Original Article

Low dose effects of dichlorodiphenyltrichloroethylene (DDT) on gene transcription and DNA methylation in the hypothalamus of young male rats: implication of hormesis-like effects

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ABSTRACT — To verify the relationship between oxidative stress and DNA methylation in the young brain, dichlorodiphenyltrichloroethylene (DDT) was administered by gavage to male young rats at doses of 0, 0.006, 0.06, 0.6, 6, and 60 mg/kg/day for a period of 4 weeks. The most conspicuous decrease in the lipid peroxidation level was observed in the 0.06 mg/kg/day group compared with controls. Microarray analysis of brain samples from the control and 0.06 mg/kg/day groups revealed that the expression of 40 genes was changed in the hypothalamus, whereas mRNA expression was unaltered in the hippocampus. This result suggests that the hypothalamus is more susceptible to low-level oxidative stress at the young period. We further examined this possibility by selecting 10 genes from the hypothalamic microarray data. RT-PCR analysis revealed that expression of 7 of these 10 genes was significantly changed in the 0.06 mg/kg/day group, compared with controls. Furthermore, RT-PCR analysis showed that mRNA expressions of Dnmt1, Hsp90 and Hsp70 in the hypothalamus were significantly lower in the 0.06 mg/kg/day group than in controls. Methylated DNA-PCR analysis in the hypothalamus revealed that 6 CpG islands were significantly hypomethylated compared with controls. Thus, we speculate that the DNA methylation machinery malfunctions under low levels of oxidative stress, thereby leading to incomplete methylation of specific gene regions. Our data indicate that a low level of oxidative stress appears to correlate positively with transcriptional down-regulation and hypomethylation, but the precise mechanisms underlying these processes are unclear.

Key words: DDT, Hypothalamus, Transcriptional down-regulation, Hypomethylation, Hormesis-like effect, Young rats

INTRODUCTION

Dichlorodiphenyltrichloroethylene (DDT) is a well-known insecticide and has been used since 1940 primarily for the prevention of malaria, yellow fever, and sleeping sickness, and also in agriculture for the control of insects (International Agency for Research on Cancer, 1991). DDT has been studied extensively and shown to have a carcinogenic effect on the liver (International Agency for Research on Cancer) as well as a neurotoxic effect (Eriksson and Talts, 2000). Another characteristic of DDT is the hormetic effect of low doses on liver carcinogenesis (reviewed by Fukushima et al., 2005). Hormesis is defined as a dose-response relationship in which there is biological activation at low doses but inhibition at high doses. Hormesis has been shown by many toxicants that produce benefits, rather than harm, at low-level exposure (Calabrese, 2002). A study on the neurotoxicity of DDT showed that exposure of infant mice to a low dose of DDT changed their susceptibility to other pesticides in adulthood (Eriksson and Talts, 2000). This report indicates that effect of low dose of DDT in the infant period persisted for a long time. Previously, we reported that DDT and its metabolites in the brain were excreted gradually over a
few weeks and were then deposited in the adipose tissue (Tomiyama et al., 2003, 2004). Thus, we speculate that low dose of DDT may have epigenetic effects including DNA methylation in the young brain.

DNA methylation is the major epigenetic modification in the eukaryotic genome and occurs predominantly at the carbon-5 position of cytosine residues when followed by a guanine in so-called CpG dinucleotides (Bestor, 2000). DNA methylation has been studied extensively in development and has long been considered a static process following cell differentiation (Szyf et al., 1991; Monk et al., 1987; Goto et al., 1994; Deng and Szyf, 1999; Bestor, 2000). Consequently, the two main roles of DNA methylation are thought to be maintenance of genome stability (Eden et al., 2003) and control of gene transcription (Bird, 2002). In higher organisms, which have functionally different cell types, DNA methylation is thought to be important in the control of gene expression, and changes in DNA methylation can dramatically affect chromatin structure and genome stability (Yauk et al., 2008). Franco et al. (2008) indicated that reactive oxygen species (ROS) induced by oxidative stress contribute to the multistage process of carcinogenesis by both genetic and epigenetic mechanisms. Shahrzad et al. (2007) indicated that a low level of the methyl group donor S-adenosyl-L-methionine (SAM) induced by hypoxia may cause DNA hypomethylation in solid tumors exposed to ischemia and reperfusion. These results indicate that oxygen consumption and oxidative stress can markedly affect the methylation status of the mammalian genome.

The brain has the ability to consume oxygen in large quantities and, neuronal cells are highly plastic. DNA methylation plays an important role in regulating the induction of synaptic plasticity (Levenson et al., 2006) and memory formation (Miller and Sweatt, 2007) in the young adult central nervous system (CNS). Typically, DNA methyltransferase (Dnmt) expression greatly diminishes when cell differentiation has terminated (Szyf et al., 1991; Goto et al., 1994; Deng and Szyf, 1999), but the young adult CNS has relatively high levels of Dnmt mRNA and enzyme activity (Goto et al., 1994; Brooks et al., 1996). Alterations in DNA methylation induce detrimental disorders of the brain. For example, Dnmt enzyme in the brain might be involved in DNA repair and neurodegeneration (Brooks et al., 1996; Endres et al., 2000; Fan et al., 2001). Also, hypomethylation in the hippocampus due to dietary choline deprivation during pregnancy can decrease memory performance in offspring (Niculescu et al., 2006). Misregulation of DNA methylation is also thought to participate in a number of cognitive disorders, including several autism spectrum disorders (Samaco et al., 2004). Taken together, these findings suggest that Dnmt enzyme and DNA methylation in the brain are crucial processes in young adults as well as during the perinatal period.

