Sexually dimorphic gene expression profile of the developing mouse embryonic brain exposed in utero to 2,3,7,8-tetrachlorodibenzo-p-dioxin

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Introduction

Perinatal developmental exposure of dioxin and dioxin-like compounds are known to cause reproductive, neurological, and nueroendocrinological disruptions in human and laboratory animals. So far, majority of environmental endocrine disruptor studies were to understand the mechanisms of reproductive endocrine disruption by the hormone-like chemicals. However, an increasing number of the research reports that indicate aberrations in non-reproductive sex-linked behavioral responses of laboratory animals implies that the causes of alterations in non-reproductive behavior should be further studied at gene expression level.[1, 2, 3] Thus, we exposed female C57BL/6 pregnant mice to 5µg/kg-body weight of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by single gavage at gestational day (GD) 12.5, and sexually differentiated gene expression profiles of the whole embryonic brain at GD18.5 were analyzed by CodeLink^{TM}UniSet Mouse I Bioarrays (10,012 gene probes, Amersham Biosciences). The data analysis by ANOVA (analysis of variance) statistics and subsequent gene ontology classification revealed that 52.7% of the genes spotted on the CodeLink^{TM}Bioarray were found to be up-regulated in male but down-regulated in female, whereas 10.6% of the genes were found to be up-regulated in female but down-regulated in male. The genes up-regulated in both sexes and the genes down-regulated in both sexes accounted for 19.9% and 16.8%, respectively. The result showed very obvious sexual dimorphic responses to TCDD in the developing mouse embryonic brain, and the signal transducer genes in particular appeared to play very important roles to this sexual dimorphism. Among the signal transducer genes, rhodopsin-like G-protein-coupled receptors were down-regulated more in male than in female, and on the other hand, heterotrimeric G-protein GTPase genes were down-regulated more in female than in male.

Materials and Methods

C57BL/6 pregnant female mice (SPF grade) were prepared to obtain selected 12 male (six TCDD-exposed and six unexposed) and 12 female (six TCDD-exposed and six unexposed) embryonic brain samples. To one group of the pregnant mice 5µg TCDD/kg-body-weight was given by single gavage at gestational day (GD) 12.5, and to the other non-exposed control group corn oil (vehicle) was given in the same manner. Intrauterine positions of the selected embryos fitted in such a way that a sample embryo was not positioned next to the opposite sex on either side. Embryonic brain sampling was carried out at GD 18.5. RNeasy Mini Kit (QIAGEN) was used for the extraction and the purification of total RNA from the brain samples, and 2µg of the total RNA was reverse-transcribed with T7-oligo dT primer. Subsequently ds-cDNA was synthesized and *in vitro* transcribed with biotinylated-UTP to generate biotin-labeled cRNA target samples (CodeLink^{TM} Expression Assay Kit, Amersham Biosciences). Each of the purified cRNA samples were fragmented and hybridized with the oligo-probes on two (for duplicated analysis) CodeLink^{TM}UniSet Mouse I Bioarrays (Amersham Biosciences) at 37°C for 18 hours. Post-hybridization wash and affinity binding of streptavidin-Cy5 to the hybridized cRNA target (biotinylated) were carried out, and then CodeLink^{TM}Bioarrays were scanned with GenePix 4000B scanner (Axon Instruments) to obtain expression level of each of the genes. Statistical Analysis was performed with ArrayStat z-test (Imaging Research Inc.) and significance determination of $p < 0.05$. Gene ontology charts were generated by DAVID that was provided on-line by the National Institute of Allergy and Infectious Diseases (NIAID).[4]

Results and Discussion

Numerous reports have shown that *in utero* exposure to dioxin and dioxin-like compounds causes endocrine-disruption and adverse developmental consequences, e.g., physiological disorders and reproductive

abnormalities.[5, 6] However, only a small number of the studies of those compounds' effects on non-reproductive behavior was available.[3] As we believed that risk assessment of the potential endocrine disruptors needed a novel method which includes gene expression profiling at the time of endocrine disruption (early endpoint) as well as behavioral assay (late endpoint), we were particularly interested in investigating whether non-reproductive behavioral aberrations have roots in the alterations made by the chemicals at gene expression level during embryonic development.

