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Original Article

Ameliorating effect of postweaning exposure to antioxidant on disruption of hippocampal neurogenesis induced by developmental hypothyroidism in rats

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ABSTRACT — Developmental hypothyroidism as a model of autism spectrum disorders disrupts hippocampal neurogenesis through the adult stage. The present study investigated the ameliorating effect of postweaning exposure to antioxidant on the hypothyroidism-induced disruptive neurogenesis. Mated female Sprague-Dawley rats were treated with 0 or 10 ppm 6-propyl-2-thiouracil (PTU) as an anti-thyroid agent in drinking water from gestational day 6 to postnatal day (PND) 21 on weaning. PTU-exposed male offspring were fed either basal diet, diet containing α-glycosyl isoquercitrin (AGIQ) at 5,000 ppm or α-lipoic acid (ALA) at 1,000 ppm as an antioxidant from PND 21 to PND 77. PTU-exposure decreased DCX^+ and $NeuN^+$ granule cell lineage subpopulations, synaptic plasticity-related FOS^+ granule cells, and hilar $PVALB^+$ and $GAD67^+$ GABAergic interneurons, increased hilar SST^+ and $CALB2^+$ interneurons, and upregulated *Gria3*, *Otx2*, and antioxidant enzyme genes in the dentate gyrus on PND 77. These results suggest disruption of neurogenesis remained in relation with increase of oxidative stress and compensatory responses to the disruption at the adult stage. AGIQ recovered expression of some antioxidant enzyme genes and was effective for restoration of $NeuN^+$ postmitotic granule cells and $PVALB^+$ and SST^+ interneurons. In contrast, ALA was effective for restoration of all interneuron subpopulations, as well as postmitotic granule cells, and upregulated *Grin2a* that may play a role for the restoration. Both antioxidants recovered expression of *Otx2* and AGIQ-alone recovered *Gria3*, suggesting a reversal of disruptive neurogenesis by compensatory responses. Thus, postweaning antioxidant exposure may be effective for ameliorating developmental hypothyroidism-induced disruptive neurogenesis by restoring the function of regulatory system.

Key words: α-Glycosyl isoquercitrin (AGIQ), α-Lipoic acid (ALA), Antioxidant, Developmental hypothyroidism, Hippocampal neurogenesis, Oxidative stress

INTRODUCTION

In the hippocampal formation of the brain, the subgranular zone (SGZ) of the dentate gyrus retains the capacity to produce new neurons throughout adult life (Hodge *et al.*, 2008; Sibbe and Kulik, 2017; Fig. 1). Adult neurogenesis in the SGZ is a highly regulated process starting from type-1 neural stem cells, which produce proliferative progenitor cells in the order of type-2a, type-2b, and type-3 (Hodge *et al.*, 2008; Sibbe and Kulik, 2017). Type-3 pro-

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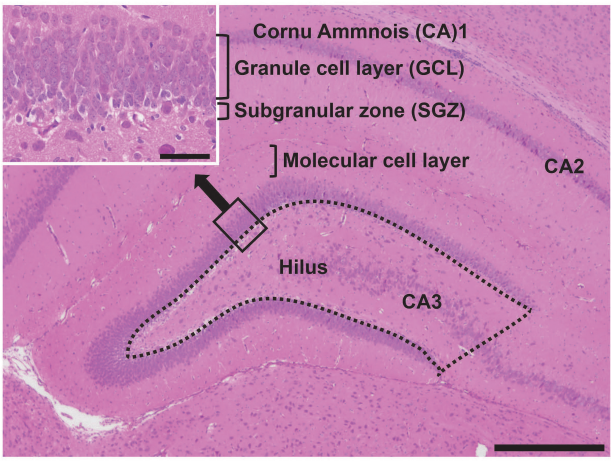

Fig. 1. Overview of the hippocampal formation of a male rat stained with hematoxylin and eosin. The numbers of cells in the hilus of the dentate gyrus (as enclosed by the dotted line) displaying immunoreactivity for parvalbumin (PVALB), somatostatin (SST), calretinin (CALB2), reelin (RELN), glutamic acid decarboxylase 67 (GAD67), glial fibrillary acidic protein (GFAP), and ionized calcium-binding adaptor molecule 1 (IBA1) were counted and normalized for the unit area. Only small-sized cells with positive immunoreactivity for these antigens were counted, and larger Cornu Ammonis (CA) 3 neurons were excluded. (Inset) Higher magnification of the granule cell layer (GCL) and subgranular zone (SGZ). The distribution of immunoreactive cells for GFAP, sex determining region Y (SRY)-box 2 (SOX2), T box brain 2 (TBR2), doublecortin (DCX), neuronal nuclei (NeuN), Fos proto-oncogene, AP-1 transcription factor subunit (FOS), activity-regulated cytoskeleton-associated protein (ARC), cyclooxygenase 2 (COX2), and proliferating cells or apoptotic cells were measured in the SGZ and/or GCL. Magnification × 40; bar 400 µm. (Inset) Higher magnification of the granule cell layer. Magnification × 400; bar 50 µm.

genitor cells differentiate into post-mitotic immature granule cells and finally into mature granule cells that populate the granule cell layer (GCL) (Hodge *et al.*, 2008; Sibbe and Kulik, 2017). As a regulatory system to control neurogenesis processes in the SGZ, γ-aminobutyric acid (GABA)-ergic interneurons in the hilus of the dentate gyrus innervate granule cell lineage populations (Freund and Buzsáki, 1996; Sibbe and Kulik, 2017). A subpopulation of GABAergic interneurons produces reelin (RELN), an extracellular protein that is essential for neuronal migration, including granule cell lineage (Gong *et al.*, 2007). In addition to inputs of GABAergic interneurons, various types of neurons outside the SGZ also create a synaptic connection with neurons in the dentate gyrus, such as glutamatergic neurons in the entorhinal cortex providing axonal projections to the dentate gyrus (Leranth and Hajszan, 2007) and cholinergic neurons originating from the septal nucleus and nucleus of the diagonal band of Broca innervating neurons in the dentate hilus (Leranth and Hajszan, 2007). Glutamatergic inputs to the SGZ are important for maintaining proper proliferation and differentiation of the granule cell lineage (Freund and Buzsáki, 1996).

Thyroid hormones are crucial for normal brain development during fetal and neonatal periods. They play important roles in neuronal proliferation and migration, neuritogenesis, synaptogenesis and myelinogenesis (Moog *et al.*, 2017). Experimental induction of developmental hypothyroidism in rats causes aberrant brain growth involving diverse cellular populations and also impairs inherent brain structures and functions (Moog *et al.*, 2017). Developmental hypothyroidism impairs neuronal migration and results in subcortical band heterotopia in the corpus callosum, affects postnatal neurogenesis in the hippocampus (Moog *et al.*, 2017; Shiraki *et al.*, 2016), and causes white matter hypoplasia with suppression of both axonal myelination and oligodendrocytic accumulation (Schoonover *et al.*, 2004). It has been considered that maternal hypothyroidism is associated with autism spectrum disorders (ASD; Moog *et al.*, 2017). Multiple studies have highlighted the involvement of key processes, including neurogenesis, neurite growth, synaptogenesis and synaptic plasticity in the pathophysiology of neurodevelopmental disorders, such as ASD (Gilbert and Man, 2017). Therefore, an experimental induction of developmental hypothyroidism could provide a reasonable model for ASD (Román, 2007).