Thus, we speculate that oxidative stress at the young period may alter DNA methylation and gene transcription in the brain. We investigated this notion in the present study by administering DDT by gavage to male young Wistar Hannover GALAS (Brilln: WIST@Jcl) rats at doses of 0, 0.006, 0.06, 0.6, 6, or 60 mg/kg/day for a period of 4 weeks. After the treatment, we assessed oxidative stress, gene transcription and DNA methylation in samples of rat brain by using the lipid peroxidation (LPO) assay, microarray analysis, quantitative RT-PCR, methylated RNA (MeD)-PCR analysis, and global DNA methylation assay. In addition, we performed a toxicokinetics analysis using serum samples in order to confirm DDT intake.

**MATERIALS AND METHODS**

**Chemicals and route of exposure**

DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; p,p' DDT, purity > 98%] was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and administered by gavage to male infant rats at doses of 0, 0.006, 0.06, 0.6, 6, and 60 mg/kg/day for a period of 4 weeks. This dosing range was chosen because the dose of 60 mg/kg/day does not show any clinical effect, whereas the dose of 0.06 mg/kg/day is almost equivalent to No-observed adverse effect level (NOAEL) determined by the Japanese Ministry of Environment in 2003.

**Animals housing conditions**

The animals used in the present study were reared, treated, and sacrificed in accordance with provisions for animal welfare at the Institute of Environmental Toxicology, which follows the guidelines on animal experimentation issued by the Japanese Association for Laboratory Animal Science (1987). Thirty-six specific-pathogen-free (SPF) male Wistar Hannover GALAS rats were purchased from the Fuji Breeding Center, Clea Japan, Inc. (Shizuoka, Japan) at 3 weeks of age. All animals were acclimatized to the testing environment for 3 days after receipt, and administration of the test substance was initiated at 3 weeks of age. Each dose group consisted of 6 animals. Animals were housed in wire-mesh stainless steel cages held in an animal room at a temperature of 22 ± 3°C, a humidity of 50% ± 20%, a ventilation cycle of 10 times or more per hour, and illumination time of 12 hours per day. Animals were allowed free access to a cer-
Low-dose effects of DDT in hypothalamus of young rats

tified diet of MF pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) and to filtered tap water that was confirmed to have no contaminants.

Tissue sampling
After 4 weeks of treatment, animals were sacrificed for toxicokinetics analysis, LPO assay, and molecular analyses. At necropsy, blood, liver and brain were collected from each animal. Each brain was divided into three portions (fore, mid, and hind), and then immediately frozen using an ethanol-dry ice bath. These samples were then stored at −80°C until analysis. Serum samples were used for the toxicokinetics analysis. The hypothalamus and hippocampus of the mid-brain portions were used for molecular analyses, and the other brain portions were used for LPO assay.

Toxicokinetics analysis of Dichlorodiphenyldichloroethylene (DDE) and DDT in serum
The concentration of DDE and DDT was measured in serum samples using the DDE/DDT Assay kit (AbraGen LLC, Warminster, PA, USA) according to the protocol provided by the manufacturer.

LPO assay
The LPO content in the brain except for mid-portion was measured by the formation of thiobarbituric acid-reaction substrates (Ohkawa et al., 1979). The frozen samples were homogenized, and the reaction of malondialdehyde with thiobarbituric acid was detected by using a spectrophotometer (Shimazu Corporation, Kyoto, Japan).

Extraction of RNA and DNA
Frozen mid-brain samples were immersed in RNA-Later-Ice (Ambion, Austin, TX, USA) for isolation of the hippocampus and hypothalamus. Total RNA was extracted from one side of each hippocampus and from half of each hypothalamus by using the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). DNA was extracted from the other side of each hippocampus and from the other half of each hypothalamus by using the DNeasy Tissue Kit (QIAGEN K.K.). The concentrations of DNA and RNA were measured by using a spectrophotometer (GeneQuant pro; GE Healthcare Japan, Tokyo, Japan). These samples were stored at −80°C until required.

Microarray analysis
We performed the comprehensive gene expression analysis in the hypothalamus and hippocampus, respectively. Each microarray analysis was performed for the comparison between the 0.06 mg/kg/day and control group with 4 array slides. To adjust for scale differences among the slides, a pooled control RNA sample was prepared from control samples and used as a reference for each sample, according to the method of Yang et al. (2002). Cyanine 3 (Cy3)-labeled reference probes were synthesized from pooled control RNA samples, and cyanine 5 (Cy5)-labeled sample probes were synthesized from RNA derived from the 0.06 mg/kg/day group and control group by using the Fluorescent Direct Label Kit (GE Healthcare Japan) according to the protocol recommended by the manufacturer. Cy3-labeled reference probes and Cy5-labeled sample probes were mixed and hybridized to a Rat Oligo Array (Agilent Technologies, Tokyo, Japan) containing about 20,000 genes according to manufacturer’s recommended protocol. The microarray slides were scanned with a microarray scanner (ScanArray lite, Perkin-Elmer Bioscience Japan, Tokyo, Japan). The criteria for gene expression were set in accordance with Yang et al. (2002).

