

Alterations in cerebrocortical synaptosomal acetylcholinesterase activity and histomorphology of thyroid gland of adult rat during different thyroidal conditions

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The role of thyroid hormone (TH) in adult mammalian brain function including cholinergic neurotransmission is illusive, and its clinical application in recovery of mood disorder during altered thyroidal conditions appears to be dispersed. In this study, we explored whether TH supplementation helps in recovery of adult brain status during thyroid dysfunction. We observed a dose-dependent stimulation of acetylcholinesterase (AChE) activities by triiodothyronine (T3) in the synaptosomes prepared from adult rat cerebral cortex. The AChE activation in both propylthiouracil (PTU)-treated and T3-treated animals remained elevated indicating the presence of an autoregulatory mechanism for thyroid hormone levels in altered thyroidal conditions. Additive effects were also found with gradual decreases in the enzyme activities at 24, 48 and 72 h after single injection of T3 to the PTU-treated rats. It appears that T3 exhibit a stimulatory effect on cholinergic neurotransmission in adult mammalian brain. Moreover, the histomorphological observation of thyroid gland revealed the disappearance of colloids and changes of follicular structures in 14-day PTU-treated condition. But the colloids reappeared and follicular structures normalized after a single injection of T3 to PTU-treated animals gradually at 24, 48 and 72 h. The results therefore, indicates that T3 supplementation can augment hypothyroid induced alterations of central cholinergic neurotransmission *vis-à-vis* peripheral thyroid morphology.

Keywords: Mood disorder, Propylthiouracil, Synaptosomes, Thyroid morphology, Triiodothyronine

Thyroid dysfunction is associated with the cognitive and affective disorders in adult humans that can be cured reversibly by thyroid hormone treatment¹⁻³. Although tetraiodothyronine (T4)-monotherapy adjust circulatory T4/T3 molar ratio and/or TSH level in hypothyroid patients, it is not sufficient to recover mood disorder for certain hypothyroid cases, particularly for those patients (16% of hypothyroid patients) who have deiodinase-type-2 polymorphism (Thr92Ala). The combine therapy with T4 and T3 is suggested to be beneficial for these groups of hypothyroid patients for improving the mood disorder in addition to gaining the peripheral euthyroidism. However, the clinical significance of combined T4 and T3 therapy for hypothyroidism is still a controversy⁴⁻⁶. In addition, the T3 action in adult mammalian brain is still not clear^{7,8}.

Adult brain^{9,10} and nerve terminals¹¹⁻¹³ accumulate T3 and maintain T3 level in altered thyroid conditions. Both, genomic and non-genomic

(adrenergic) pathways, are reported to be involved in synaptic T3 homeostasis in adult brain^{14,15}. Thyroid hormone influences adult brain neurotransmission^{16,17} and T3 supposedly acts as neurotransmitter in association with adrenergic neurons¹⁸. Thyroid hormone acts non-genomically at synaptic level through activation of G-protein linked pathway, alters Na/K-ATPase activity¹⁹, modulates protein phosphorylation²⁰, and thus remains involved in regulation of synaptic function. Our previous *in vitro* studies with intact synaptosomes, an enucleated model of synaptic regions, isolated from cerebral cortex of adult male rat brain, under depolarized conditions indicate that T3 acts non-genomically at synaptic level to enhance Ca²⁺/NO-dependent cholinergic neurotransmission and subsequently rapid synaptic Ca²⁺ recovery²¹⁻²³.