Schedule-controlled operant behavior (SCOB) has been a powerful tool for analyzing spatial discrimination abilities (e.g., learning, performance, and memory) to detect and interpret the effects of chemical exposure during developmental or non-developmental period.[7] For instance, one study with SCOB showed that unexposed male rodents responded better than unexposed female rodents, and this was the result of sexually differentiated food-motivated function (i.e., behavioral perseverance) in males. However, in the same study *in utero* TCDD-exposed males and females moved generally in opposite directions, meaning that the exposed females responded better than the exposed males.[1] In addition, the performance of castrated males resembles the lower response rates more typical of the unexposed females, and this suggested an influence of testosterone.[8]

Thus, developmental endocrine disruptive influences by the *in utero* TCDD-exposure lead to behavioral sex differences, and with DNA microarray technology we tried to identify the alterations made to the gene expression profile of developing embryonic brain by the *in utero* TCDD-exposure (exposure at GD 12.5 and analysis at GD18.5). It should also be noted that we performed a very careful selection of the embryo samples in terms of their intrauterine positions so that individual variation in sexual characteristics among the samples in the same sex were kept minimal.[9, 10] Among the 10,012 gene probes contained on CodeLink^{TM}UniSet Mouse I Bioarray, 5376 genes were regulated by TCDD in the mouse embryonic brains with statistical significance. Our result showed very clear sexual dimorphism in TCDD-affected gene responses (figure 1), and we found that 2,832 genes (52.7%) were up-regulated in male but down-regulated in female (group B), and that 571 genes (10.6%) were down-regulated in male but up-regulated in female (group C) (figure 2). Additionally, the number of the genes that were up-regulated in both male and female embryonic brains was 1,071 (19.9%, group A), and the number of the genes that were down-regulated in both male and female was 902 (16.8%, group D) (figure 2). For the differentially affected and sexually dimorphic genes (groups B and C), gene ontology classification by molecular gene function revealed that the percentage share of signal transducer genes in group B was only 6.8%, but on the other hand, that of signal transducer genes in group C was 18.6% (figure 3). Very interestingly, further categorization of the molecular function of these TCDD-affected signal transducer genes indicated that the percentage share of rhodopsin-like G-protein-coupled receptors that were down-regulated in male but up-regulated in female (group C, 23.6%) was much higher than that of rhodopsin-like G-protein-coupled receptors that were down-regulated in female but up-regulated in male (group B, 6.3%) (figure 4). Furthermore, heterotrimeric guanine nucleotide binding protein genes that were up-regulated in male but down-regulated in female (group B) shared 5.2%, but none of this gene was found to be up-regulated in female but down-regulated in male (group C) (figure 4).

Table 1 showed that some of the genes among the rhodopsin-like G-protein-coupled receptors in group C displayed larger difference between the TCDD-responses of male and female embryonic brains, and they were cholecystokinin A receptor (*cckar*, down-regulated 0.89-fold in male but up-regulated 1.46-fold in female), dopamine receptor 4 (*drd4*, down-regulated 0.49-fold in male but up-regulated 1.42-fold in female), lysophosphatidic acid G-protein-coupled receptor 4 (*edg4*, down-regulated 0.70-fold in male but up-regulated 1.40-fold in female), and somatostatin receptor 2 (*smstr2*, down-regulated 0.72-fold in male but up-regulated 1.18-fold in female). Cholecystokinin A receptor is present in a few brain areas, and it seems to be mediating the short-term inhibition of food consumption.[11] It appeared to be involved in the mechanisms of stress or anxiety on social-aggressive encounter.[12] Dopamine receptor 4 is relatively new to the dopamine D_2 receptor family and its function is somewhat controversial, but gene knockout mouse study indicated that it was involved in spontaneous locomotoracitivity, rearing behavior, and exploratory behavior.[13] G-protein-coupled receptor 4 was studied *in vitro*, and it had functions of mediating Ca^{2+} signaling, adenylylcyclase inhibition, inositol phosphates production, MAP kinase activation, and arachidonic acid release.[14] Somatostatin receptor 2 is expressed in the cortex and hippocampus, and it is thought to be involved in glutamate-dependent plasticity and spatial learning.[15] Additionally, table 2 showed that among the heterotrimeric guanine nucleotide binding proteins in group B, G-protein alpha o (Go-α, down-regulated 0.70-fold in female but up-regulated 1.13-fold in male) and G-protein alpha stimulating (GNAS, down-regulated 0.77-fold in female but up-regulated 1.18-fold in male) displayed larger difference between the TCDD-responses of male and female