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defense system in an organism and involved in a variety of disorders such as neurodegenerative diseases and malignant tumors. It is known that neural injury in the central and peripheral nervous system by some kinds of neurotoxicants is considered to be related to the induction of oxidative stress (Li *et al.*, 2013), but it is unclear how neurotoxicants cause oxidative stress and neurotoxicity. Importantly, SGZ cells in the hippocampal dentate gyrus generate ROS, because these cells have a high cellular activity for proliferation and differentiation requiring high oxygen demand (Walton *et al.*, 2012). With regard to the effect of hypothyroidism on brain tissues, induction of oxidative stress has been reported in rat hippocampus by administration of anti-thyroid agent during developmental stage or adult stage (Cano-Europa *et al.*, 2008; Cattani *et al.*, 2013). It seems that deleterious effect

of hypothyroidism during neonatal and juvenile growth on learning and memory is at least in part due to brain tissues oxidative damage (Farrokhi *et al.*, 2014). Furthermore, co-exposure of extracts of a medicinal plant, Nigella sativa, with 6-propyl-2-thiouracil (PTU), as an antithyroid agent, from gestation period to adult stage in rats reduces apoptosis in the hippocampal dentate gyrus, Cornu Ammonis (CA) 1 and CA3 area (Asiaei *et al.*, 2017). Therefore, there is a possibility that antioxidant treatment may prevent or ameliorate the hypothyroidism-related disruption of hippocampal neurogenesis.

The present study was performed to clarify whether exposure to antioxidant after the induction of developmental hypothyroidism has a potential to ameliorate hypothyroidism-related disruption of hippocampal neurogenesis in rats. For this purpose, we used PTU as an antithyroid agent and two kinds of antioxidant, α-glycosyl isoquercitrin (AGIQ) and α-lipoic acid (ALA), in the present study. AGIQ, also known as enzymatically modified isoquercitrin, is a flavonol glycoside derived by enzymatic glycosylation of rutin. AGIQ is a mixture of quercetin glycoside, consisting of isoquercitrin and its α-glucosylated derivatives, with 1-10 or more of additional linear glucose moieties, and has greater water-solubility and bioavailability (Akiyama *et al.*, 2000). AGIQ has been reported to exert anti-oxidative (Nishimura *et al.*, 2010), anti-inflammatory (Kangawa *et al.*, 2017), anti-hypertensive (Gasparotto *et al.*, 2011), anti-allergic (Makino *et al.*, 2013) and tumor suppressive (Nishimura *et al.*, 2010) properties. ALA, a natural compound that is chemically named 5-(1,2-dithiolan-3-yl) pentanoic acid and is also known as thioctic acid (Gruzman *et al.*, 2004), is another metabolic antioxidant. In addition to direct antioxidant activity, ALA and its endogenous counterpart dihydrolipoic acid (DHLA), which is rapidly formed after uptake into the body's cells, contributes to the non-enzymatic regeneration of reduced glutathione, vitamin C, vitamin E, and coenzyme Q10 *in vivo* (Biewenga *et al.*, 1997). Moreover, DHLA stimulates glutathione synthesis by enhancing cellular cysteine uptake. As DHLA is a supplier of reducing equivalents for the regeneration of detoxication enzymes, it is capable of supporting repair of oxidative damage (Biewenga *et al.*, 1997). ALA has been reported to prevent or ameliorate several ailments such as cardiovascular diseases, diabetic complications including retinopathy and neuropathy, and hypertension because of its antioxidant properties (Rochette *et al.*, 2013).

MATERIALS AND METHODS

Chemicals and animals

6-Propyl-2-thiouracil (PTU; CAS No. 51-52-5, purity > 99%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Alpha-glycosyl isoquercitrin (AGIQ, purity > 97%) was provided from San-Ei Gen F.F.I., Inc. (Osaka, Japan). Alpha-lipoic acid (ALA; CAS No. 1077-28-7, purity > 99%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Mated female Slc:SD rats at gestational day (GD) 1 (the appearance of vaginal plugs was designated as GD 0) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in an air-conditioned animal room (temperature: 23 ± 2°C, relative humidity: 55 ± 15%) with a 12-hr light/dark cycle. Mated female rats were provided *ad libitum* a pelleted basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water until the start of exposure to PTU on GD 6. From GD 6 to day 21 after delivery, animals were provided the same CRF-1 diet and the drinking water with or without PTU. From postnatal day (PND) 21 (where PND 0 was the day of delivery) onwards, offspring were reared and provided a powdered CRF-1 diet with or without antioxidant and tap water *ad libitum* throughout the experimental period until PND 77.

Experimental design

Mated female rats were housed individually with their offspring and randomly divided into two groups. One group of 12 animals was selected as untreated controls and another group of 25 animals was treated with 10 ppm PTU in the drinking water from GD 6 to day 21 after delivery on weaning (Fig. 2). Concentration of PTU was determined in accordance with the previous study revealing hypothyroidism in both dams and offspring (Shiraki *et al.*, 2016). On PND 4, the litters were culled randomly 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 eight pups per litter.

On PND 21, 2 male and 2-5 female pups per dam in untreated controls and PTU-exposed animals were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and subjected to prepubertal necropsy for other experimental purposes. Dams in untreated controls were euthanized in the similar way to their offspring at this time point, and dams in PTU-exposed group were further maintained for additional 7 days without PTU-treatment for further nursing of offspring showing growth suppression due to hypothyroidism. In PTU-exposed group, dams with offspring were randomly divided into three groups, i.e., antioxidant (–), AGIQ and ALA groups

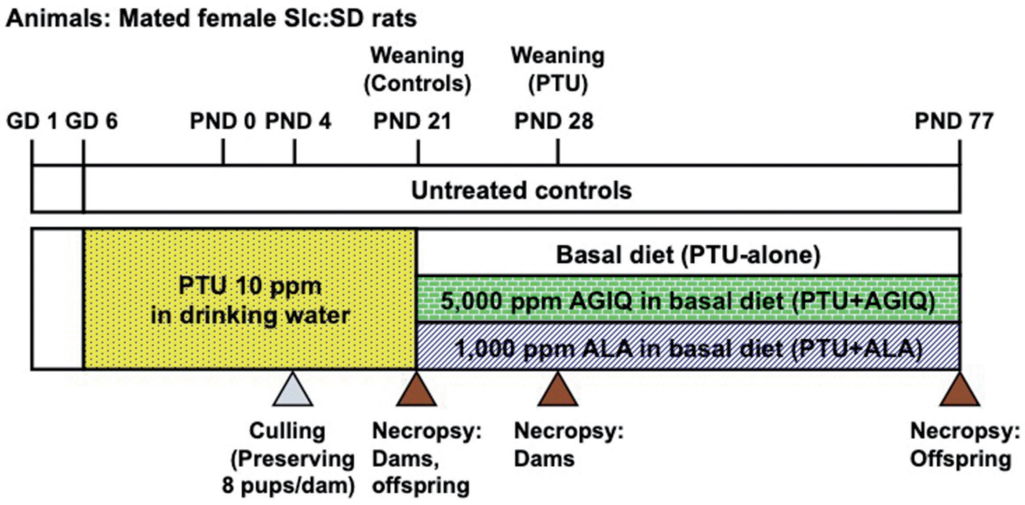

Fig. 2. Experimental design of maternal exposure to 6-propyl-2-thiouracil (PTU) from gestational day (GD) 6 to postnatal day (PND) 21 and following exposure to α-glycosyl isoquercitrin (AGIQ) or α-lipoic acid (ALA) from PND 21 to PND 77 of male offspring in rats. Dams in untreated controls and PTU-exposed group were euthanized on PND 21 and PND 28, respectively. Offspring in both of untreated controls and PTU-exposed group were euthanized on PND 21 and PND 77.

on PND 21. AGIQ and ALA were dietary administered to dams and offspring until PND 28 and to offspring until PND 77 at 5,000 ppm and 1,000 ppm, respectively. Both AGIQ and ALA were powdered compound and mixed with powdered basal diet to the determined concentration. The chosen dosage of AGIQ or ALA has been shown to suppress the promotion of hepatic preneoplastic lesions in rats (Fujii *et al*., 2013a, 2013b; Hara *et al*., 2014; Kuwata *et al*., 2011). All dams of PTU-exposed group were euthanized on PND 28. Body weight and food and water consumptions of all dams were measured twice weekly from PND 4 to PND 21 and once at PND 28 in PTU-exposed dams. Body weight of offspring was measured twice weekly from PND 4 to PND 21, and once weekly from PND 27 until adult stage on PND 77. Food and water consumptions were measured once weekly from PND 21 to PND 77 in offspring of untreated controls and from PND 28 to PND 77 in PTU-exposed offspring.