Involvement of T3 to cholinergic neurotransmission is evident in adult mouse brain²⁴ and rat sympathetic superior cervical ganglion²⁵. Alteration of thyroid states is found to be related with modulation of presynaptic D₂ receptor for release of dopamine and acetylcholine in adult brain²⁶. Further, n-propylthiouracil

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(PTU)-treated hypothyroidism induces a significant decrease of acetylcholine content^{27,28} and acetylcholinesterase²⁸ activity in hippocampus of adult rats, which are restored to control level by T4 administration for two weeks after PTU-treatment. The studies with neuroblastoma cells (Neuro-2a) indicate that thyroid hormone activates serine/threonine protein-kinase pathway through activation of thyroid nuclear receptors and stabilizes mRNA of AChE²⁹. But the definite role of thyroid hormone on cholinergic function of adult brain cerebral cortex is ambiguous as reports on both unchanged³⁰ and increased^{31,32} activity of the AChE either in thyroidectomized or PTU-induced hypothyroid rats are available. Both, PTU-induced hypothyroidism and T3-supplementation with single dose, stimulates AChE activity in cerebrocortical synaptosomes of adult rats³³.

However, knowledge on the role of thyroid hormone on cholinergic neurotransmission in adult rat brain is still not clear. The present investigation is an attempt to understand the same with different thyroidal states including T3 supplement after PTU-induced hypothyroidism. The status of the thyroid histology had been compared with central cholinergic neurotransmission during different thyroidal conditions.

Materials and Methods

T3 was obtained from Sigma Chemical Company (USA). Haematoxylin and Eosin were purchased from Gurr, Germany and B.D.H Poole, England respectively. All other chemicals used were of analytical grade and deionized doubled distilled water was used throughout the study. The experiments were performed on brain tissues prepared from adult (3 months old) male albino rats (approximate 180-200 g body wt.) of Charles Foster Strain. The animals were maintained in a temperature controlled room ($24 \pm 1^\circ\text{C}$) with a 12:12 h light/dark cycle and fed *ad libitum* with standard diet and gave free access to drinking water. All experimental protocols were approved by the Institutional Animal Ethics Committee at the Department of Physiology, University of Calcutta, India.

Preparation of T3 solution

For *in vivo* treatment, required amount of 3-5-3'-triiodo-L-thyronine (T3) was suspended in minimum amount of 0.9% NaCl solution and dissolved in 0.1N NaOH solution. Final volume was adjusted by 0.9%

NaCl solution. For *in vitro* treatment, required amount of 3-5-3'-triiodo-L-thyronine was suspended in minimum amount of double distilled water and dissolved in 0.1N NaOH solution. Final volume was adjusted by doubled distilled water.

Preparation of synaptosomes

Synaptosomes were prepared from the cerebral cortex according to the method of Whittaker with slight modifications²¹⁻²³. Briefly, cell debris and nuclei were removed from the 10% cortical homogenate in 0.32M sucrose by centrifugation at $2000 \times g$ for 5 min at 4°C . The supernatant was layered on 1.2M sucrose and centrifuged at $50000 \times g$ for 50 min using ultracentrifuge (Beckman-L7). The fraction collected between 0.32M and 1.2M sucrose layer was diluted to 1:1.5 with ice-cold, doubled distilled water, further layered on 0.8M sucrose and re-centrifuged at $50000 \times g$ for 30 min. The pellet (synaptosomal fraction) obtained was washed, repelleted at $20000 \times g$ for 5 min and finally suspended in 0.32M sucrose solution.

Assay of the acetylcholinesterase (AChE) activity

The AChE activity was assayed by following the method Ellman *et al.*³⁴ with some modifications. The enzyme activity was measured by following the rate of formation of the yellow colour produced by thiocholine (SCh), the AChE hydrolysis product of the substrate acetylthiocholine (AcSCh) and the reagent dithiobis-2-nitrobenzoic-acid (DTNB). The yellow colour formed due to production of 5-thio-2-nitrobenzoate that absorbs at 412 nm. In an assay medium of 0.5 mL, containing 60 mM Tris-HCl (pH 7.4), 5 mM AcSCh, 20-50 μg synaptosomal protein was incubated for 30 min at 37°C . Before incubation synaptosomal suspension was added to the buffer and the mixture was allowed to equilibrate at 37°C for 5 min and then the reaction was started by addition of the substrate to the mixture. The reaction was terminated by the addition of 0.1 mL of 15% perchloroacetic acid (PCA). Appropriate blanks in a total volume of 0.45 mL, containing 67 mM Tris-HCl buffer (pH 7.4) and the synaptosomal protein (20-50 μg) were run simultaneously. After addition of PCA the final volume of the blanks (0.6 mL) were adjusted by the substrate. Samples and blanks were centrifuged at 3000 rpm (REMI-R8C) for 10 min to precipitate protein. A supernatant of 0.1 mL was taken with 4 mL of the diluted DTNB solution to develop the colour of the product formed in the reaction. The intensity of the developed colour was