Quantitative real-time RT-PCR (Quantitative RT-PCR)
Quantitative RT-PCR was performed using hypothalamic samples. Predicted genes were excluded and ten genes having CpG islands in the related region were selected on the basis of microarray analysis data (Table 1). We used quantitative RT-PCR to assess the mRNA expression of these 10 genes. And 5 mRNAs of Dnmt-related genes or stress markers were measured additionally in order to clarify the mechanism. TaqMan probe sets were purchased from Bio Technologies Japan (Tokyo, Japan). The sequences of these probe sets are shown in Table 2. The concentration of RNA was adjusted to 0.01 μg/μl, and a 0.05-μg sample of the RNA was reverse transcribed in a 50-μl reaction using TaqMan RT reagents (Applied Biosystems Japan, Tokyo, Japan). Standards for quantitative real-time PCR analyses were made from PCR products amplified using primer sets corresponding to each gene. In each case, the molar concentration was calculated. Quantitative PCR was performed with diluted standards and sample cDNA using an ABI PRISM 7700 (Applied Biosystems Japan). Data were represented as copy number per RNA content from molecular concentration and Avogadro’s constant.

DNA methylation analysis by quantitative real-time PCR (quantitative MeD-PCR)
Quantitative MeD-PCR was performed using hypothalamic samples. Methylated DNA was isolated by using anti-5-methylcytidine monoclonal antibodies (EURO-
Table 1. Data of microarray

<table>
<thead>
<tr>
<th>Gene name (symbol)</th>
<th>Function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatostatin (Sst)</td>
<td>inhibiting the secretion of several pituitary, pancreatic, and gastrointestinal hormones</td>
<td>0.38</td>
</tr>
<tr>
<td>Galanin (Gal)</td>
<td>binding G-protein coupled receptors GalR1, GalR2, and GalR3; involved in negative regulation of immature thymocyte proliferation; plays a role in regulation of the immune system</td>
<td>0.26</td>
</tr>
<tr>
<td>ADP-ribosylation factor 1 (Arf1)</td>
<td>playing a role in the activation of non-voltage-activated Na(+)-selective current</td>
<td>0.47</td>
</tr>
<tr>
<td>Transthyretin (Ttr)</td>
<td>binding thyroxine (T4) and 3,5,3' triiodothyronine (T3); plays a role in thyroid hormone transport in serum</td>
<td>0.16</td>
</tr>
<tr>
<td>Homeo box, msh-like 1 (Mxl1)</td>
<td>transcription factor; may mediate dental and craniofacial development; mutations in human associated with tooth agenesis and cleft palate</td>
<td>0.49</td>
</tr>
<tr>
<td>insulin-like growth factor 2 (Igf2)</td>
<td>a mitogenic growth factor; may have a role in fetal development</td>
<td>0.39</td>
</tr>
<tr>
<td>Ectodermal-neural cortex 1 (Encl)</td>
<td>mouse homolog binds actin and may play a role in nervous system induction and development</td>
<td>0.49</td>
</tr>
<tr>
<td>Preproenkephalin 1 (Penkl)</td>
<td>neuropeptide hormone; plays a role in the responses to stress and pain perception</td>
<td>0.30</td>
</tr>
<tr>
<td>Gamma-aminobutyric acid B receptor 2 (Gabbr2)</td>
<td>combining with GABA-B1 to form functional GABA-B receptors; inhibits high voltage activated calcium ion channels</td>
<td>0.43</td>
</tr>
<tr>
<td>Galectin-related interferon protein (Grifin)</td>
<td>having similarity to the galectin family but, unlike galectins, does not bind betagalactoside sugars</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Fold change means the average ratio of 0.06 mg/kg/day group vs control group.

GENTEC, Seraing, Belgium) and the METHYL Kit (Diagenode, Liede, Belgium). Briefly, 0.1 µg/µl DNA prepared in 55 µl TE (pH 8.0) was sheared by using a Bioruptor™ (Diagenode) on high, 15 sec ON and 15 sec OFF for 8 min. A 50-µl aliquot was used for immunoprecipitation (IP) with antibody, and 5 µl was used for input DNA (Input). For immunoprecipitation of methylated DNA, the IP sample and 2 µg of antibody was incubated with rotation at 4°C overnight, and then washed with washing buffers (provided in this kit). DNA was extracted from the IP and Input samples by using a PCR Purification Kit (QIAGEN K.K.). Quantitative PCR was performed as described above. CpG islands were identified by using the Methyl Primer Express (Applied Biosystems Japan) and CpGenome with MethPrimer (http://www.uogene.org/methprimer/index1.html) online services. TaqMan probe sets were purchased from Bio Technologies Japan. Sequences of the TaqMan probe sets and locations of the identified CpG islands are shown in Table 3.

