Effect of thyroxine on synaptotagmin 1 and SNAP-25 expression in dorsal hippocampus of adult-onset hypothyroid rats

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ABSTRACT. Adult-onset hypothyroidism causes cognitive dysfunctions of learning and memory, in which many synaptic proteins in hippocampus are involved. In our work, we studied the effect of adult-onset hypothyroidism on the expression of synaptotagmin 1 (syt 1) and SNAP-25 in dorsal hippocampus as well as its recovery by levothyroxine (L-T4) replacement therapy. Rats were divided into 4 groups: control, hypothyroidism, and hypothyroid rats treated with 5 μg T4/100 g body weight (BW) and 20 μg L-T4/100 g BW, respectively. Protein levels of syt 1 and SNAP-25 in dorsal hippocampus were determined by Western blot and immunohistochemistry. The immuno blot analysis indicated that syt 1 was expressed at a significantly lower level in hypothyroid rats, while the level of SNAP-25 was much higher compared to controls. Furthermore, using immunostaining, we found that on the one hand, expression of syt 1 was significantly down-regulated in the examined layers of CA1 and CA3 sub-regions but not dentate gyrus (DG); however, on the other hand, expression of SNAP-25 was up-regulated in the layers of CA1, CA3, and DG. Two-week treatment with 20 μg L-T4/100 g BW fully restored the levels of syt 1 and SNAP-25 to the normal level, which was more effective than 5 μg L-T4/100 g BW that partially restored the levels of both proteins. These results suggest that adult-onset hypothyroidism caused down-regulation of syt 1 and up-regulation of SNAP-25 level in dorsal hippocampus, which could be restored by L-T4 treatment, and the recovery degree is related to the L-T4 dosage. (J. Endocrinol. Invest. 34: 280-286, 2011) ©2011, Editrice Kurtis

INTRODUCTION

Adult-onset hypothyroidism causes a wide range of central nervous system dysfunctions, including learning and memory impairment (1-4). Previous studies revealed that thyroid hormone deficiency in adulthood affected performances in hippocampal-dependent tasks of learning and memory (4, 5), in which synaptic proteins that mediate the release of neurotransmitters were usually involved (6). Synaptotagmin 1 (syt 1), a Ca²⁺-dependent synaptic vesicle protein, can bind to SNAP-25 (synaptosomal-associated protein of 25 kDa) on pre-synaptic membrane to facilitate neurotransmitter release (7). Although a few reports have shown that thyroid hormone regulates the expression of syt 1 and SNAP-25 during rat brain development (8, 9), the expression of both proteins in the brain of adult-onset hypothyroid rats has not been studied well.

T₄ replacement therapy, the standard treatment of hypothyroidism, appears to be effective in restoring euthyroidism biochemically as evidenced by T₄ and T₃ concentrations within the normal range (10), and thereafter ameliorates the brain impairment induced by the adult-onset hypothyroidism (4), though there are variable results from clinical researches with regard to whether the hormone substitution therapy fully restored the impaired cognitive functions (10-13). By looking into expression of synaptic proteins upon T₄ replacement therapy, Alzoubi et al. reported that T₄ treatment which resulted in euthyroidism could normalize hypothyroidism-induced reduction of phosphorylated (P)-CAMKII, neurogranin, and calmodulin to basal levels but not protein kinase C-γ levels in hippocampal CA1 region of adult rats (14). This asynchronous recovery of the synaptic proteins may be attributed to the administrated dosage of T₄ and/or the different sensitivities of proteins to T₄.

In our study, we investigated the expression of syt 1 and SNAP-25 in the dorsal hippocampus of adult-onset hypothyroidism and the efficiency of levothyroxine (L-T₄) replacement therapy on the altered proteins expression in the 6-n-propyl-2-thiouracil (PTU)-induced hypothyroid rats.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague-Dawley rats weighing 280-300 g were used in current experiments. All rats were purchased from Nanjing Experimental Animal Center (Nanjing, China). They were maintained under standard laboratory conditions with a natural light-dark cycle and had free access to food or water. Procedures involving animals and care of them were performed according to the Animal Care and Use Committee of Anhui Medical University.

Forty-five healthy rats were divided into 4 groups randomly as indicated below.

1) Hypo group: 12 rats with hypothyroidism induced by daily ip injection of PTU [dissolved in saline solution, 1 mg/100 g body weight (BW)] for 6 weeks;
2) T₄-5 group: 11 rats were treated with PTU for 6 weeks as de-
scribed above. But since the 5th week, they were also treated with ip-injected L-T2 (dissolved in saline solution, 5 μg/100 g BW) every day for 2 weeks;
3) T2-20 group: 11 rats were treated according to the same protocols as the T2-5 group except increasing the dosage of L-T2 to 20 μg/100 g BW;
4) C group: 11 control rats were given the same volume of saline solution for 6 weeks.