read at 412 nm within 3 min of the addition of diluted DTNB solution to the supernatant. The standard curve was calibrated with reduced form of glutathione at the final concentration range of 10–50 nmol. The assay was adjusted to allow the reaction to occur in the linear region for both synaptosomal concentration and incubation time. The enzyme activity was expressed as μmol product formed (SCh)/ min/ mg protein.

Treatment of animals

To evaluate dose response relationship various doses of T3 (0.1–5.0 $\mu\text{g/g}$ body wt.) were injected intraperitoneally (i.p.) at a single dose with a maximum of 400 μL volume to different groups of animals and sacrificed after 24 h of the injection³⁵. The control rats received equal volume of vehicle (0.9% saline alkalined with 0.1N NaOH). Animals were made hyperthyroid by a single injection of T3 (2 $\mu\text{g/g}$ body wt.) i.p. and were sacrificed after 24 h of T3 injection. Rats were made hypothyroid by single injection of n-propylthiouracil (PTU, 2 mg/100g body wt.) i.p. each day for 14 days³¹. Some of the hypothyroid rats were injected i.p. with a single dose of T3 (2 $\mu\text{g/g}$ body wt.) to counteract the effect produced by hypothyroidism. Animals were sacrificed after 24 h of the last injection of T3 or PTU or PTU plus T3. Also, a group of animals treated with PTU plus T3 were sacrificed after 48 and 72 h of the last injection of T3. The control rats received equal volume of vehicle (0.9% saline alkalined with 0.1N NaOH).

Thyroid histology

The thyroid glands were removed from sacrificed animals and immediately fixed in Bouin's fixative. Fixed tissues were processed through graded (30–70%) series of alcohols, dehydrated in xylene and finally embedded in paraffin block as the routine procedure. Sections of thyroid tissues were cut by microtome at 7 μm thickness and stained with haematoxylin and eosin.

Determination of cellular thickness and lumen area of thyroid follicles in histological sections

The digital images of histological sections were retrieved in Image J (National Institute of Mental Health, Bethesda, Maryland, USA) software program in computer for measurements and quantifications. The thyroid follicles having complete outer boundaries appeared in the histological sections were selected for measurements. The follicular lumen areas of two adjacent follicles were measured separately followed by taking average of two measures as “mean follicular lumen area”. The distance of the inner

layers of follicular cells of two adjacent follicles was considered as cellular thickness in histological sections. The average of three measures of cellular thickness in same adjacent follicles was considered as “mean follicular cellular thickness”. The ratio of “mean follicular lumen area” versus “mean follicular cellular thickness” was represented as “Thyroid Tissue Index” in the present study.

Determination of Protein

The protein concentrations were estimated by the method of Vera³⁶ using bovine serum albumin as standard.

Statistical analysis

Data are expressed as mean \pm SEM of the number of experiments indicated. Statistical significance was analyzed by two tailed Student's t-test with a significance cut off at $P < 0.05$.