embryonic brains. Go-α protein is found most expressed in neurons, and plays a major role in motor control, motor behavior, pain perception, and Ca^{2+} channel regulation.[16] GNAS generates several gene products, including Gs-α (coupling seven-transmembrane receptors to the cAMP-generating enzyme adenylylcyclase) and XL alpha s (expressed from the paternal GNAS allele).[17] Mice with mutations in XL alpha s have poor postnatal growth and survival and a spectrum of phenotypic effects that indicate that XL alpha s controls a number of key postnatal physiological adaptations, including suckling, blood glucose and energy homeostasis.[18]

Overall, finding and categorizing the TCDD-responsive genes in mouse embryonic brain by our cDNAmicroarray analysis and gene ontology classification were valuable, and this work surely started revealing the links between the developing brain-expressed signal transducer genes and TCDD-induced sexually dimorphic behaviors. Although the precise molecular causes of the TCDD-induced sexually dimorphic behaviors could not be identified in this study, those particular signal transducer genes would become the foci of our further research. Also, amongst the signal transducers, rhodopsin-like G-protein-coupled receptors and heterotrimeric G-proteins appeared to have potentials to be biomarkers that indicate the risks of endocrine disruptors to human and wildlife health.

References

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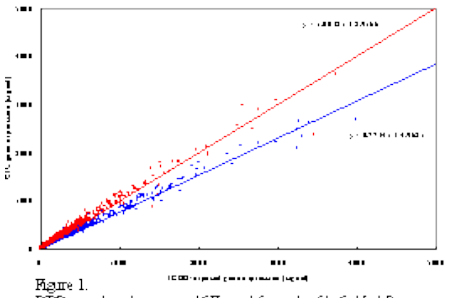

Figure 1.

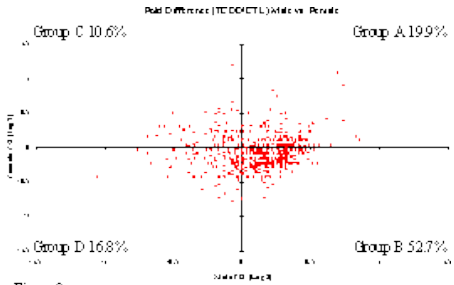

Figure 2.

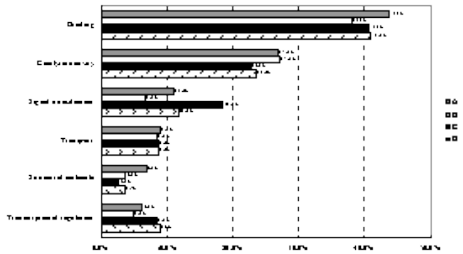

Figure 3.

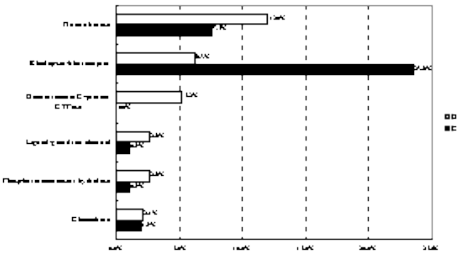

Figure 4.

Table 1
TCDD-affected genes categorized and outstanding in group C:
Up-regulated in female and down-regulated in male

[illegible]	[illegible]	[illegible]	[illegible]

Table 2
TCDD-affected genes categorized and outstanding in group B:
Up-regulated in male and down-regulated in female

[illegible]	[illegible]	[illegible]	[illegible]