13.8 (male), 78.0 ± 10.9

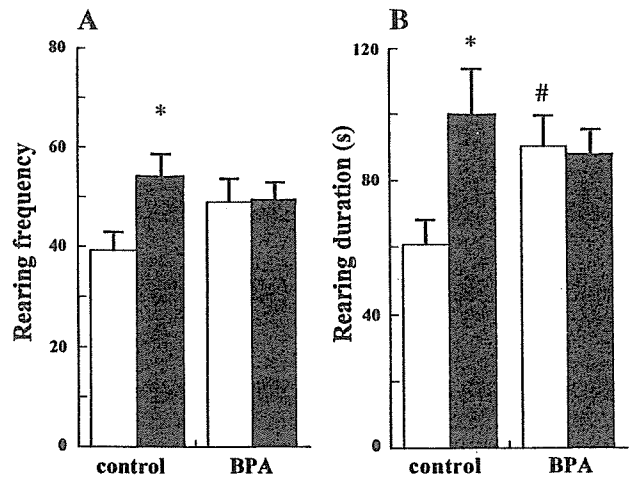

Fig. 1 – The effect of BPA on the rearing behavior in the open-field test, rearing frequency (A) and duration (B). The white and black columns are expressed as mean ± SEM for the males and females data, respectively (n = 22–24 animals per group). *$P < 0.05$, two-way ANOVA followed by Fisher's PLSD test between the data of males and females in same group. #$P < 0.05$, two-way ANOVA followed by Fisher's PLSD test between the data of control group and BPA group in same sex. Results demonstrate that BPA impaired the sex difference of the exploratory behavior.

Table 1 – The effect of BPA on the elevated plus maze test (mean ± SEM)

	Control group		BPA group	
	Male (23)	Female (21)	Male (21)	Female (23)
Time spent in open arms (s)	25.2 ± 5.1	23.1 ± 6.0	15.0 ± 4.6	16.9 ± 3.5
Number of open arms entries	2.78 ± 0.58	2.67 ± 0.45	2.14 ± 0.37	2.83 ± 0.38
Number of total entries	8.57 ± 1.06	10.24 ± 0.84	9.43 ± 0.90	11.65 ± 1.17
Open/Total entries	0.32 ± 0.040	0.26 ± 0.038	0.22 ± 0.038	0.25 ± 0.035

Number of rats in each group in parentheses.

(female)), the total distance moved (control: 3194 ± 169 (male), 3658 ± 173 (female); BPA: 3269 ± 238.4 (male), 3668 ± 188.0 (female)) and the number of rapid movement (control: 85.2 ± 6.0 (male), 103.0 ± 5.8 (female); BPA: 86.5 ± 8.3 (male), 104.9 ± 7.8 (female)) showed a main effect of sex ($F_{1,88} = 5.47$, $P < 0.05$, $F_{1,88} = 4.93$, $P < 0.05$, $F_{1,88} = 6.58$, $P < 0.05$, respectively) but not treatment.

2.2. *Elevated plus maze test*

The time spent in open arms in BPA groups was shorter than that of control groups in both sex but the difference did not reach the significance level ($F_{1,84} = 2.88$, $P = 0.094$) (Table 1). The number of total entries of female rats also missed significance level (main effect of sex: $F_{1,84} = 3.72$, $P = 0.057$). All other parameters such as number of open arm entries and percentage of open arm entries did not show any significant difference by two-way ANOVA.

2.3. *Passive avoidance test*

To evaluate the effect of BPA on learning and memory (avoidance learning), rats were tested using the passive avoidance test. In the control group, latency was significantly longer in males than in females ($P < 0.05$). This sexually dimorphic pattern of the avoidance behavior was also shown in the BPA groups as well as control groups (Fig. 2).