On PND 77, 10 male offspring per group (1 male offspring per dam) were subjected to perfusion fixation for brain immunohistochemistry through the left cardiac ventricle with ice-cold 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) at a flow rate of 35 mL/min under CO_2/O_2 anesthesia. For transcript expression analysis, brain samples from 6 male offspring per group (1 male offspring per dam) were prepared. The remaining brain samples from 16 male offspring per group (1-2 male offspring per dam) were prepared for other purposes of analysis.

All dams and offspring were checked for general conditions in terms of the appearance of abnormal gait and behaviors every day.

All procedures of this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology. All efforts were made to minimize animal suffering.

Immunohistochemical analysis, and apoptotic cell detection

For immunohistochemistry analysis, perfusion-fixed brains were additionally fixed with the same solution overnight. Coronal slices were prepared at −3.5 mm from bregma in PND 77 offspring brains (N = 10/group). Brain slices were further fixed with 4% (w/v) PFA overnight at 4°C. Brain slices were routinely processed for paraffin embedding and were sectioned into 3-μm-thick sections.

Brain sections from offspring at PND 77 were subjected to immunohistochemistry using primary antibodies shown in Supplementary Table 1: glial fibrillary acidic protein (GFAP), which is expressed in type-1 stem cells (radial glial cells) in the SGZ and astrocytes (Sibbe and Kulik, 2017); sex determining region Y (SRY)-box 2 (SOX2), which is expressed in type-1 stem cells and type-2a progenitor cells in the SGZ (Hodge *et al.*, 2008);

T-box brain 2 (TBR2), which is expressed in type-2b progenitor cells in the SGZ (Hodge *et al.*, 2008); doublecortin (DCX), which is expressed in type-2b and type-3 progenitor cells and immature granule cells in the SGZ and GCL (Sibbe and Kulik, 2017); neuronal nuclei (NeuN), which is expressed in postmitotic neurons of both immature and mature granule cells in the SGZ and GCL (Sibbe and Kulik, 2017); and RELN, parvalbumin (PVALB), calretinin (CALB2) and somatostatin (SST), which are expressed in GABAergic interneurons in the hilus of the dentate gyrus (Freund and Buzsáki, 1996); glutamic acid decarboxylase 67 (GAD67), a GABA-producing enzyme in GABAergic interneurons (Houser, 2007); ionized calcium-binding adapter molecule 1 (IBA1), a microglia-specific molecule in the brain (Ito *et al.*, 1998); proliferating cell nuclear antigen (PCNA), a cell proliferation marker in the SGZ; Fos proto-oncogene, AP-1 transcription factor subunit (FOS), activity-regulated cytoskeleton-associated protein (ARC) and cyclooxygenase 2 (COX2), which are members of the immediate-early genes involved in synaptic plasticity (Guzowski, 2002; Chen *et al*., 2002) in the GCL. The respective primary antibodies were applied to brain sections for incubation overnight at 4°C. One section per animal was subjected to immunohistochemistry of each molecule.

To block endogenous peroxidase, deparaffinized sections were incubated in 0.3% (v/v) H_2O_2 solution in absolute methanol for 30 min. The antigen retrieval conditions that were applied for some antibodies are listed in Supplementary Table 1. Immunodetection was conducted using a Vectastain® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine (DAB)/H_2O_2 as the chromogen. Hematoxylin counterstaining was then performed, and coverslips were mounted on immunostained sections for microscopic examination.

Double immunohistochemical staining of RELN and NeuN was carried out using Vectastain ABC-AP kit (Vector Laboratories Inc.) with a Vector® Red Alkaline Phosphatase Substrate Kit (Vector Red; Vector Laboratories Inc.) to visualize RELN and DAB to visualize NeuN.

To evaluate apoptosis in the SGZ of the dentate gyrus in the offspring, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed using the ApopTag *In situ* Apoptosis Detection Kit (Merck Millipore, Burlington, MA, USA) according to the manufacturer's instructions, with DAB/H_2O_2 as the chromogen. One section per animal was subjected to TUNEL assay.

Evaluation of immunoreactive cells and apoptotic cells

Immunoreactive cells, i.e., GFAP$^+$, SOX2$^+$, TBR2$^+$, DCX$^+$, NeuN$^+$, FOS$^+$, ARC$^+$, COX2$^+$, and PCNA$^+$ proliferating cells, or TUNEL$^+$ apoptotic cells in the SGZ and/or GCL were bilaterally counted and normalized for the length of the SGZ (Fig. 1). Immunoreactive cells distributed within the entire hilar region of the hippocampal dentate gyrus, i.e., RELN$^+$, PVALB$^+$, SST$^+$, CALB2$^+$ and GAD67$^+$ cells were bilaterally counted and normalized per area unit of the hilus area (Fig. 1). These immunolocalized and apoptotic cells were analyzed by blind trial for the treatment conditions. Immunoreactive neurons located inside of the CA3, consisting of large pyramidal neurons that can be morphologically distinguished from relatively small interneurons, were excluded from counting immunoreactive cells in the hilus of the dentate gyrus. Number of each immunoreactive cellular population (except for NeuN$^+$ cells in the GCL) or TUNEL$^+$ apoptotic cells was manually counted under microscopic observation using a BX53 microscope (Olympus Corporation, Tokyo, Japan). In case of NeuN$^+$ cells in the GCL, digital photomicrographs at × 100-fold magnification were taken using a BX53 microscope attached to a DP72 Digital Camera System (Olympus Corporation), and positive cell counting was performed applying the WinROOF image analysis software package (version 5.7; Mitani Corporation, Fukui, Japan). The length of the SGZ and the hilar area were measured in microscopic images at × 40-fold magnification by applying the cellSens image analysis software package (standard package 1.9; Olympus Corporation).

Transcript expression analysis

Transcript expression levels in the hippocampal dentate gyrus were examined using real-time RT-PCR. Brain tissues were dissected and processed using methacarn solution as previously described (Akane *et al.*, 2013). In brief, 2-mm-thick coronal cerebral slices were prepared at the position of −3.0 mm from bregma. Tissues of the hippocampal dentate gyrus were collected from the slice using punch-biopsy devices with a pore size of 1 mm in diameter (Kai Industries Co., Ltd., Gifu, Japan). Total RNA was extracted from tissue samples of six animals in each group using RNeasy Mini kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using SuperScript® III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) in a 20-μL total reaction mixture from 1 μg of total RNA. Analysis of the transcript levels for gene targets shown in Supplementary Table 2 was performed using the PCR

primers designed with Primer Express software (Version 3.0; Thermo Fisher Scientific). Real-time PCR with Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) was conducted using a StepOnePlus™ Real-time PCR System (Thermo Fisher Scientific). The relative differences in gene expression between four groups were calculated using threshold cycle (C_T) values that were first normalized to those of the hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) or glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene as the endogenous control in the same sample and then relative to a control C_T value using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Numerical data are expressed as the mean ± SD. Differences between untreated controls, PTU-alone group, PTU+AGIQ group and PTU+ALA group were evaluated as follows. Data were analyzed by Levene's test for homogeneity of variance. If the variance was homogenous, numerical data were assessed using Tukey's test to compare four groups. For heterogeneous data, Aspin-Welch's *t*-test with Bonferroni correction was performed. Numerical data consisting of two sample groups were analyzed by the F-test for homogeneity of variance. Student's *t*-test was applied when the variance was homogenous between the groups, and Aspin-Welch's *t*-test was performed when the variance was heterogeneous. All analyses were performed using the IBM SPSS Statistics ver. 25 (IBM Japan, Ltd., Tokyo, Japan).