Global DNA methylation assay

Global DNA methylation was assessed by using the Methylamp™ Global DNA Methylation Quantification Ultra Kit (Epigentek Group Inc., Brooklyn, NY, USA) according to the protocol provided by the manufacturer.

Statistical analysis

Data are represented as the mean and standard deviation (S.D.). The experimental data were analyzed by Student t-test or Aspin-Welch test following F-test. A p value less than 0.05 or 0.01 was considered significant.

RESULTS

General observations

There were no remarkable clinical signs in any of the animals used for these experiments. The appearance, motility, and growth rate were comparable between the control and treated groups.

Toxicokinetics analysis of DDE and DDT in serum

Fig. 1 shows the ratio of the serum concentrations of DDE and DDT. These concentrations appear to represent a dose-response effect. DDE and DDT were above the limit of detection in 2 samples in the 0.006 mg/kg/day group, 4 samples in the 0.06 mg/kg/day group, and all samples for other doses.
### Table 2. TaqMan probe sets for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession No.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srt</td>
<td>NM_012689</td>
<td>AGAAACGGAAGCTGCCAAGT</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>91</td>
</tr>
<tr>
<td>Gad</td>
<td>NM_013237</td>
<td>GAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>79</td>
</tr>
<tr>
<td>Thr</td>
<td>NM_012681</td>
<td>GAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>68</td>
</tr>
<tr>
<td>Atp5a1</td>
<td>NM_013151</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>85</td>
</tr>
<tr>
<td>Atp5b1</td>
<td>NM_013152</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>104</td>
</tr>
<tr>
<td>Hif2</td>
<td>NM_001086401</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>72</td>
</tr>
<tr>
<td>Enol</td>
<td>NM_017139</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>76</td>
</tr>
<tr>
<td>Gd1</td>
<td>NM_013810</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>90</td>
</tr>
<tr>
<td>Gdh2</td>
<td>NM_013797</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>79</td>
</tr>
<tr>
<td>Gdh1</td>
<td>NM_013797</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>81</td>
</tr>
<tr>
<td>Gdh2</td>
<td>NM_013237</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>88</td>
</tr>
<tr>
<td>Hsp70b2</td>
<td>NM_013237</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>151</td>
</tr>
<tr>
<td>Hsp70a1</td>
<td>NM_001086401</td>
<td>AGAAAGCGGAGGAGGGACTC</td>
<td>CTGCAAGAGGCACACTCT</td>
<td>84</td>
</tr>
<tr>
<td>Gene symbol</td>
<td>Location</td>
<td>Sequence</td>
<td>Amplicon size (bp)</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>----------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>Sst</td>
<td>(-)152- (+)418</td>
<td>TCGTAAAGCACGCTGGTGAGAT CTCTCCACGGTCTCCCCCTTT CGCCTCCTTGGCTGACGTCAGA</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Gal</td>
<td>(-)134- (+)164</td>
<td>TAAACAGCTAGGCGCGGT GAGGACAGGCAGGCTCACC</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Arf1</td>
<td>(-)334- (+)655</td>
<td>GCTCTCTGAGCCGAAGCT CCCACTGACAGACCTCA</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Ttr</td>
<td>(-)2882- (-)2774</td>
<td>GCCCTCTGTCCAAACATGCTT GATGTAAGAAAGATGAGTGCTT</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>Mshl</td>
<td>(-)561- (+)64</td>
<td>AGTCGTCTCCACCGTCTT GATCAGCGGACCATAAGAGA</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Igf2</td>
<td>(-)199- (-)1152</td>
<td>CGTCTCCTGCTCTCGAAGT CAGAGGGAGGCTAGGAAA</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Encl</td>
<td>(-)1469- (-)794</td>
<td>CTGAGCTCCTCAATAATCCACCA CTGGTACGGGAGGCGTCTCA ACAAGCCCAAGAAAGCGCGATTCG</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Penkl</td>
<td>(-)613- (-)18</td>
<td>TGGCGAGCTCCCTCGTGT GACACCGAGCAGACATTGAGGAA</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Gabbr2</td>
<td>(-)643- (+)358</td>
<td>TCGGTAGGATCTCTGCTAGA CCGGTGACGAGATAGTCTGA AGCAGGTTGGCTCAGGTTGAATTT</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Grfin</td>
<td>(+)427- (+)563</td>
<td>GAGGTAGGCGCGTTCAGTCA CAGACATGGGAGGCGGATGTC TCCTGACCCGACGACACTCTCCAC</td>
<td>105</td>
<td></td>
</tr>
</tbody>
</table>

Location indicates the position of analyzed-CpG island from the transcription start site.
Low-dose effects of DDT in hypothalamus of young rats

**LPO assay in the cerebrum**

Fig. 2 shows the results for the LPO assay. LPO levels decreased significantly in the 0.06 mg/kg/day group and in the 0.6 mg/kg/day group compared with controls. This reduction in LPO in the brain may be hormesis-like, because the dose-response curve was U-shaped, and a reduction in LPO is beneficial in many cases. A dose of 60 mg/kg/day is known to induce hepatocarcinogenesis (Harada et al., 2003). In the present study the LPO contents significantly increased in the livers at this dose compared with control livers (data not shown) and were more than 2 times higher in the cerebroms of control rats than in the livers of those (data not shown).