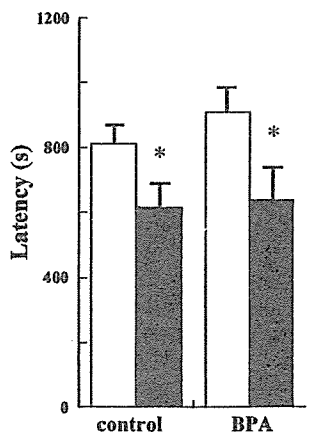

Fig. 2 – The effect of BPA on the avoidance learning in the passive avoidance test. The white and black columns are expressed as mean ± SEM for the males and females data, respectively (n = 20–24 animals per group). *P < 0.05, Mann–Whitney's U test between the data of males and females in same group. Results show that BPA did not affect the avoidance learning.

2.4. *Forced swimming test*

A main effect of sex on struggling behavior was significant ($F_{1,92} = 5.28$, $P < 0.05$), but sex × treatment interaction did not reach the significance level ($F_{1,92} = 3.51$, $P = 0.064$). Sex difference was revealed only in control groups ($P < 0.01$) but not in BPA groups (Fig. 3). A main effect of treatment on immobility revealed a significance ($F_{1,92} = 9.60$, $P < 0.01$). BPA exposure significantly increased immobility time (males: $P < 0.01$). There was no sex difference in immobility.

Active behaviors such as diving and limb movement had significantly affected by BPA (sex × treatment in diving: $F_{1,92} = 4.62$, $P < 0.05$; treatment in limb movement: $F_{1,92} = 10.66$, $P < 0.01$) (Table 2). These active behaviors did not show sex difference in either groups, but BPA increased duration of diving in females ($P < 0.05$) and decreased the duration of limb movement in males ($P < 0.01$). A main effect of

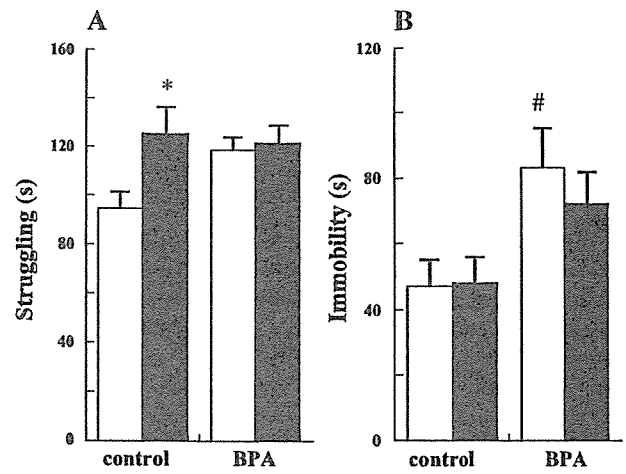

Fig. 3 – The effect of BPA on the forced swimming test, duration of struggling (A) and that of immobility (B). The white and black columns are expressed as mean ± SEM for the males and females data, respectively (n = 24 animals per group). *P < 0.05, two-way ANOVA followed by Fisher's PLSD test between the data of males and females in same group. #P < 0.05, two-way ANOVA followed by Fisher's PLSD test between the data of control group and BPA group in same sex. Results demonstrate that BPA impaired the sex difference of the struggling behavior and increased the duration of immobility.

Table 2 – The effect of BPA on the forced swimming test (mean ± SEM)

	Control group		BPA group	
	Male (24)	Female (24)	Male (24)	Female (24)
Head shaking (s)	28.1 ± 2.2	21.6 ± 1.5*	26.3 ± 1.8	20.1 ± 2.0*
Diving (s)	4.73 ± 1.63	1.79 ± 0.84	3.09 ± 1.28	6.06 ± 1.59**
Limb movement (s)	725 ± 11.4	703 ± 12.9	669 ± 14.4**	680 ± 12.9

Number of rats in each group in parentheses.
* $P < 0.05$ vs. male of same group.
** $P < 0.05$ vs. same sex of control group by Fisher's PLSD test followed by two-way ANOVA.

sex showed significance in head shaking ($F_{1,92} = 11.46$, $P < 0.01$). This sex difference was seen in both groups and BPA did not affect this parameter ($P < 0.05$).