Results

Dose response relationship between T3 and AChE activity in synaptosomes of pre-treated animals: *in vivo* effects of T3

The AChE activity increased in synaptosomes prepared from animals injected with different doses (0.1–5.0 $\mu\text{g/g}$ body wt.) of T3. The enzyme activity significantly increased at 0.2 $\mu\text{g/g}$ body wt. dose of T3 ($P < 0.01$) with maximum 1.44 fold higher effect at 2.0 $\mu\text{g/g}$ body wt. dose of T3, in comparison to untreated control value (0.242 ± 0.0030 μmol SCh/min/mg protein, $P < 0.001$). Significant dose dependent activation of the enzyme was found between 0.1 and 0.15 $\mu\text{g/g}$ body wt. (0.232 ± 0.0046 vs. 0.249 ± 0.0041 μmol SCh/min/mg protein, $P < 0.02$), 0.20 and 0.25 $\mu\text{g/g}$ body wt. (0.257 ± 0.0031 vs. 0.278 ± 0.0038 μmol SCh/min/mg protein, $P < 0.001$), 0.25 and 1.0 $\mu\text{g/g}$ body wt. (0.278 ± 0.0038 vs. 0.331 ± 0.0025 μmol SCh/min/mg protein, $P < 0.001$), 1.0 and 2.0 $\mu\text{g/g}$ body wt. (0.331 ± 0.0025 vs. 0.349 ± 0.0027 μmol SCh/min/mg protein, $P < 0.001$) doses of T3. There was no significant difference in the enzyme activity between 2.0 and 3.0 $\mu\text{g/g}$ body wt. (0.343 ± 0.00093 μmol SCh/min/mg protein) of T3 doses. The enzyme activity decreased significantly with higher doses i.e. 4.0 and 5.0 $\mu\text{g/g}$ body wt. (0.288 ± 0.0028 μmol SCh/min/mg protein and 0.281 ± 0.0046 μmol SCh/min/mg protein) of T3 than maximum value obtained but remained higher than untreated value. These results of the dose-dependent *in vivo* effects of T3 on synaptosomal AChE activities are summarized in Fig. 1.

Synaptosomal AChE activity during different thyroidal conditions: *In vivo* effects

The rats rendered both hyper- and hypo-thyroid showed 1.4 fold ($0.364 \pm 0.0070 \mu\text{mol SCh}/\text{min}/\text{mg}$ protein, $P < 0.001$) and 1.42 fold ($0.368 \pm 0.0070 \mu\text{mol SCh}/\text{min}/\text{mg}$ protein, $P < 0.001$) increase in enzyme activity, respectively, compared to the euthyroid controls ($0.260 \pm 0.0030 \mu\text{mol SCh}/\text{min}/\text{mg}$ protein). The enzyme activity decreased gradually when hypothyroid rats were treated with single injection of T3 for 24 h ($0.344 \pm 0.0054 \mu\text{mol SCh}/\text{min}/\text{mg}$ protein, $P < 0.02$), 48 h ($0.322 \pm 0.0050 \mu\text{mol SCh}/\text{min}/\text{mg}$ protein, $P < 0.001$) and 72 h ($0.302 \pm 0.0042 \mu\text{mol SCh}/\text{min}/\text{mg}$ protein, $P < 0.001$) compared to hypothyroid value. The enzyme activities were significantly different between 24 and 48 h ($P < 0.01$), 48 and 72 h ($P < 0.01$) of T3 treatment to hypothyroid rats. The enzyme activities of hypothyroid rats treated with T3 injection for 72 h remained significantly higher ($P < 0.001$) than euthyroid control value. These results of the effects of different thyroidal conditions on synaptosomal AChE activities are depicted in Fig. 2.

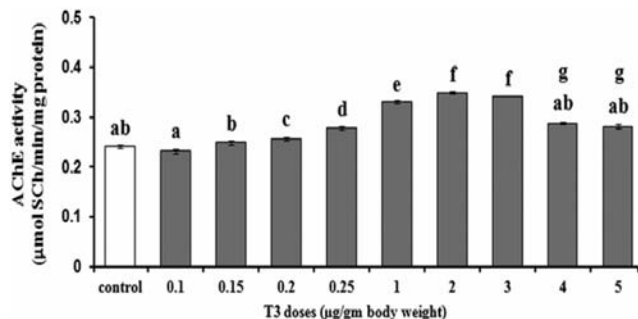


Fig. 1—*In vivo* effects of different doses of T3 on acetylcholinesterase (AChE) activity in adult rat cerebrocortical synaptosomes.