**Microarray analysis**

The LPO assay revealed a significant decrease in the brain LPO level in the 0.06 mg/kg/day group compared with controls. Since oxidative stress may be a modulator of epigenetics and gene transcription, we compared gene expression in the 0.06 mg/kg/day group with that in the control group by microarray analysis. The results of the microarray analysis revealed a change in the expression of 40 genes (39 down-regulated genes and 1 up-regulated gene) in the hypothalamus, whereas gene expression was unaltered in the hippocampus. This result suggests that the hypothalamus is more susceptible to low-lev-

![Fig. 1. Toxicokinetic analysis of the concentrations of DDE and DDT in the serum. DDE and DDT were detected in two samples in the 0.006 mg/kg/day group, four samples in the 0.06 mg/kg/day group, and in all samples for all other doses. Changes in the concentrations of DDE and DDT in serum appear to represent a dose-response reaction. These data were normalized by considering values of the untreated group to be a background.]

![Fig. 2. LPO contents in the cerebrum. LPO levels decreased significantly in the 0.06 mg/kg/day group and in the 0.6 mg/kg/day group compared with controls. This reduction in LPO in the cerebrum may be hormesis, because the dose-response curve was U-shaped. Values are represented as mean and SD. * P < 0.05, ** P < 0.01, when compared with the control group, as demonstrated by Student t-test or Aspin-Welch test following F-test.]

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Table 4. Expression levels of mRNAs in the hypothalamus (copies/μg RNA)

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>Sst ($\times 10^3$)</th>
<th>Gal ($\times 10^3$)</th>
<th>Arfl ($\times 10^3$)</th>
<th>Trt ($\times 10^3$)</th>
<th>Msx1 ($\times 10^3$)</th>
<th>Enc1 ($\times 10^3$)</th>
<th>Igf2 ($\times 10^3$)</th>
<th>Penk1 ($\times 10^3$)</th>
<th>Gabbr2 ($\times 10^3$)</th>
<th>Grifin ($\times 10^3$)</th>
<th>Dnmt1 ($\times 10^3$)</th>
<th>Dnmt3a ($\times 10^3$)</th>
<th>Dnmt3b ($\times 10^3$)</th>
<th>Hsp90 ($\times 10^3$)</th>
<th>Hsp70 ($\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.13 ± 0.93</td>
<td>4.52 ± 0.21</td>
<td>1.10 ± 0.28</td>
<td>1.98 ± 0.36</td>
<td>4.96 ± 0.28</td>
<td>1.10 ± 0.28</td>
<td>1.67 ± 0.28</td>
<td>4.58 ± 0.28</td>
<td>1.60 ± 0.28</td>
<td>2.50 ± 0.28</td>
<td>0.33 ± 0.28</td>
<td>2.61 ± 0.28</td>
<td>3.17 ± 0.28</td>
<td>0.81 ± 0.28</td>
<td>8.62 ± 0.28</td>
</tr>
<tr>
<td>0.006</td>
<td>1.22* ± 0.34**</td>
<td>0.79* ± 0.33**</td>
<td>0.79* ± 0.35</td>
<td>0.70 ± 0.35</td>
<td>3.23 ± 0.35</td>
<td>1.00 ± 0.35</td>
<td>0.71 ± 0.35</td>
<td>1.82 ± 0.35</td>
<td>0.71 ± 0.35</td>
<td>2.18 ± 0.35</td>
<td>0.59 ± 0.35</td>
<td>2.11 ± 0.35</td>
<td>2.82 ± 0.35</td>
<td>0.53 ± 0.35</td>
<td>6.13 ± 0.35</td>
</tr>
<tr>
<td>0.06</td>
<td>0.87** ± 0.34**</td>
<td>0.68* ± 0.31</td>
<td>0.28 ± 0.22</td>
<td>0.68* ± 0.23</td>
<td>0.41** ± 0.23</td>
<td>0.63 ± 0.23</td>
<td>0.69 ± 0.23</td>
<td>1.47* ± 0.23</td>
<td>0.78 ± 0.23</td>
<td>0.72 ± 0.23</td>
<td>1.51* ± 0.23</td>
<td>2.27 ± 0.23</td>
<td>3.48* ± 0.23</td>
<td>0.78* ± 0.23</td>
<td>4.83* ± 0.23</td>
</tr>
<tr>
<td>0.6</td>
<td>1.64 ± 0.34</td>
<td>2.05 ± 0.31</td>
<td>2.22 ± 0.22</td>
<td>4.59 ± 0.22</td>
<td>1.20 ± 0.22</td>
<td>0.91 ± 0.22</td>
<td>3.07 ± 0.22</td>
<td>3.08 ± 0.22</td>
<td>0.66* ± 0.22</td>
<td>2.31 ± 0.22</td>
<td>3.07 ± 0.22</td>
<td>0.90 ± 0.22</td>
<td>7.10 ± 0.22</td>
<td>1.54 ± 0.22</td>
<td>3.62 ± 0.22</td>
</tr>
<tr>
<td>6</td>
<td>2.65 ± 3.82</td>
<td>0.87* ± 3.82</td>
<td>1.15 ± 0.82</td>
<td>0.99 ± 0.82</td>
<td>2.83 ± 0.82</td>
<td>1.82 ± 0.82</td>
<td>0.75 ± 0.82</td>
<td>2.57 ± 0.82</td>
<td>2.67 ± 0.82</td>
<td>0.54 ± 0.82</td>
<td>2.49 ± 0.82</td>
<td>3.82 ± 0.82</td>
<td>1.46 ± 0.82</td>
<td>6.38 ± 0.82</td>
<td>1.09 ± 0.82</td>
</tr>
<tr>
<td>60</td>
<td>1.17 ± 3.82</td>
<td>1.29 ± 0.86</td>
<td>0.86 ± 0.97</td>
<td>0.92 ± 0.92</td>
<td>2.63 ± 0.92</td>
<td>1.00 ± 0.92</td>
<td>2.18 ± 0.92</td>
<td>3.95 ± 0.92</td>
<td>1.13** ± 0.92</td>
<td>2.10 ± 0.92</td>
<td>2.63 ± 0.92</td>
<td>0.89 ± 0.92</td>
<td>6.06 ± 0.92</td>
<td>1.00 ± 0.92</td>
<td>3.00 ± 0.92</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D.
Significantly different from control: *, p ≤ 0.05; **, p ≤ 0.01.
el oxidative stress at the infant period. To further investigate this notion, we selected 10 genes contained CpG islands in 5' flanking or coding regions based on microarray data for the hypothalamus. Data of these 10 genes that are described in Table 1.