RESULTS

Clinical signs, body weight, food and water consumption and necropsy data of dams

Throughout the PTU-treatment period, no dams died and there was no abnormal behavior of all dams in this experiment. Body weight of dams was not statistically different between untreated controls and PTU group during PTU-exposure period (Supplementary Fig. 1). Food consumption of PTU group was significantly decreased from GD 16 to PND 5 and from PND 12 to PND 21 compared with untreated controls. Water consumption was significantly increased from GD 6 to GD 9, but decreased on PND 5, PND 12, PND 20 and PND 21 compared with untreated controls. At the necropsy of dams on PND 21 or PND 28, numbers of implantation sites and living offspring and male ratio were not statistically different between untreated controls and PTU group (Supplementary Table 3).

Clinical signs, body weight, food and water consumption, antioxidant intake and necropsy data of offspring

During early postweaning period, most of PTU-exposed animals were hyperactive, aggressive and restless in nature. Body weight of offspring was significantly decreased from PND 4 to PND 21 in both males and females of PTU-exposed group compared with untreated controls (Supplementary Table 4). At necropsy on PND 21, PTU-exposed offspring of both males and females exhibited significantly lower body and brain weights as compared with untreated controls (Supplementary Table 5). After starting administration of AGIQ or ALA, all the PTU-exposed groups significantly decreased body weight compared with untreated controls, and PTU+AGIQ group did not change body weight compared with PTU-alone group (Supplementary Table 6). In contrast, PTU+ALA group significantly decreased body weight from PND 62 to PND 77 compared with PTU-alone group. Food consumption of male offspring was significantly decreased from PND 35 to PND 56 in PTU-alone group and PTU+AGIQ group compared with untreated controls (Supplementary Table 7). PTU+ALA group significantly decreased food consumption from PND 35 to PND 69 compared with untreated controls and from PND 56 to PND 77 compared with PTU-alone group. Water consumption was significantly decreased from PND 35 to PND 77 in all PTU-exposed groups compared with untreated controls, except for no statistically significant difference from PND 63 to PND 69 in PTU+AGIQ group (Supplementary Table 7). There were no statistically significant differences between PTU-alone group and any of the PTU + antioxidant groups. The mean postweaning exposure level of exogenously administered AGIQ or ALA in PTU-exposed offspring was 516.1 and 103.4 mg/kg body weight/day, respectively. At necropsy on PND 77, body and brain weights were significantly decreased in all PTU-exposed groups compared with untreated controls and PTU+ALA group significantly decreased body weight compared with PTU-alone group (Supplementary Table 8).

Number of granule cell lineages in the SGZ and/or GCL

No significant differences in the number of $GFAP^+$, $SOX2^+$ and $TBR2^+$ cells in the SGZ were observed between untreated controls and any of PTU-exposed groups (Fig. 3, Supplementary Table 9). The number of DCX^+ cells was significantly fewer in all PTU-exposed groups than in untreated controls, but there were no statistically significant differences between PTU-alone

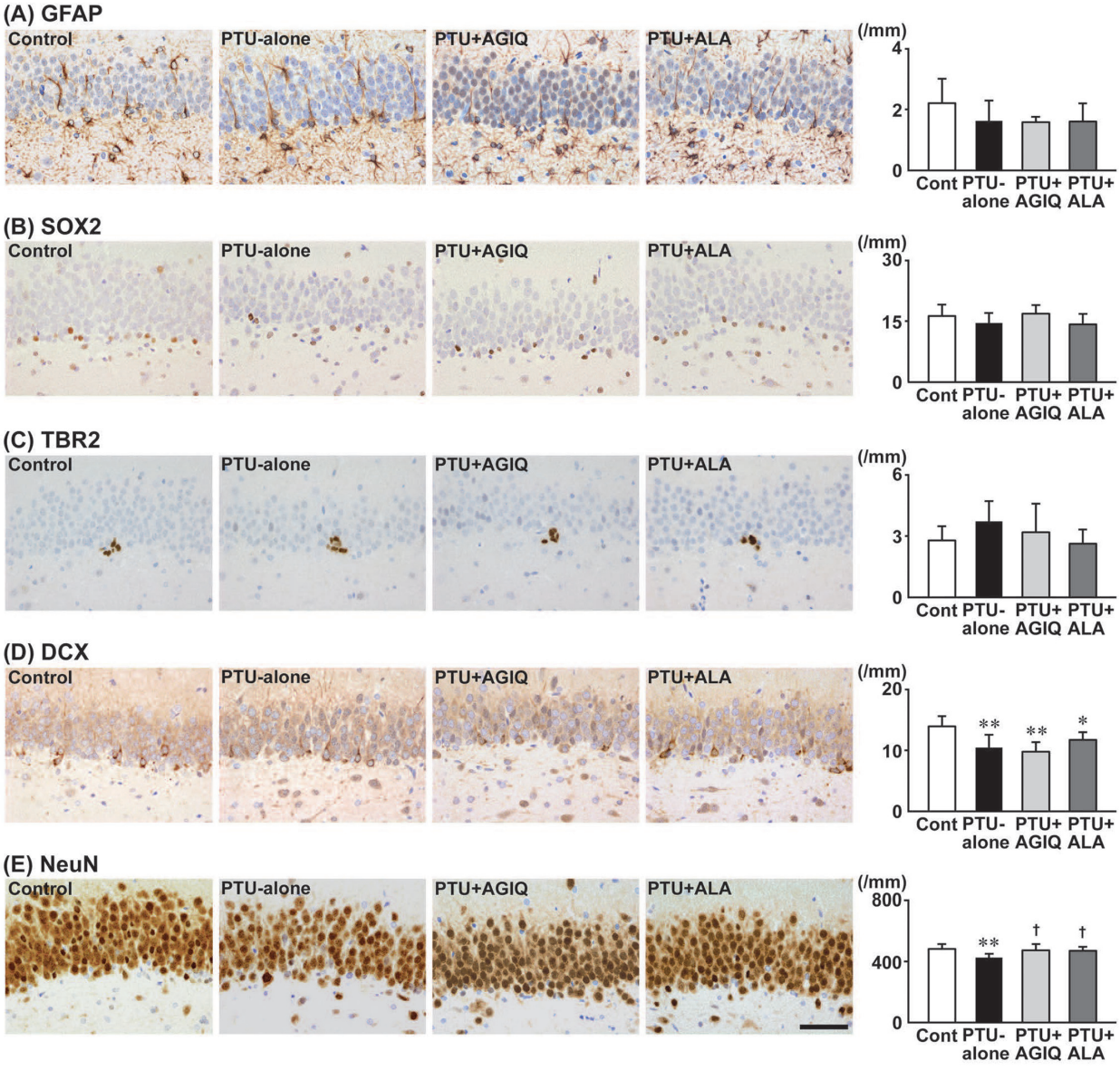

Fig. 3. Number of immunoreactive cells for (A) GFAP, (B) SOX2, (C) TBR2 in the SGZ, (D) DCX and (E) NeuN in the SGZ and GCL of the hippocampal dentate gyrus of male offspring maternally exposed to PTU and then directly exposed to AGIQ or ALA through the adult stage. Representative images of untreated controls, PTU-alone group, PTU+AGIQ group and PTU+ALA group (from left to right). Magnification × 400; bar = 50 μm. Graphs show the numbers of immunoreactive cells in the SGZ and/or GCL. Data are expressed as mean + SD. N = 10/group. *$P < 0.05$, **$P < 0.01$, significantly different from untreated controls and $^{\dagger}P < 0.05$, significantly different from PTU-alone group by Tukey's test or Aspin-Welch's *t*-test with Bonferroni correction.

group and any of PTU+antioxidant groups. The number of NeuN^{+} cells was significantly fewer in PTU-alone group than in untreated controls. Both of PTU+AGIQ and PTU+ALA groups significantly increased NeuN^{+} cells compared with PTU-alone group.

