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Expression alterations of genes on both neuronal and glial development in rats after developmental exposure to 6-propyl-2-thiouracil

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HIGHLIGHTS

- Target gene profiles of DNT were examined using rat hypothyroidism model.
- Region-specific global gene expression profiling was conducted in brain regions.
- Ephrin signaling and glutamate transmission were commonly affected in the brain.
- Cerebral cortex and corpus callosum disrupted glial development and myelination.
- Hippocampal dentate gyrus disrupted COX2-mediated synapse function on neurogenesis.

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ABSTRACT

The present study was performed to determine target gene profiles associated with pathological mechanisms of developmental neurotoxicity. For this purpose, we selected a rat developmental hypothyroidism model because thyroid hormones play an essential role in both neuronal and glial development. Region-specific global gene expression analysis was performed at postnatal day (PND) 21 on four brain regions representing different structures and functions, i.e., the cerebral cortex, corpus callosum, dentate gyrus and cerebellar vermis of rats exposed to 6-propyl-2-thiouracil in the drinking water at 3 and 10 ppm from gestational day 6 to PND 21. Expression changes of gene clusters of neuron differentiation and development, cell migration, synaptic function, and axonogenesis were detected in all four regions. Characteristically, gene expression profiles suggestive of affection of ephrin signaling and glutamate transmission were obtained in multiple brain regions. Gene clusters suggestive of suppression of myelination and glial development were specifically detected in the corpus callosum and cerebral cortex. Immunohistochemically, immature astrocytes immunoreactive for vimentin and glial fibrillary acidic protein were increased, and oligodendrocytes immunoreactive for oligodendrocyte lineage transcription factor 2 were decreased in the corpus callosum. Immunoreactive intensity of myelin basic protein was also decreased in the corpus callosum and cerebral cortex. The hippocampal dentate gyrus showed downregulation of *Ptgs2*, which is related to synaptic activity and neurogenesis, as well as a decrease of cyclooxygenase-2-immunoreactive granule cells, suggesting an impaired synaptic function related to neurogenesis. These results suggest that multifocal brain region-specific microarray analysis can determine the affection of neuronal or glial development.

Abbreviations: COX2, cyclooxygenase 2; C_T, threshold cycle; DNT, developmental neurotoxicity; GD, gestational day; Gfap, glial fibrillary acidic protein; Mbp, myelin basic protein; Olig2, oligodendrocyte lineage transcription factor 2; PND, postnatal day; PTU, 6-propyl-2-thiouracil; RSA, retrosplenial agranular cortex; RSGb, retrosplenial granular b cortex; RT-PCR, reverse transcription-polymerase chain reaction; SBH, subcortical band heterotopia; SGZ, subgranular zone; THs, thyroid hormones; TSH, thyroid-stimulating hormone; T_4, thyroxine; T_3, triiodothyronine.

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1. Introduction

Developmental neurotoxicity (DNT) testing is a field that is in need of a rapid screening system because testing one chemical with the current guidelines is time consuming and requires hundreds of animals to conduct one study (OECD 2007; US EPA, 1991). Therefore, a new, more efficient screening system for the testing of untested chemicals with the use of a minimal number of animals may be established. For this purpose, application of global approaches on the effect of DNT concurrently in different brain regions may be essential for providing a comprehensive understanding of the mechanism of DNT. One approach may be to establish a set of DNT markers based on the understanding of the pathological mechanisms of the disruption of brain development specific to and commonly across the inherent brain structures and functions.

Thyroid hormones (THs) play a crucial role in normal brain development during fetal and neonatal periods. They have many effects relating to neuronal proliferation, cell migration, neuritogenesis, and synaptogenesis (Bernal and Nunez, 1995). Experimentally, rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) and methimazole show aberrant brain growth (Lavado-Autric et al., 2003), resulting in an impaired neuronal migration to form subcortical band heterotopia (SBH) in the corpus callosum (Goodman and Gilbert, 2007; Shibutani et al., 2009), as well as white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation (Schoonover et al., 2004). In addition, developmental PTU administration in rats affects motor activity, function in learning and memory, and auditory function of offspring (Axelstad et al., 2008; Kobayashi et al., 2005). Therefore, rat developmental hypothyroidism could provide a reasonable model for the evaluation of DNT.

Approaches of toxicogenomics, transcriptomics, and proteomics have recently been applied in many organs in toxicity studies using rodents (Heijne et al., 2005). Gene expression profiling using microarrays provides a global view of molecular changes associated with the mechanisms underlying diseases or toxicity development following chemical exposure. The central nervous system has an anatomically elaborate architecture with region-specific differences in the distribution of neuronal and glial cell populations. To examine DNT, function and differentiation potentials of both neuronal and glial cell populations should be analyzed. We have recently established a high-throughput tissue sampling method that enables us to identify molecular profiles of RNAs and polypeptides simultaneously in anatomically different brain regions by applying a whole brain fixation method using methacarn fixative (Akane et al., 2013).