Quantitative RT-PCR

The results of quantitative RT-PCR for the hypothalamus are described in Table 4. The expression of 7 of the 10 selected genes was significantly lower in the 0.06 mg/kg/day group than in controls. These genes include Sst, Gal, Arf1, Mx1, Enc1, Penk1, and Gabbr2. For the remaining 3 genes, Tlr, Igf2 and Griffin, there were no significant changes in mRNA expression in the 0.06 mg/kg/day group because of the variability of the individual samples. The mRNA expressions of Sst and Gal was significantly decreased even in the 0.006 mg/kg/day group, whereas the expression of Gal was also significantly decreased in the 6 mg/kg/day group. It is interesting that Gal mRNA was down-regulated to less than one-tenth of the level of expression in the 0.06 mg/kg/day group compared with controls (0.33 vs. 4.52). Expression of Griffin was significantly increased in the 0.6 mg/kg/day group and in the 60 mg/kg/day group compared with controls.

To clarify the mechanism in the hypothalamus, we performed further quantitative RT-PCR analyses for the mRNA expression of Dnmt-related genes, including Dnmts (1, 3a and b) and stress markers including, heat shock proteins (Hsp90 and Hsp70). The results are described in Table 4. In the hypothalamus, the mRNA expressions of Dnmt1, Hsp90 and Hsp70 were significantly decreased in the 0.06 mg/kg/day group compared with controls, and mRNA expression amounts of Dnmt3a were approximately at the same level as those of Dnmt1 in the control animals.

Quantitative MeD-PCR

Quantitative MeD-PCR was performed by using DNA extracted from the hypothalamus from the 0.006 mg/kg/day, 0.06 mg/kg/day, 60 mg/kg/day and the control groups. The results of the MeD-PCR analysis are summarized in Table 5.

The CpG islands analyzed in this study are listed in Table 3. We found that 6 CpG islands were hypomethylated in the 0.06 mg/kg/day group. There is 1 CpG island in the 5' flanking or coding region for Sst, Gal or Arf1. In each case, the CpG island is located on the transcription start site. Of the two CpG islands in the 5' flanking or coding region for Tlr, we analyzed the island in the 5' flanking region. There are 6 CpG islands in the 5' flanking or coding region for Mx-1, and the island on the transcription start site was analyzed. There is 1 CpG island within exon 3 for Griffin.

There were no significant changes in the methylation status for the CpG islands analyzed in the regions associated with the remaining 4 genes. There are 4 CpG islands in the 5' flanking or coding region for Igf2, and the island on the transcription start site was analyzed. There are 4 CpG islands in the 5' flanking or coding region for Enc1, and the island in the 5' flanking region was analyzed. There are 3 CpG islands in the 5' flanking or coding region for Penk1, and the island near the transcription start site was analyzed. There is 1 CpG island in the 5' flanking or coding region for Gabbr2, and the island on the transcription start site was analyzed.

The islands on the transcription start site for Gal and that within exon 3 for Griffin were significantly hypomethylated in the 60 mg/kg/day group. By comparison, there was no significant difference in methylation status in the 0.006 mg/kg/day group, compared with controls.

Global DNA methylation assay

The global DNA methylation assay was performed using hypothalamic DNA samples from the 0.06 mg/kg/day, 60 mg/kg/day and the control groups. The results of the global methylation assay are summarized in Table 5. There was no significant difference in methylation in the 0.06 and 60 mg/kg/day groups, compared with controls.