Number of GABAergic interneurons in the hilus of the dentate gyrus

There were significantly fewer PVALB^{+} cells and GAD67^{+} cells and more SST^{+} cells and CALB2^{+} cells in PTU-alone group than in untreated controls (Fig. 4,

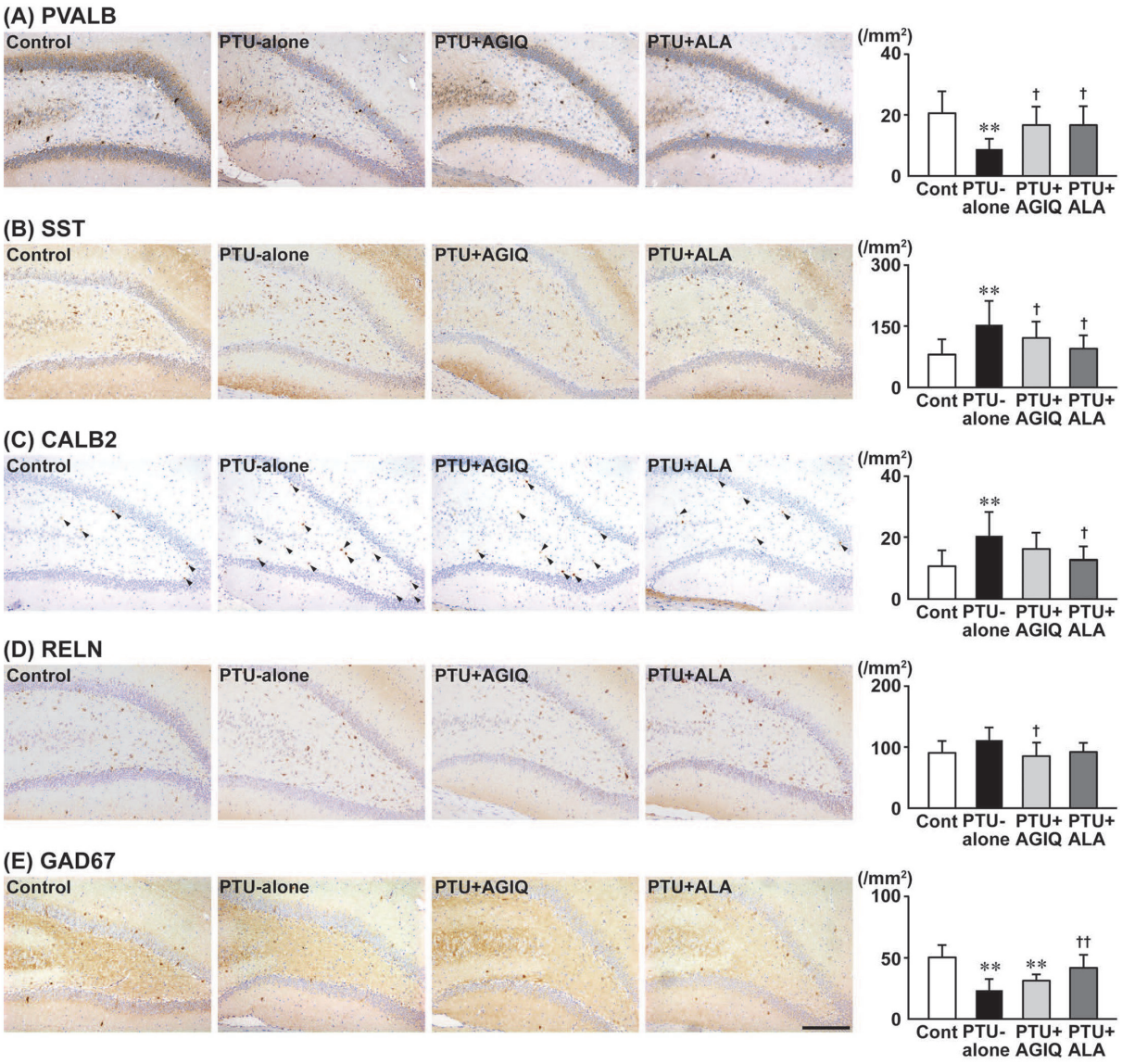

Fig. 4. Number of immunoreactive cells for (A) PVALB, (B) SST, (C) CALB2, (D) RELN and (E) GAD67 in the hilus of the hippocampal dentate gyrus of male offspring maternally exposed to PTU and then directly exposed to AGIQ or ALA through the adult stage. Representative images of untreated controls, PTU-alone group, PTU+AGIQ group and PTU+ALA group (from left to right). *Arrowheads* indicate immunoreactive cells for CALB2. Magnification × 100; bar = 200 µm. Graphs show the numbers of immunoreactive cells/unit area (mm²) in the hilar region. Data are expressed as mean + SD. N = 10/group. $^{**}P < 0.01$, significantly different from the untreated controls and $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, significantly different from the PTU-alone group by Tukey's test or Aspin-Welch's *t*-test with Bonferroni correction.

Supplementary Table 9). PTU+AGIQ group also showed significantly fewer GAD67$^+$ cells. Both of PTU+AGIQ and PTU+ALA groups significantly increased PVALB$^+$ cells and significantly decreased SST$^+$ cells compared with PTU-alone group. PTU+AGIQ group showed significantly fewer RELN$^+$ cells than in PTU-alone group. PTU+ALA group showed significantly fewer CALB2$^+$ cells and more GAD67$^+$ cells than in PTU-alone group.

Double immunohistochemistry of NeuN and RELN revealed that there were no statistically significant difference in the number of NeuN-expressing and RELN-expressing cells, NeuN-expressing and RELN-lacking cells,

NeuN-lacking or weakly-expressing and RELN-expressing cells between untreated controls and any of PTU-exposed groups and between PTU-alone group and any of PTU+antioxidant groups (Supplementary Fig. 2).

Number of proliferating and apoptotic cells in the SGZ

There were significantly fewer PCNA$^+$ proliferating cells in PTU-alone group and PTU+ALA group than in untreated controls (Supplementary Fig. 3, Supplementary Table 9). There were no significant difference in the number of PCNA$^+$ cells between PTU-alone group and any of PTU+antioxidant groups.

There were no significant difference in the number of TUNEL$^+$ apoptotic cells between untreated controls and any of PTU-exposed groups and between PTU-alone group and any of PTU+antioxidant groups (Supplementary Fig. 3, Supplementary Table 9).

Number of synaptic plasticity-related molecule-immunoreactive cells in the GCL

There were significantly fewer FOS$^+$ cells in PTU-alone group and both of PTU+antioxidant groups than in untreated controls (Supplementary Fig. 4, Supplementary Table 9). There were no significant difference in the number of FOS$^+$ cells between PTU-alone group and any of PTU+antioxidant groups.

There were no significant difference in the number of ARC$^+$ or COX2$^+$ cells between untreated controls and any of PTU-exposed groups and between PTU-alone group and any of PTU+antioxidant groups (Supplementary Fig. 4, Supplementary Table 9).

Number of GFAP$^+$ astrocytes and IBA1$^+$ microglia in the hilus of the dentate gyrus

There were no statistically significant difference in the number of GFAP$^+$ astrocytes and IBA1$^+$ microglia between untreated controls and any of PTU-exposed groups and between PTU-alone group and any of PTU+antioxidant groups (Supplementary Fig. 5, Supplementary Table 9).

Transcript expression in the hippocampal dentate gyrus

In PTU-alone group, there was significantly more transcript level of *Calb2*, *Otx2*, *Gria3*, *Gpx1*, *Gpx4*, *Gsta5*, *Nqo1*, *Prdx1*, *Txn1* and *Vldlr* after normalization with either or both of *Gapdh* and *Hprt1* compared with untreated controls (Tables 1 and 2, Supplementary Tables 10 and 11). There were no genes to show significantly less transcript level in this group after normalization with either or both of *Gapdh* and *Hprt1* compared with untreated controls.