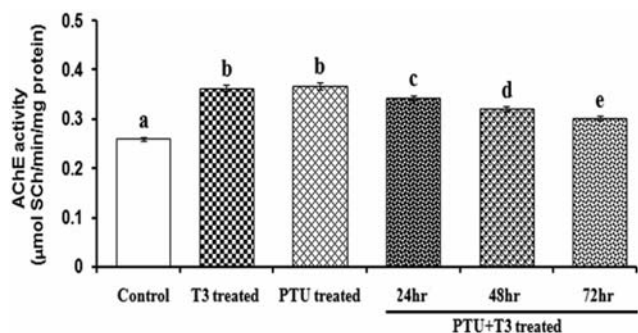


Fig. 2—Changes in acetylcholinesterase (AChE) activity of adult rat cerebrocortical synaptosomes during different thyroidal conditions.

Changes in histomorphological structure of thyroid gland

Histomorphological assessments performed on the sections of thyroid gland from the different groups of thyroidal conditions are presented in Fig. 3 (A-F). In control animals, the follicles of the thyroid section appeared to be filled with dense homogeneous colloid. The histological demonstrations of sections of thyroid gland from hypothyroid animals showed hyperplastic and hyperactive epithelial cells. The follicles remained empty with thickened follicular walls, infolded and collapsed in lumen. The follicular walls were prominent with more separate and round shaped large nuclei. Due to single injection of T3 to hypothyroid rats, reappearance of colloid and normalization of the follicular cells with compact, small and elongated nuclei occurred within 24 h and were almost normalized within 72 h, respectively. The quantitative measurements of “thyroid tissue index” (Fig. 3G) also indicate the gradual recovery of thyroid morphology with single dose of T3 administration in hypothyroid conditions.

Discussion

The enzyme acetylcholinesterase (AChE) is predominantly present in membrane bound form and plays important role in brain cholinergic neurotransmission^{37,38}. It hydrolyses acetylcholine (ACh) released from presynaptic terminals and thus terminates the action of this neurotransmitter. Therefore, the changes in synaptosomal AChE activity during different thyroidal states represent the status of the cholinergic neurotransmission in the present investigation. Animals treated with various doses of T3 showed increase in AChE activity with maximum effect (44.2% over control) at the dose of 2 µg/g body wt., (Fig. 1) that had been used in subsequent treatment for other experiments, *in vivo*. Both PTU-treated and T3-treated animals showed elevated AChE activity (Fig. 2). PTU-induced (14 days) hypothyroidism has been proved by histomorphological observations of thyroid gland that demonstrated disappearance of colloids and changes in follicular structures with lowering of the thyroid tissue index (decrease in follicular lumen area versus increase in cellular thickening) in our experimental condition (Fig. 3C & G). Although animals were rendered hypothyroid peripherally as revealed by lowering of T4/T3 level in serum in other study¹² and thyroid gland structural alteration in the present experiment, the AChE activity in cerebrocortical synaptosomes increased (41.5% over untreated)