DISCUSSION

Based on our speculation that DNA methylation has a strong relationship with oxidative stress in the infant brain, the present study was performed on male young rats receiving DDT at doses of 0, 0.006, 0.06, 0.6, 6 or 60 mg/kg/day for 4 weeks. LPO analysis revealed that less oxidative stress was found in the 0.06 mg/kg/day group. This result led us to perform microarray analyses, and we compared between the control and 0.06 mg/kg/day groups about hypothalamic and hippocampus samples, respectively. Since the hypothalamus was found to be more susceptible to low-level oxidative stress, we conducted further molecular analyses of the hypothalamus samples. LPO levels in the liver increased significantly in the 60 mg/kg/day group compared with controls (data not shown), but significant increases in LPO were not observed in the brain differently from the liver. So, the effect of less oxidative stress was investigated as a result.

We observed a significant decrease in the cerebral LPO level in the 0.06 and 0.6 mg/kg/day groups. This reduction in the LPO level may reflect a hormesis-like effect, although the detailed mechanism underlying this response
Table 5. Genomic methylation status in the hypothalamus (%)

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>Global methylation</th>
<th>CpG island</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sst</td>
<td>Gal</td>
</tr>
<tr>
<td>0</td>
<td>2.08</td>
<td>±</td>
</tr>
<tr>
<td>0.20</td>
<td>±</td>
<td>5.61</td>
</tr>
<tr>
<td>0.006</td>
<td>N.D.</td>
<td>14.37</td>
</tr>
<tr>
<td></td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>0.06</td>
<td>2.05</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>60</td>
<td>2.01</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>0.21</td>
<td>4.77</td>
<td>±</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D..
Significantly different from control: * p ≤ 0.05; ** p ≤ 0.01.
N.D.: Not determined.
is obscure. Kinoshita et al. (2003) reported that low-dose treatment with phenobarbital, which is a nongenotoxic carcinogen like DDT (Harada et al., 2003), induced a hormetic effect in hepatocarcinogenesis via detoxification and enzyme induction.

Of the 7 genes with altered expression in the 0.06 mg/kg/day group, 3 are estrogen-related. Gal, one of the neuropeptide genes, is involved in the neuroendocrine-induced control of the hypothalamic-pituitary-gonadal axis, and its gene expression is regulated via the estrogen receptor (ER)-α (Howard et al., 1997; Shen et al., 1998, 1999). Sst is also a neuropeptide gene and is reportedly expressed in the rat hypothalamus at the time of estrogen-dependent development (Orikasa et al., 2007). Penk1 is a neuropeptide hormone gene expressed in the limbic-hypothalamic circuit and is reported to be up-regulated by estrogen (Holland et al., 1998).

It is known that p, p’ DDT has estrogenic activity (Soto et al., 1995) despite its relatively weaker binding to the ER than other xenoestrogens (Bolger et al., 1998). Di Lorenzo et al. (2002) reported that a high dose of p, p’ DDT (5 mg/kg) induced ER transcriptional activation one-fifth that induced by estrogen (50 μg/kg) and that estrogen pre-treatment induced low level of this activation (one-fifth of 50 μg/kg estrogen) by the low dose of DDT (50 μg/kg) in the male mouse brain. Monje et al. (2007) reported that control female and control male levels of serum estrogen were similar at postnatal day (PD) 21. These data indicate that a low dose of DDT can bind ER and induce the low level of transcriptional activity in the infant cerebrum. Taken together, these results suggest that the down-regulation of these 3 genes in the infant male hypothalamus may be related to the anti-estrogen effect in appearance by DDT binding to ER in substitution for estrogen. In addition, the hypothalamic down-regulation of Sst mRNA observed at the lowest DTT dose is quite interesting, because the mRNA expression of Sst in the sexually dimorphic nucleus of the preoptic area is crucial for sex differentiation in the infant brain (Orikasa et al., 2007).

Although the mechanisms underlying the transcriptional down-regulation are still unclear, it is interesting that Gal mRNA was down-regulated by DDT to less than one-tenth at the hormesis-like dose. As the cerebrum maintains homeostasis under a high level of oxidative stress, it is possible that silencing activity via unidentified factors might be enhanced by a low level of oxidative stress. The influence of oxidative stress on the ER seems to be important. We also observed significant down-regulation of Hsp90 at this dose. Hsp90 is a molecular chaperone and known to be a highly conserved and essential stress protein that is expressed in all eukaryotic cells (Pratt and Toft, 2003). Hsp90 is reported to function in maintaining the ER in a high-affinity hormone-binding conformation; hormone binding by the ER was reduced at a low level of geldanamycin, which is an inhibitor of Hsp90 (Fliss et al., 2000; Ruden et al., 2005). Furthermore, Hsp90, an epigenetic capacitor, affects morphological development when modulated by stress by altering chromatin remodeling (Rutherford and Lindquist, 1998; Sollars et al., 2003). Thus, it is possible that oxidative stress alters the function of the ER by modulating chromatin structure via Hsp90.