In PTU+AGIQ group, there was significantly more transcript level of *Il1a* after normalization with *Gapdh* compared with PTU-alone group (Tables 1 and 2, Supplementary Tables 10 and 11). In this group, there was significantly less transcript level of *Otx2*, *Gria3*, *Nqo1*, *Txn1*, *Fos* and *Nfkb1* after normalization with either or both of *Gapdh* compared with PTU-alone group.

In PTU+ALA group, there was significantly more transcript level of *Gad1*, *Grin2a*, *Reln*, *Dab1*, *Il1a*, and *Tgfb2* after normalization with either or both of *Gapdh* and *Hprt1* compared with PTU-alone group (Tables 1 and 2, Supplementary Tables 10 and 11). In this group, there was significantly less transcript level of *Otx2*, *Fos* and *Nfkb1* after normalization with either or both of *Gapdh* and *Hprt1* compared with PTU-alone group.

DISCUSSION

Developmental hypothyroidism causes sustained disruption of hippocampal neurogenesis in rats (Saegusa *et al.*, 2010; Shiraki *et al.*, 2016). We previously found that PTU at 10 ppm affects differentiation and cell survival of granule cell lineages that involve type-1 stem cells on PND 21, and most of the changes in neurogenesis were reversed on PND 77 (Shiraki *et al.*, 2016). In the present study, we observed decreased number of DCX$^+$ cells and NeuN$^+$ cells, suggestive of type-2b and type-3 progenitor cells and immature and mature granule cells, by 10 ppm PTU at the adult stage. While the reason for this discrepancy between the previous and present studies conducted under the identical experimental design was not clear, a similar decrease of DCX$^+$ cells to the present study was reported at the adult stage after developmental hypothyroidism (Gilbert *et al.*, 2017). It has also been shown that hypothyroidism targets to decrease postmitotic populations of granule cell lineage (Sánchez-Huerta *et al.*, 2016), and causes decrease of NeuN$^+$ granule cells similar to the present study at the end of developmental PTU exposure (Cattani *et al.*, 2013). With regard to the GABAergic interneurons, hypothyroidism also strongly affects these cell populations (Moog *et al.*, 2017). In the present study, developmental exposure to PTU at 10 ppm decreased the number of PVALB$^+$ cells and increased SST$^+$ cells and CALB2$^+$ cells in the hilar region at the adult stage, similar to the previous studies (Shiraki *et al.*, 2016; Uchida *et al.*, 2014; Wallis *et al.*, 2008). With regard to synaptic plasticity-related neuronal cell populations, the present study revealed decrease of FOS$^+$ granule cells by 10 ppm PTU at the adult stage, similar to the previous study of

Table 1. Transcript expression changes in the hippocampal dentate gyrus of male offspring normalized by *Gapdh*.

	Control	PTU-alone	PTU+AGIQ	PTU+ALA
No. of animals examined	6	6	6	6
GABAergic interneuron-related genes				
Pvalb	1.01 ± 0.14	0.91 ± 0.32	0.80 ± 0.12	1.00 ± 0.13
Sst	1.05 ± 0.30	1.07 ± 0.33	1.07 ± 0.36	1.53 ± 0.35*
Calb2	1.05 ± 0.38	3.35 ± 1.08**	2.47 ± 1.20	2.31 ± 0.50**
Otx2	1.27 ± 0.88	3.15 ± 0.75**	2.09 ± 0.99	1.48 ± 0.55††
Gad1	1.05 ± 0.39	1.11 ± 0.25	1.05 ± 0.37	1.55 ± 0.28
Glutamate receptors				
Gria1	1.10 ± 0.51	0.82 ± 0.22	0.94 ± 0.35	1.34 ± 0.35
Gria2	1.10 ± 0.55	1.02 ± 0.19	1.12 ± 0.38	1.51 ± 0.35
Gria3	1.05 ± 0.35	1.44 ± 0.30	1.07 ± 0.12	1.30 ± 0.34
Grin2a	1.09 ± 0.51	0.71 ± 0.17	0.96 ± 0.34	1.28 ± 0.25†
Grin2b	1.04 ± 0.33	1.17 ± 0.34	1.11 ± 0.20	1.52 ± 0.32
Grin2d	1.05 ± 0.42	1.22 ± 0.59	1.12 ± 0.37	1.04 ± 0.39
Antioxidant enzyme genes				
Cat	1.02 ± 0.19	1.28 ± 0.22	1.13 ± 0.20	1.43 ± 0.33*
Gpx1	1.12 ± 0.66	1.64 ± 0.34	1.61 ± 0.57	1.30 ± 0.29
Gpx2	1.04 ± 0.32	0.99 ± 0.48	0.95 ± 0.42	1.16 ± 0.31
Gpx4	1.04 ± 0.33	1.53 ± 0.31	1.32 ± 0.20	1.61 ± 0.83
Gsta5	1.03 ± 0.27	1.56 ± 0.36	1.47 ± 0.55	1.51 ± 0.25
Hmox1	1.02 ± 0.22	1.16 ± 0.45	0.94 ± 0.27	1.20 ± 0.23
Nqo1	1.02 ± 0.20	1.80 ± 0.41**	1.17 ± 0.30†	1.49 ± 0.38
Prdx1	1.03 ± 0.27	1.58 ± 0.45	1.56 ± 0.43	1.34 ± 0.28
Sod1	1.02 ± 0.21	1.15 ± 0.51	1.21 ± 0.28	0.98 ± 0.19
Sod2	1.01 ± 0.15	0.94 ± 0.25	1.13 ± 0.33	0.88 ± 0.09
Txn1	1.07 ± 0.33	2.21 ± 0.46**	1.40 ± 0.28††	1.77 ± 0.38*

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; *Calb2*, calretinin; *Cat*, catalase; GABA, γ-aminobutyric acid; *Gad1*, glutamate decarboxylase 1; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Gpx1*, glutathione peroxidase 1; *Gpx2*, glutathione peroxidase 2; *Gpx4*, glutathione peroxidase 4; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Gria3*, glutamate ionotropic receptor AMPA type subunit 3; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; *Grin2b*, glutamate ionotropic receptor NMDA type subunit 2B; *Grin2d*, glutamate ionotropic receptor NMDA type subunit 2D; *Gsta5*, glutathione *S*-transferase alpha 5; *Hmox1*, heme oxygenase 1; *Nqo1*, NAD(P)H quinone dehydrogenase 1; *Otx2*, orthodenticle homeobox 2; *Prdx1*, peroxiredoxin 1; PTU, 6-propyl-2-thiouracil; *Pvalb*, parvalbumin; *Sod1*, superoxide dismutase 1; *Sod2*, superoxide dismutase 2; *Sst*, somatostatin; *Txn1*, thioredoxin 1. Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, significantly different from untreated controls and †$P < 0.05$, ††$P < 0.01$, significantly different from PTU-alone group by Tukey's test or Aspin-Welch's *t*-test with Bonferroni correction.

developmental hypothyroidism (Dong *et al.*, 2005). These results suggest that developmental hypothyroidism caused sustained effects on GABAergic interneuron subpopulations, granule cell lineage subpopulations and synaptic plasticity of granule cells.

It is well known that oxidative stress is produced as a result of routine adult neurogenesis in the hippocampus, and it has been hypothesized that the energetic demands of highly proliferative progenitors generate localized oxidative stress that contributes to ROS-mediated damage within the neuropoietic microenvironment (Walton *et al.*, 2012). Therefore, it can be postulated that hippocampal neurogenesis can be disrupted by inducing excess oxidative stress. In fact, we previously found an increase of SGZ cells immunoreactive for malondialdehyde, a lipid peroxidation end product, at the end of maternal exposure to mycotoxin, T-2 toxin or ochratoxin A, and suggested the relationship with disruption of neurogenesis (Tanaka *et al.*, 2016a, 2016b). Hypothyroidism causes oxidative stress in the brain during the exposure to anti-thyroid agent (Cattani *et al.*, 2013; Rahaman *et al.*, 2001), and PVALB$^+$ interneurons are quite vulnerable to oxidative stress early in development, resulting in permanent decrease in number in adulthood (Powell *et al.*, 2012). Therefore, decreased number of PVALB$^+$ interneurons in the present study may be due to excess oxidative stress

Table 2. Transcript expression changes in the hippocampal dentate gyrus of male offspring normalized by *Hprt1*.