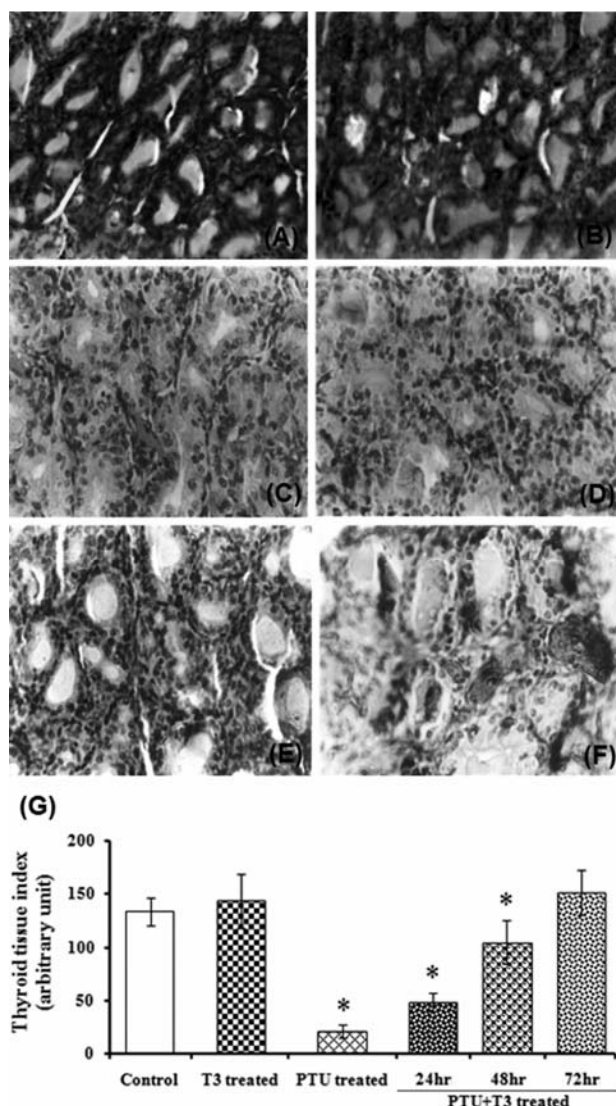


Fig. 3—Histomorphology of thyroid gland of treated adult rats with different thyroidal conditions. [(A) Vehicle treated control (Euthyroid); (B) 24 h after single injection of T3 (2 $\mu\text{g/g}$ body wt.) treatment (hyperthyroid); (C) PTU treated (2 mg/100 g body wt. for 14 days (hypothyroid); (D, E and F) 24, 48 and 72 h after a single injection of T3 (2 $\mu\text{g/g}$ body wt.) to PTU treated animals. All images were taken in Olympus microscope with X250 magnification; and (G) “Thyroid tissue index” calculated as the ratio of follicular lumen area versus follicular cellular thickness as described in “Materials and Methods” section. The follicles of the thyroid section of control groups appeared to be filled with dense homogeneous colloid. The follicles of the thyroid section of PTU-treated animals remained empty with thickened follicular walls, infolded and collapsed in lumen. PTU-treated animals showed hyperplastic and hyperactive epithelial cells. The follicular walls were prominent with more separate and round shaped large nuclei in PTU-treated animals. A single injection of T3 to hypothyroid rats causes reappearance of colloid, normalization of the follicular cells with compact, small and elongated nuclei occurred within 24 h and were almost normalized within 72 h. The “Thyroid tissue index” indicates quantitatively the gradual recovery of thyroid morphology with single dose of T3 administration in hypothyroid conditions]

significantly in the same condition in the present study. T3 (2 $\mu\text{g/g}$ body wt.)-treated animals also maintained similar level of elevated AChE activity (40% over control) as found in PTU-treated animals. The increase in AChE activity in both PTU-treated and T3-treated condition could reflect the increase in ACh release probably through neurotransmission. The synaptosomal T3 content was reported to increase at different levels during both hypothyroid and hyperthyroid condition compared to euthyroid animals^{10,12}. Rapid and selective accumulation of T3 in the nerve-ending fraction of brain i.e. in synaptosomes was reported after intravenous administration of radio-active- T3/T4^{9,11}. Elevation of T3 level in synaptosomes during hypothyroidism might be due to conversion of T4 to T3 by PTU-insensitive 5'-deiodinase-II (5'-D-II) enzyme^{10,39,40}. Although we cannot rule out the possibilities of onset of any stress in the early period of hypothyroidism (14-days PTU-induced), in which a number of events may also contribute to the elevated levels of AChE at this condition. Therefore, the elevated T3 level in nerve ending of adult rat cerebral cortex during PTU-treated and T3-treated conditions in our experiment might have increased the ACh release that was reflected to an increase in AChE activity. The increase in AChE activity in both hypothyroid and hyperthyroid states may be related to some presynaptic events *viz.*, (i) elimination of ACh-induced reduction of ACh release⁴¹; and/or (ii) reuptake of choline for formation of further releasable ACh⁴²; and/or (iii) co-release of ACh with other neurotransmitters⁴³. Reciprocally, the elevated AChE activity decreases availability of ACh to postsynaptic receptors. The net effect of the both presynaptic and postsynaptic events could be related to the maintenance of normal postsynaptic cholinergic neurotransmission during PTU-treated and T3-treated condition in the present investigation.