Unfortunately, we could not measure LPO level in the specific brain position for the limitation of number of animals used in this study. However, we revealed that hypothalamic down-regulations of Hsp90, Hsp70 and Penk1 were observed in 0.06 mg/kg/day group. As mentioned above, Hsp90 is essential stress protein (Pratt and Toft, 2003) and Hsp70 is known as a stress marker in the brain (Goldbaum and Richter-Landsberg, 2004; Xing et al., 2008). Furthermore, Penk1 mRNA is reported to be up-regulated by oxidative stress. Thus, we think that oxidative stress was reduced in the hypothalamus of 0.06 mg/kg/day group.

DNA methylation in mammals is catalyzed by three Dnmts, Dnmt1, Dnmt3a, and Dnmt3b, which have different capacities for maintenance (Dnmt1) and de novo methylation (Dnmt3a and Dnmt3b) (Leonhardt et al., 1992; Okano et al., 1999; Robertson et al., 1999). Dnmt3a and Dnmt3b are two major de novo Dnmts that are essential for embryonic development (Turek-Plewa and Jagodziński, 2005; Oka et al., 2006), and it is thought that these Dnmt expression diminishes greatly in most cell types once terminal differentiation has occurred (Szyf et al., 1991; Goto et al., 1994; Deng and Szyf, 1999). Our data revealed that mRNA expression amounts of Dnmt3a involved in de novo DNA methyltransferase were approximately at the same level as those of Dnmt1 in the control animals. In the rat brain, proliferation, migration, and setting take place mainly before PD 22, whereas differentiation, synaptogenesis, and myelination mainly occur between PD 22 and 60 (Kaufmann and Grüters, 2006). These findings indicate that the hypothalamus in rats at the age used in the present study may be in the process of epigenetic development.

From the report of Eriksson and Talts (2000), we have thought of a possibility that acetylcholine might become hard to accumulate in the synapse by the pretreatment of DDT. This report is as follows: Mice were given a single dose of DDT (0.5 mg/kg) when they were 10 days old, and then at the adult age of 5 months they were given paraoxon (1.4 mg/kg), an organophosphorus compound,
four times at 48-hr intervals (giving 45% AChE inhibition). Twenty-four hours after the last injection of paraoxon, no abnormalities were observed in the animals pretreated with DDT. Our data revealed that the mRNA of galanin involved in inhibition of acetylcholine release (Fisone et al., 1987) was significantly down-regulated and that the Cpg island on the transcription start site was significantly hypomethylated in the 0.06 mg/kg/day group. Previously, we reported that DDT and its metabolites in the brain were excreted gradually over a few weeks and were then deposited in the adipose tissue (Tomiyama et al., 2003, 2004). Taken together, we speculate that the effect of hypomethylation induced by a low dose of DDT in the young period may appear after direct transcriptional down-regulation by DDT has disappeared. Unexpectedly, hypomethylation and transcriptional down-regulation were observed for several genes in this study. In general, hypomethylation is known to cause transcriptional activation, so hypomethylation and transcriptional down-regulation in this study seemed to occur simultaneously without interaction.

Methylation analysis revealed that 6 CpG islands were significantly hypomethylated in the 0.06 mg/kg/day group. Furthermore, we observed significant down-regulation of Dnmt1 and a tendency for down-regulation of the other genes of Dnmt family in this group. Dnmt3a and b are de novo DNA methyltransferase and Dnmt3b was reduced markedly but not significantly because of the variability of the individual samples. This variability is obscure, but Dnmt3b seems to be in the fluctuation process of gene expression in this period. We speculate that the DNA methylation machinery malfunctions under low levels of oxidative stress, thereby leading to incomplete methylation of specific gene regions as a result. Therefore, the mild but significant hypomethylation in the specific gene regions observed at the hormesis-like dose are suggestive of the importance of oxidative stress during development.

Abele (2002) described oxygen as a double-edged sword in that it provided a fuel that would allow the evolution of complex organisms with high energy demands, but also represented a new source of toxins. Furthermore, Hedges et al. (2004) reported that oxygen concentration in the atmosphere may have relationship to evolution such as variation of cell types. Interestingly, Bloomfield and Pears (2003) reported about Dictyostelium being social amoeba that the single cells aggregated to form a multicellular organism by superoxide signaling. Therefore, we hypothesize that oxidative stress is essential for epigenetic modification including DNA methylation in the higher organisms having functionally different cell types. In this paper, we revealed that Dnmt1 was significantly down-regulated in the hypothalamus of 0.06 mg/kg/day group. It is well known that the brain has the ability to consume oxygen in large quantities and it was reported that the young adult CNS has relatively high levels of Dnmt mRNA and enzyme activity (Goto et al., 1994; Brooks et al., 1996). Although detail mechanisms are obscure, these indicate a possible relationship between oxidative stress and transcriptional activity of Dnmt1.

In conclusion, we revealed that a low dose of DDT (0.06 mg/kg/day) that is considered to be hormetic induced transcriptional down-regulation and DNA hypomethylation in the young hypothalamus. Areas of research that need to be clarified in future investigations include the duration of hypomethylation, the mechanism of transcriptional down-regulation, and the mechanism of hypomethylation. The brain has a high level of plasticity in the regulation of dynamic changes in DNA methylation (Miller and Swett, 2007), and the DNA methylation pattern in the brain changes dynamically throughout life (Siegmund et al., 2007). Therefore, we are planning further long-term experiments to examine these issues.

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