3. Discussion

The present study demonstrated that prenatal exposure to BPA (below the level of RfD in US and TDI in Japan, 50 μg/kg/day) impaired sexual differentiation of rearing behavior in open-field test and increased immobility in forced swimming test. Our previous study showed that rearing behavior is more sensitive to perinatal exposure to BPA than locomotor activity (Kubo et al., 2003). The present study also showed vulnerability of the rearing behavior to BPA even in 1-week prenatal exposure. The present study is different from previous experiments in that it restricted the exposure period to further define the critical period for disruption of the development of sex differences in behavior by this chemical. Important points of the present study were short time of exposure and the very low dose that was used relative to the dose predicted to cause no effect. The final week of prenatal period is critical for the sex differentiation of rearing behavior. In rats, both pre- and postnatal periods are important for sexual differentiation of brain via androgen action (MacLusky and Naftolin, 1981). The sex difference of open-field behavior was abolished by androgen or estrogen in the neonatal period (Blizard et al., 1975; Stevens and Goldstein, 1981; Stewart et al., 1975), but the effects of prenatal exposure of sex hormones were not well studied. Prenatal actions of sex hormones have been shown to be essential for sexual differentiation of LC (Guillamon et al., 1988; Pinos et al., 2001; Rodriguez-Zafra et al., 1993) and catecholamine systems (Siddiqui and Gilmore, 1988; Stewart and Rajabi, 1994) which are deeply involved in regulation of the open-field behavior (Britton et al., 1984; Kubo et al., 2001, 2003).

Prenatal exposure to BPA did not affect development of avoidance behavior and anxiety level. In our previous study, we found that sex difference in the latency of entry into the dark compartment in the passive avoidance test was abolished by 5 ppm BPA exposure (Kubo et al., 2001). In the present study, however, 0.1 ppm BPA was not sufficient to impair sexual differentiation of the avoidance behavior. These findings suggest that the neural system controlling the avoidance behavior was less sensitive to BPA than that of the rearing behavior. Farabollini et al. (1999) found that BPA exposure to Sprague–Dawley rats during the perinatal period at a dose of 40 μg/kg/day but not 400 μg/kg/day reduced anxiety. The differences between Farabollini's result and our findings may be due to the difference in experimental designs including dose and strain.

In the forced swimming test, we found that struggling behavior was a sensitive marker to evaluate the effect of BPA on sexual differentiation as rearing behavior. Struggling, swimming and diving behaviors were classified as active behavior (Detke et al., 1995; Lucki, 1997). Although their emotional roles are still not well understood, these active behaviors have been shown to be enhanced by treatment of either noradrenergic or serotonergic antidepressants in type-dependent manner (Detke et al., 1995; Lucki, 1997). In present study, BPA increased the diving in females, while it decreased limb movement including swimming in males. In contrast to these active behaviors, immobility has been shown to represent a type of depression-like behavior called behavioral despair (Detke et al., 1995; Lucki, 1997; Porsolt et al., 1977). Although there was no sex difference in the immobility, BPA increased this depressive response in both males and females in which statistical significance appeared only in males. Because BPA reduced the active movement of limbs and increased the depression-like behavior in males, male rats appeared to be more sensitive to an enhancing effect of BPA on depressive response. Altered development of monoamine systems is attribute to depressive behavior in rodents (Ansorge et al., 2004; Fernandez-Pardal and Hilakivi, 1989; Hansen et al., 1997; Mirmiran et al., 1981; Vogel etal., 1990). Neonatal treatment of monoamine uptake inhibitors increases the incidence of depressive behavior (Fernandez-Pardal and Hilakivi, 1989; Hansen et al., 1997). Although few studies investigated effects of EEDs on depressive behavior, our result is similar to Vessey's report in which DES administration in pregnant women correlated with higher incidence of psychiatric diseases such as depression in their offspring (Vessey et al., 1983).