The single injection of T3 (2 $\mu\text{g/g}$ body wt.) to PTU-treated animals was found to have additive effects with significant decrease in AChE activity compared to that found in only PTU-treated condition. This condition was persisted for 72 h as observed in this study (Fig. 2). In an earlier study¹², similar condition of injection of 2 $\mu\text{g/g}$ body wt. of T3 to rat showed a synaptosomal T3 concentration of 1.07 ± 0.24 ng/mg protein after 24 h of T3 injection. The hypothyroid condition also demonstrated a synaptosomal level of T3 at 4.10 ± 0.06 ng/mg protein

at 24 h of last injection of PTU¹². Thus, it may be speculated that despite an induction of profound peripheral hypothyroidism, a condition of central hyperthyroidism had been created due to adaptive changes for T4 to T3 through induction of deiodinase type-II activity in brain in such PTU-treated condition, in our present experiment. Therefore, one may easily expect a stimulated AChE activity in the PTU-treated rats similar to only T3-treated (2 µg/g body wt.) condition. When a single injection of T3 (2 µg/g body wt.) was given to PTU-treated rats in the similar experimental condition, a synaptosomal T3 level was found to be at 2.56 ± 0.14 ng/mg protein after 24 h of T3 injection¹². It might be contemplated that such a PTU+T3-treated condition could compensate the stimulated deiodinase type-II system for generation of T3 towards normalization and therefore, showed an attenuated AChE activity with the fall of T3 level in comparison to the AChE activity found in only PTU-treatment in our experimental condition. Although the synaptosomal T3 content has not been measured at 48 and 72 h of single injection of T3 (2 µg/g body wt.) to PTU-treated rats, the gradual fall in AChE activity at this duration were most probably due to subsequent lowering of synaptosomal T3 levels owing to attenuate deiodinase type-II activity and its metabolic clearance. We cannot rule out the possibility of involvement of any stress factor originated from the onset of early peripheral hypothyroidism (after 14 days of PTU-treatment) to contribute to the alterations in the AChE activity as found in our PTU-treated and PTU+T3-treated conditions as described earlier. The synaptosomal AChE activity remained higher (16.2%) than control value after 72 h of T3 treatment.

This compensatory effect of T3 on hypothyroid condition also had been found in thyroid histology with gradual reappearance of colloids and regaining the normal follicular shape as indicated by regaining thyroid tissue index within 72 h of T3 injection (Fig. 3 C-F & G). It is reported that PTU is eliminated from serum within three days after discontinuation of PTU treatment in rats. After PTU withdrawal, thyroid gland initially accumulates PTU in correlation with the transient increase of thyroglobulin concentration in the follicular epithelium followed by elimination of PTU from thyroid gland for longer (1 week to 1 month) period⁴⁴. Histomorphological studies indicate that the follicles restore round shaped and the area of epithelium layer reduces until one week after PTU

withdrawal⁴⁵. Therefore, our present study infers that single dose of T3 administration might accelerate the regaining of thyroid histology from hypothyroid condition. T3 may act through pituitary-thyroid axis^{46,47} or directly on thyrocytes⁴⁷ for normalization of thyroid structure, which need further investigations.

Overall, T3 has stimulatory effect on cholinergic neurotransmission through elevation of adult rat cerebrocortical synaptosomal AChE activity in early altered thyroidal states. T3 administration may be beneficial for recovery of hypothyroid condition both in peripherally at thyroid level and centrally at synaptic level, the exact evidence of which is illusive.

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