	Control	PTU-alone	PTU+AGIQ	PTU+ALA
No. of animals examined	6	6	6	6
GABAergic interneuron-related genes				
Pvalb	1.02 ± 0.21	0.93 ± 0.25	0.78 ± 0.18	1.02 ± 0.08
Sst	1.05 ± 0.32	1.10 ± 0.27	1.04 ± 0.34	1.55 ± 0.24*
Calb2	1.08 ± 0.49	3.58 ± 1.43*	2.30 ± 0.90	2.34 ± 0.38**
Otx2	1.26 ± 0.95	3.27 ± 0.72**	1.99 ± 0.80†	1.51 ± 0.51††
Gad1	1.01 ± 0.16	1.15 ± 0.23	1.00 ± 0.30	1.59 ± 0.27** †
Glutamate receptors				
Gria1	1.09 ± 0.45	0.85 ± 0.22	0.90 ± 0.28	1.37 ± 0.35
Gria2	1.08 ± 0.45	1.06 ± 0.18	1.06 ± 0.27	1.53 ± 0.24
Gria3	1.03 ± 0.29	1.51 ± 0.37*	1.04 ± 0.13†	1.33 ± 0.29
Grin2a	1.07 ± 0.42	0.75 ± 0.20	0.91 ± 0.28	1.34 ± 0.39†
Grin2b	1.03 ± 0.28	1.24 ± 0.47	1.08 ± 0.22	1.58 ± 0.43
Grin2d	1.02 ± 0.22	1.31 ± 0.74	1.06 ± 0.30	1.08 ± 0.47
Antioxidant enzyme genes				
Cat	1.06 ± 0.36	1.32 ± 0.18	1.09 ± 0.17	1.46 ± 0.31
Gpx1	1.06 ± 0.35	1.70 ± 0.27*	1.54 ± 0.50	1.31 ± 0.20
Gpx2	1.11 ± 0.50	1.05 ± 0.59	0.88 ± 0.28	1.18 ± 0.31
Gpx4	1.03 ± 0.28	1.58 ± 0.26*	1.27 ± 0.16	1.61 ± 0.64
Gsta5	1.06 ± 0.35	1.64 ± 0.40*	1.40 ± 0.45	1.53 ± 0.12*
Hmox1	1.03 ± 0.28	1.26 ± 0.52	0.88 ± 0.18	1.22 ± 0.20
Nqo1	1.01 ± 0.18	1.87 ± 0.39**	1.13 ± 0.28††	1.50 ± 0.23*
Prdx1	1.02 ± 0.22	1.63 ± 0.44*	1.50 ± 0.36	1.36 ± 0.17
Sod1	1.02 ± 0.24	1.24 ± 0.54	0.76 ± 0.25	0.68 ± 0.13
Sod2	1.07 ± 0.46	1.01 ± 0.25	0.68 ± 0.12	0.62 ± 0.13
Txn1	1.08 ± 0.42	2.28 ± 0.25**	1.33 ± 0.13††	1.80 ± 0.31*

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; *Calb2*, calretinin; *Cat*, catalase; GABA, γ-aminobutyric acid; *Gad1*, glutamate decarboxylase 1; *Gpx1*, glutathione peroxidase 1; *Gpx2*, glutathione peroxidase 2; *Gpx4*, glutathione peroxidase 4; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Gria3*, glutamate ionotropic receptor AMPA type subunit 3; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; *Grin2b*, glutamate ionotropic receptor NMDA type subunit 2B; *Grin2d*, glutamate ionotropic receptor NMDA type subunit 2D; *Gsta5*, glutathione *S*-transferase alpha 5; *Hmox1*, heme oxygenase 1; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Nqo1*, NAD(P)H quinone dehydrogenase 1; *Otx2*, orthodenticle homeobox 2; *Prdx1*, peroxiredoxin 1; PTU, 6-propyl-2-thiouracil; *Pvalb*, parvalbumin; *Sod1*, superoxide dismutase 1;*Sod2*, superoxide dismutase 2; *Sst*, somatostatin; *Txn1*, thioredoxin 1. Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, significantly different from untreated controls and $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, significantly different from PTU-alone group by Tukey's test or Aspin-Welch's *t*-test with Bonferroni correction.

responses. However, little is known about redox status after the cessation of developmental exposure to anti-thyroid agent. In adult rats, increase of oxidative stress in the brain sustains for at least 8 weeks after achieving hypothyroidism for 4 weeks by exposure to anti-thyroid agent (Santi *et al.*, 2014). In the present study, we observed transcript upregulation of antioxidant enzyme genes, *Gpx1*, *Gpx4*, *Gsta5*, *Nqo1*, *Prdx1* and *Txn1*, in the dentate gyrus at the adult stage after developmental hypothyroidism. While pattern in the response of activity and expression level of antioxidant enzymes differ among organs and oxidative damage-inducing stimuli, oxidative damage to the brain causes upregulation of many antioxidant enzyme genes (Baranowska-Bosiacka *et al.*, 2012; Halder *et al.*, 2018; Tejada *et al.*, 2007). Therefore, upregulation of many antioxidant enzyme genes by developmental hypothyroidism may be the signature of increased oxidative stress in the brain even at the adult stage.

In the present study, mean postweaning exposure level of exogenously administered AGIQ or ALA in PTU-exposed offspring was 516.1 and 103.4 mg/kg body weight/day, respectively. While antioxidant effect of AGIQ has not been reported in the brain injury model until now, 5,000 ppm dietary dose of AGIQ has shown to suppress hepatocellular tumor promotion in rats (Fujii *et al*., 2013a; Hara *et al*., 2014; Kuwata *et al*., 2011). These

animals showed either increase or decrease in the level of thiobarbituric acid-reactive substance (TBARS) in the liver, probably depending on the tumor promotion mechanism. It is well known that antioxidants exerts either antioxidant or prooxidant effect, and in particular, prooxidant state known as "antioxidant stress state" can be produced at high doses (Pisoschi and Pop, 2015). In fact, we found that 5,000 ppm AGIQ can exert either antioxidant or prooxidant effect to suppress hepatocellular tumor promotion (Fujii *et al*., 2013a; Hara *et al*., 2014; Kuwata *et al*., 2011). With regard to ALA, we have found suppression of hepatocellular tumor promotion at 1,000 ppm dietary dose; however, involvement of prooxidant effect was not clear (Fujii *et al*., 2013b). On the other hand, dose-response studies of ALA in chemically-induced brain injury models revealed ameliorating effect accompanying suppression of TBARS levels by ALA at ≥ 50 or at 100 mg/kg body weight/day (Jalali-Nadoushan and Roghani, 2013; Macêdo *et al*., 2012). Considering that both AGIQ and ALA can pass the blood-brain barrier and are distributed in the brain (Gilgun-Sherki *et al*., 2001; Valentová *et al*., 2014), these results suggest that dietary concentration of AGIQ at 5,000 ppm and ALA at 1,000 ppm may be sufficient to ameliorate the imbalance of redox state in diseased brain.

With regard to the exogenously administered antioxidant on the hypothyroidism-induced disruption of hippocampal neurogenesis, the present study revealed that postweaning exposure to AGIQ or ALA restores the number of GABAergic interneuron subpopulations and $NeuN^+$ postmitotic granule cells. ALA was superior to AGIQ in the restoration in number of GABAergic interneuron subpopulations. Interestingly, recovery of transcript level of antioxidant enzyme genes was found with *Nqo1* and *Txn1* only by AGIQ exposure, suggesting an antioxidant effect of AGIQ involving expression changes of antioxidant enzyme genes in the hippocampal dentate gyrus. Similar to the present study, quercetin treatment reversed increased antioxidant enzyme activity and their gene expression in the brain of cadmium-exposed mouse offspring (Halder *et al.*, 2018). In contrast, ALA did not apparently recover the transcript expression level of antioxidant enzyme genes in the present study. As aforementioned, ALA is known to exert antioxidant effect with multiple mechanisms (Biewenga *et al.*, 1997), and there are reports showing that ALA exerts antioxidant effect accompanying upregulation or no change in expression of antioxidant-related genes in the brain inducing oxidative damage (Liu *et al.*, 2017; Saleh *et al.*, 2017).