BPA is known to have both estrogenic and anti-androgenic activities in vitro (Krishnan et al., 1993; Sohoni and Sumpter, 1998). Concerning estrogenic action, our previous study demonstrated that DES disrupts the sexual differentiation of an exploratory behavior and LC (Kubo et al., 2003). In the present study, effects of BPA were seen mainly in male rats. If BPA acts on the estrogen receptors in the developing brain, it might induce brain masculinization. Because BPA has anti-androgenic activity, and ARs are also important for brain masculinization (Garcia-Falgueras et al., 2005; Morris et al., 2005), feminizing effect of BPA may be caused by its anti-androgenic action. AR has been shown to be involved in the masculinization of rats LC (Garcia-Falgueras et al., 2005) and

BPA inverted the sex difference of a size of LC (Kubo et al., 2003), therefore, BPA impaired the brain masculinization via anti-androgenic action. The development of monoamine systems is under the control of gonadal hormones. Gonadal hormones modify the brain monoamine systems in the fetal period (Kaylor et al., 1984) and affect monoamine levels in the neonatal period (Giulian et al., 1973; Hardin, 1973; Siddiqui and Gilmore, 1988). Since BPA has been shown to affect development of the monoamine systems (Ishido et al., 2004; Suzuki et al., 2003), including the noradrenergic LC cells (Kubo et al., 2001, 2003), behavioral effects of BPA were expressed through monoamine systems at least in part. Further study is necessary to elucidate the precise mechanisms of BPA actions that contribute to these behavioral abnormalities.

In conclusion, the sexual differentiation of exploratory behavior and an active stress response such as struggling behavior were impaired by only 1-week exposure of low-dose BPA (below RfD) at the final prenatal period. In addition, depression-like behavior was enhanced by this treatment.

4. Experimental procedures

4.1. *Animals*

Female and male Wistar rats at 11 weeks of age were purchased from Kyudo Corp. (Tosu, Japan) and were housed in our laboratory for 1 week. After copulation, six mother rats were exposed to vehicle as control, and another six dams were exposed to BPA. Pregnant day was judged by detecting the sperm in vaginal smear. The animals were kept in a closed colony room under a 12:12-h light–dark cycle (light period 08:00–20:00 h) in a constant air-conditioned environment of 23 ± 1 °C and 60 ± 10% relative humidity. They had free access to laboratory chow (CE-2, CLEA Japan, Inc., Tokyo) and tap water. All experiments were performed under the Guidelines for Animal Experiments of the Graduate School of Life Science and Systems Engineering, Kyushu Institute of technology, and Law (No.105) and Notification (No. 6) of the Government of Japan.

4.2. *Treatment*

BPA was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and dissolved in distilled water. Both groups were given tap water until gestational day 13, then BPA group was administered BPA solution (0.1 ppm), and control group was administered vehicle until the day of birth (postnatal day 0, PND0). All of dams were allowed to feed and drink ad libitum from the day that sperm was detected in a vaginal smear in the morning, until their pups were weaned on PND 21. On PND1 at 0–3 p.m., the body weight of all offspring was measured, and litters were standardized to both four males and females by removing any excess offspring. On PND21 at 0–3 p.m., all offspring were weaned then classified and housed in the same sex and litter groups and were allowed food and tap water ad libitum until the end of the experiment. As a result, mother rats were administered BPA approximately 15 μg/kg/day. The route of administration by drinking is probably the most relevant method of exposure with regard to how humans are exposed to BPA.

4.3. *Behavioral test*

4.3.1. *General*

The following behavioral tests were examined: open-field test at 6th, elevated plus maze test at 7th, passive avoidance test at 8th, forced swimming test at 9th week of age. All tests were conducted between 13:00 and 19:00 h. Each apparatus was cleaned after the test session of each subject to prevent olfactory cues from affecting the behavior of subsequently tested rats. In the forced swimming test, water was continuously exchanged at a flow rate of 2 l/min and changed to fresh tap water every 4 rats. All sessions, except that of the passive avoidance test, were manually or automatically recorded with a computer-based videotracking system (Ethovision v1.96, Noldus Info. Tech., Netherlands).