Previous studies using microarray analysis have revealed gene expression profiles of many brain regions in rats suffering from developmental hypothyroidism (Dong et al., 2005; Kobayashi et al., 2009; Royland et al., 2008). However, disruption mechanisms related to developmental hypothyroidism specific to and commonly across the inherent brain structures and functions have not been elucidated until now.

The present study was performed to determine target gene profiles of the pathological mechanism of DNT in the model of developmental hypothyroidism by applying region-specific global gene expression analysis in combination with a high-throughput tissue sampling method that we have established in representative brain regions of rat offspring. Subsequently, we performed comprehensive gene expression profiling on the different brain regions representing different structure and function. We selected the cerebral cortex and hippocampal dentate gyrus uniquely continuing neurogenesis throughout the life (Supplementary Fig. 1; Hodge et al., 2008; Kempermann et al., 2004; Knoth et al., 2010), both structures being parts of the limbic system. We also selected the corpus callosum representing cerebral white matter and cerebellar vermis representing cerebellum. Based on the obtained expression profiles, cellular localization of molecules showing altered expression were immunohistochemically examined to confirm changes in cellular phenotypes.

2. Materials and methods

2.1. Chemicals and animals

PTU (CAS No. 51-52-5) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Pregnant Crl: CD®(SD) rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestational day (GD) 2 (the appearance of vaginal plugs was designated as GD 0). Pregnant rats were housed individually with their offspring in plastic cages with wood chip bedding until postnatal day (PND) 21 (where PND 0 is the day of delivery). Animals were maintained in an air-conditioned animal room (temperature: 23 ± 2 °C, relative humidity: 55 ± 15%) with a 12-h light/dark cycle. Pregnant rats were allowed free access to a pelleted basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) throughout the experimental period and tap water until the start of exposure to PTU. Offspring were housed with three or four animals per cage and were provided ad libitum with the CRF-1 basal diet and tap water from PND 21 onwards.

2.2. Experimental design

Pregnant rats were randomly divided into four groups of 12 dams each and treated with 0, 1, 3, or 10 ppm of PTU in the drinking water from GD 6 to PND 21. Based on our previous studies that have shown apparent aberrations in neuronal development in the hippocampal structure in offspring (Shibutani et al., 2009; Saegusa et al., 2010), the highest dose was determined as abovementioned. Body weights and food and water consumption of dams were measured throughout the experimental period. After delivery, the litters were culled randomly on PND 4 to preserve six male and two female pups per litter. If dams had fewer than six male pups, more female pups were included to maintain a total of eight pups per litter. The offspring were weighed at 3- or 4-day intervals.

On PND 21, which is the prepubertal stage, 31–36 male and 11–17 female offspring per group (1–3 males and 1–2 females per dam) and all dams were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia, and subjected to necropsy. The remaining animals were maintained until PND 77, and body weights and food consumption were measured weekly. On PND 77, 27–36 male and 10–17 female offspring per group (1–3 males and 1–2 females per dam) were euthanized and subjected to adult-stage necropsy. Body and brain weights were determined for all animals on both necropsy days.

Dosing period and number of animals used for histopathological analysis of offspring were in accordance with the OECD test guideline 426 for DNT study in rats (OECD 2007). PND 21 was selected for examination of the effect on neurodevelopment at the end of developmental exposure to PTU. In addition, formation of the hippocampal dentate gyrus is completed and postnatal neurogenesis begins until PND 21 in rats (Bayer, 1980), suggesting that PND 21 is the theoretically reasonable time point to measure the developmental exposure effect on the process of postnatal neurogenesis. On the other hand, PND 77 is the time point apparently after the sexual maturation in rodents. By observation differentially these two time points, it is expected to evaluate the reversibility of aberrations appeared in the developing nervous system.

All animal experiments were conducted in accordance with the "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006), and the protocol was approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

2.3. Serum hormone analysis

Blood samples collected from all dams and 11–12 male offspring per group (one male per dam) on PND 21 were centrifuged at 1600 × *g* for 10 min, and the serum was stored at −80 °C. Serum concentrations of thyroid-stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) were measured by a rodent enzyme-linked immunoabsorbent assay test kit (Endocrine Technologies, Newark, CA, USA) with a microplate reader (FLUOstar Optima, BMG Labtechnologies, Durham, NC, USA).

2.4. Tissue sampling of specific brain regions for gene expression analysis

For microarray and reverse-transcription polymerase chain reaction (RT-PCR) analyses, whole brains were removed from euthanized male offspring at PND 21 (n = 6/group) and fixed with a methacarn solution for 5 h at 4 °C with agitation (Akane et al., 2013). Fixed brains were dehydrated in ethanol and subjected to tissue sampling of the hippocampal dentate gyrus, parietal cerebral cortex, corpus callosum and cerebellar vermis using the brain-matrix cast (Muromachi Kikai Co., Ltd., Tokyo, Japan) and punch-biopsy devices (Kai Industries Co., Ltd., Gifu, Japan) according to the previously described method (Supplementary Fig. 1; Akane et al., 2013).