In the present study, we observed the restoration in number of hilar $PVALB^+$ interneurons by exposure to AGIQ or ALA compared to PTU-alone. $PVALB^+$ interneurons have been shown to promote differentiation of type-2 progenitor cells (Freund and Buzsáki, 1996; Tozuka *et al.*, 2005), suggesting a recovery of $PVALB^+$ interneuron signals to cause an increase of DCX^+ and $NeuN^+$ cells in the SGZ and GCL. However, we did not observe recovery in number of DCX^+ cells by postweaning exposure to AGIQ or ALA, whereas both antioxidants were effective for restoring the decrease in the number of $NeuN^+$ granule cells. DCX^+ population represents type-2b and type-3 progenitor cells and immature granule cells (Sibbe and Kulik, 2017), and $NeuN^+$ population represents postmitotic neurons of both immature and mature granule cells (Sibbe and Kulik, 2017). Considering no changes in the number of $PCNA^+$ cells and $TUNEL^+$ cells in the SGZ/GCL by exposure to AGIQ or ALA compared to PTU-alone, antioxidants may facilitate differentiation process of postmitotic granule cells without influencing cell proliferation and apoptosis in the present study. Because transcript expression level of *Pvalb* in the dentate gyrus of these antioxidant-exposed groups was not differed from PTU-alone group, function of $PVALB^+$ interneurons on restoration of granule cell differentiation might be limited.

In the present study, we found transcript upregulation of *Grin2a*, which encodes one of the *N*-methyl-D-aspartate receptor (NMDAR) subunits, NR2A, by ALA exposure compared with PTU-alone group in the present study. NR2A is essential for maintaining PVALB expression in GABAergic interneurons (Kinney *et al*., 2006), suggesting that *Grin2a* transcript upregulation is the evidence for restoration in number of $PVALB^+$ interneurons as a result of antioxidant effect of ALA. In the present study, we also found transcript upregulation of *Otx2*, encoding orthodenticle homeobox 2 (OTX2), an essential morphogen for embryonic head formation (Sugiyama *et al.*, 2009), in the dentate gyrus after cessation of developmental hypothyroidism. Expression of this gene is thyroid hormone-dependent (Chen *et al.*, 2015), and OTX2 plays a role for promoting maturation of $PVALB^+$ cells in the visual cortex (Sugiyama *et al.*, 2009). In the present study, transcript upregulation of *Otx2* in PTU-alone group may be the compensatory response to facilitate maturation of $PVALB^+$ interneurons that were reduced in number due to sustained oxidative stress responses induced by developmental hypothyroidism. Postweaning antioxidant treatment recovered expression of *Otx2* due to the reduction of oxidative damage to adjust OTX2 to the normal level.

We observed decrease in number of $GAD67^+$ cells on PND 77 after cessation of developmental hypothyroidism in the present study. It is reported that the number

of GAD67$^+$ cells was not changed in the dentate gyrus at the end of developmental hypothyroidism, while the number of GAD65$^+$ cells was decreased as with PVALB$^+$ cells (Sawano *et al.*, 2013). While the reason for the discrepancy with the previous report is not clear in the present study, decline of GABAergic interneuron signals is observed in the cerebral cortex of autistic syndrome patients (Uzunova *et al.*, 2016). Mutant animals used as a model of ASD, as well as animals of an autism model induced by valproic acid, also revealed to show alterations of GABAergic signals, as evident by reductions in GAD67$^+$ and PVALB$^+$ interneuron subpopulations in multiple brain regions (Cellot and Cherubini, 2014). Furthermore, NMDA receptor antagonism induces decreases in PVALB and GAD67 immunoreactivity specifically in PVALB interneurons (Kinney *et al.*, 2006). In the present study, postweaning ALA exposure recovered the number of hilar GAD67$^+$ cells reflecting upregulation of *Gad1* transcript level in the dentate gyrus. As aforementioned, we also found transcript upregulation of *Grin2a* by ALA exposure. These results suggested that activation of NMDA receptors resulted in increased numbers of hilar GAD67$^+$ interneurons following postweaning ALA exposure. In the present study, developmental PTU-exposure also increased the transcript level of *Gria3*, encoding alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid ionotropic receptor subunit GluR3, and postweaning AGIQ exposure recovered the level. GluR3 is ubiquitously expressed in neurons and astrocytes in the hippocampus, and among GABAergic interneurons, GluR3 immunoreactivity delineates PVALB$^+$ interneurons (Moga *et al.*, 2003). Therefore, upregulation of *Gria3* by PTU-exposure may be the compensatory response to the reduction of PVALB$^+$ interneurons, and antioxidant effect of AGIQ restored the expression level to the normal level.

In the present study, postweaning exposure to AGIQ decreased the number of hilar RELN$^+$ interneurons compared with PTU-alone group, while PTU-alone group did not change their number after the cessation of developmental hypothyroidism. We previously found sustained increase of immature population (NeuN$^{\pm\sim-}$) of RELN$^+$ cells through the adult stage by developmental PTU-exposure at 12 ppm, probably reflecting sustained neuronal mismigration (Saegusa *et al.*, 2010). However, at 10 ppm, increase in number of RELN$^+$ cells observed at the end of PTU-exposure was not sustained until adult stage (Shiraki *et al.*, 2016). In the present study, we also observed no changes in number of RELN$^+$ cells by 10-ppm PTU at the adult stage. Probably, neuronal mismigration caused by developmental hypothyroidism may be mostly recovered at the adult stage at this dose. AGIQ-induced reduction of RELN$^+$ cells may be the reflection of ameliorating effect of neuronal mismigration during postweaning period. While statistically significant difference was not attained, we also observed reducing tendency of immature (NeuN$^{\pm\sim-}$) RELN$^+$ population by postweaning exposure to AGIQ or ALA.

In conclusion, developmental hypothyroidism by PTU-exposure decreased intermediate progenitor cells and postmitotic granule cells in the SGZ/GCL, synaptic plasticity in the GCL, and PVALB$^+$ and GAD67$^+$ GABAergic interneurons in the hilar region, increased hilar SST$^+$ and CALB2$^+$ interneurons, and upregulated *Gria3*, *Otx2*, and antioxidant enzyme genes in the dentate gyrus on PND 77. These results suggest disruption of neurogenesis remained in relation with increase of oxidative stress and compensatory responses to the disruption at the adult stage. AGIQ recovered expression of some antioxidant enzyme genes and was effective for restoration of postmitotic granule cells and PVALB$^+$ and SST$^+$ interneurons. In contrast, ALA was effective for restoration of all interneuron subpopulations, as well as postmitotic granule cells, and upregulated *Grin2a* that may play a role for the restoration. Expression recovery of *Gria3* and *Otx2* by antioxidants suggested reversal of the compensatory responses to disruptive neurogenesis. Thus, postweaning antioxidant exposure may be effective for ameliorating developmental hypothyroidism-induced disruptive neurogenesis by restoring the function of regulatory system.

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Conflict of interest---- Mihoko Koyanagi and Shim-mo Hayashi are employed by food additive manufacturer whose product lines include α-glycosyl isoquercitrin. The views and opinions expressed in this article are those of the authors and not necessarily those of their respective employers. Takaharu Tanaka, Yasunori Masubuchi, Rena Okada, Kota Nakajima, Kazuki Nakamura, Sosuke Masuda, Junta Nakahara, Robert R. Maronpot, Toshinori Yoshida, and Makoto Shibutani declare that no conflicts of interest exist.

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