4.3.2. *Open-field test*

The open-field apparatus was a square field (80 × 56 × 40 cm), in which an object (12 × 9 × 7 cm) was placed at the center. Each rat was placed along one side in the apparatus at the beginning of the test and was allowed to move freely for 10 min. (1) total distance moved, (2) number of rapid movement (more than 10 cm/s), (3) frequency and duration of rearing (standing on hindlegs) and (4) time spent in center area were recorded. The total distance moved, and the number of rapid movement was evaluated as an index of locomotor activity, rearing behavior and the time spent in center area were evaluated as an index of exploratory behavior, respectively.

4.3.3. *Elevated plus maze test*

The elevated plus maze was made of translucent brown acrylic material and consisted of 2 open arms (50 × 10 cm) and 2 closed arms (50 × 10 cm) which had 39-cm high walls. The arms extended from a center square platform (10 × 10 cm) and were arranged so that those of the same type were opposite to each other. The apparatus was elevated to 50 cm above the floor. Each rat was placed on the center square platform and was allowed to move freely for 5 min. We recorded the (1) time spent in open arms and (2) number of entries into the open and closed arms. We also calculated the (3) number of total entries (open + closed arm entries) and the (4) ratio of open arm entries (open arm entries/total entries). The time spent in open arms, number of entries into the open arms and the ratio of open arm entries were evaluated as an index of anxiety. The number of total entries was also evaluated as an index of locomotor activity.

4.3.4. *Passive avoidance test*

Apparatus for the step-through passive avoidance test consisted of two compartments separated by a sliding guillotine door (SGS-001, Muromachi Co., Tokyo, Japan) (Ohno and Watanabe, 1996). The starting compartment (25 × 10 × 25 cm) was illuminated with a 100-W incandescent lamp placed 35 cm above the floor. The other compartment was a dark box which measured 30 × 30 × 30 cm and had a grid floor made of 23 parallel stainless steel rods (3 mm in diameter) spaced 1 cm apart. An electrical shock generator was connected to the steel rods that induced scrambled foot shock. On day 1, rats were put in the compartments for 5 min to allow them to adapt to the experimental conditions. On day 2, rats were placed in the illuminated compartment. After a 10-s delay, the sliding door was raised so that rats could pass through the entrance (8 × 8 cm) to the dark compartment, then the door was lowered and 0.5 mA scrambled foot shock was applied for 5 s. After the foot shock, the rats were immediately removed from the dark box and were returned to their home cages. On day 3 (24 h later), rats were placed in the illuminated compartment in the same manner. The latency to enter the dark compartment was measured as an index of avoidance learning. During the test, rats were allowed to access to the dark compartment for maximum 20 min. If rats did not enter the dark box within 20 min, then they were assigned a latency of 20 min.

4.3.5. *Forced swimming test*

Each rat was exposed to one trial of forced swimming test as previously described to avoid the influence of adaptation and/or learning components (Ma et al., 1994; Pare, 1989a,b). The water

tank consisting of a cylindrical acrylic container (50 cm high, 30 cm in diameter) was filled 35 cm deep with tap water (24 ± 1 °C). Each rat was placed in the water for 15 min, and after testing, rats were dried with a towel and returned to their home cages. The duration of (1) struggling, (2) immobility, (3) head shaking, (4) diving and (5) limb movement including swimming were recorded. Behavior was characterized as struggling when rats vigorously broke the water surface with the head and forepaws or actively tried to climb the wall of the tank with their paws. Immobility was determined by floating motionlessly on the surface of the water.

4.4. Statistics

All data were analyzed using the Statview 5.0 software for Macintosh (SAS Inc). All tests except passive avoidance test were analyzed by two-way ANOVA followed by Fisher's PLSD test. Mann–Whitney's U test was used for the passive avoidance test between males and females, two groups (control and BPA treated) were analyzed separately. Statistical differences were considered significant when the P value was below 0.05. All results are presented as means ± SEM.

Acknowledgments

We thank Drs. S. Oishi, B. Lukats, O. Hangodi, S. Douglas and M. Mizuno for critically reading and correcting the manuscript. Supported by Grants-in-aid for Scientific Research (no. 16209006, S.A.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by Health Sciences Research Grant for Research on Environmental Health from the Ministry of Health and Welfare of Japan.

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