**Thyroid hormones**

Twenty-four hours after the delivery of the last dose, all rats were anesthetized by chloral hydrate (350 mg/kg BW); 1.5 ml blood was collected from abdominal aorta and centrifuged at 11,000 \( \times g \) for 15 min immediately (4). Serum was collected and quickly frozen at −80 C until assayed by a radioimmunoassay kit (North Institute of Biological Technology, Beijing, China) to measure serum T3 and T4. The detection ranges of the assay were 0.9-2.2 ng/ml for T3 and 45-135 ng/ml for T4.

**Western blot analysis**

Crude synaptosomes were isolated from the dorsal hippocampus of each rat as previously described (15). In brief, the dorsal hippocampus was homogenized in Dounce homogenizers containing ice-cold HEPES buffer (10 mM HEPES, 1 mM EDTA, 10% sucrose, pH 7.4) plus a Protease Inhibitor Cocktail (2 μl/ml buffer; Sigma, USA). The homogenate was centrifuged for the first time at 1,000 × g for 8 min. The pellet was discarded, and the supernatant was centrifuged again at 9,500 × g for 15 min. The supernatant was discarded and the pellet (crude synaptosomal fraction) was reconstituted in ice-cold HEPES buffer plus protease inhibitors as described above and stored at −80 C until use. Protein concentration was determined by the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories). Samples (containing 20 μg protein of each) were run in triplicate and transferred onto a BioTrace polyvinylidene fluoride membrane (Amersham Biosciences). The membrane was blocked in freshly prepared Tris-buffered saline (TBS), pH 7.2, with 5% nonfat dry milk for 1 h at room temperature, then incubated with primary antibodies for syt 1 (1:200; goat polyclonal, Santa Cruz, USA) or SNAP-25 (1:15,000; rabbit polyclonal, Sigma, USA) at 37 C for 1 h and overnight at 4 C, followed by washes in PBS, incubation with the biotinylated goat anti-rabbit IgG kit (Maixin-Bio ltd, China) for 15 min at 37 C and washed in PBS. Sections were further incubated with the HRP for 10 min at 37 C, washed in PBS and colored with diaminobenzidine (DAB) (Maixin-Bio ltd, China) at room temperature for 7 min. Finally, sections were counterstained with hematoxylin for 3 min, dehydrated, rinsed, and covered slipped with glycerin. Sections that were not incubated with primary antibody served as negative controls.

An image analysis system was used for quantitative analysis. The system includes MetaMorph image acquisition and processing software (JADA 801D, China), a Nikon 80 microscope (Nikon, Japan) equipped with a HP computer. The analyzed layers from different subfields of the dorsal hippocampus are the stratum oriens (SO), stratum radiatum (SR), and stratum lacunosum-moleculare (SLM) in the CA1; SO, stratum lucidum (SL) and SR in the CA3; polymorphic layer (PL) and molecular layer (ML) in the dentate gyrus (DG). First, a picture of complete hippocampal formation was obtained at low magnification of ×40. Then, pictures of higher magnification of ×200 in various subfields of the hippocampus were acquired according to the size of each subfield: 3 pictures in CA1 for SO, SR; 1 picture in CA3 and DG-PL; 2 pictures in DG-ML and CA1-SLM. Digital data were exported into MetaMorph software for analysis and processing. The average optical density (OD) represented the intensity of immunohistochemical staining.

**Statistical analysis**

All statistical analysis was performed using SPSS 16.0 software. Values were expressed as mean±SEM. T3, T4 levels in serum as well as the immunoreactivity of syt 1 and SNAP-25 in different experimental groups were analyzed by one-way analysis of variance using least-significant difference for post-hoc analysis. \( p<0.05 \) was considered to be statistically significant.

**RESULTS**

**Thyroid hormone levels**

The average levels of serum T3 and T4 were significantly low in the Hypo group (\( p<0.02 \)) but high in the T4-20 group (\( p<0.001 \)) compared to the C group, the average levels of serum T3 and T4 in the T4-5 group is similar to the C group (Table 1).

**Protein levels of syt 1 and SNAP-25 in dorsal hippocampus**

**Western blot analysis**

Western blot analysis was performed to assess if the expression level of syt 1 and SNAP-25 in the dorsal hippocampus was affected by changes in the level of thyroid hormone. Our results showed that the amount of syt 1 in the hypothyroid rats was significantly decreased by 36% (\( p=0.007 \)), but the numbers were partially recovered...