Thyroid Hormone Homeostasis in Adult Mammalian Brain: A Novel Mechanism for Functional Preservation of Cerebral T3 Content During Initial Peripheral Hypothyroidism

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Abstract

The essential role of thyroid hormone (TH) on the maturation and differentiation of the mammalian brain is well known. But the action of THs in the adult brain was not widely a focus of study by endocrinologists based on lack of increased energy metabolism and oxygen consumption with changing thyroid status and thus not widely acknowledged. Extensive research has, however, revealed interesting findings like sequestration of T3, possible release of T3 as a neurotransmitter in nerve terminals, identification of specific membrane binding sites of T3 in the synaptosomal fraction of adult rat brain and many non-genomic neurotransmitter-like actions of TH in the adult mammalian brain. Most importantly, thyroid dysfunction is associated with significant disruption of psychobehavioural system in the adult, which can however be reversed with therapeutic hormonal intervention. A complex regulatory network involving transfer of TH through the brain barriers, interactions between neurons and glial cells, and deiodinase expression works synchronously to deliver the appropriate amount of T3 to the neurons. Despite peripheral hypo- or hyper-thyroidism, brain can maintain a normal level of TH up to certain duration. Thus, presence of a novel homeostatic mechanism in the adult mammalian brain ('central homeostasis for thyroid hormone') to defend the adverse neuropsychological manifestations commonly associated with peripheral hypothyroidism has been known for a long time. Unfortunately, the exact time course and the mechanism of such central homeostasis were not determined, till we made a pioneering attempt to evaluate the same. The entire phenomenon appeared to be coupled with nuclear mediated genomic processes like mRNA and protein synthesis. Moreover, the effects of THs on some key enzymes and ions related to neurotransmission during the start and end days of this central homeostatic phenomenon point towards a dependency of the enzymes on TH and an involvement of TH in the neurobiochemical events.
Key words: Thyroid hormone, central homeostasis, synaptosome, deiodinase II, cAMP, Na^+-K^+-ATPase, AChE, $[Ca^{2+}]_i$, Ca^{2+}/Mg^{2+}-ATPase.

Introduction

Revolutionary changes in our idea of thyroid hormone (TH) action initiated from the study on the metabolic role of thyroid gland [1]. As brain did not show alteration in O_2 consumption after treatment with TH, this organ was supposed to be TH-inert. During the last few decades enormous development on the thoughts and facts of molecular biology, along with appearance of newer micro-techniques, have helped us to understand about the diverse actions of TH on almost all organs of the mammalian vertebrates including the brain [2]. More than 110 years have passed since a committee of the Clinical Society of London stressed the important role of thyroid

gland in assuring normal brain development [3]. But efforts to understand the mechanism of the effect of TH in the central nervous system (CNS) have been hampered by the enormous complexity of the brain, which is made up of about 100 billions of cells and 100 trillions of synapses. The presence of thyroid hormone receptor (TR) in neural tissue at early stages of development suggests that foetal brain is an important target for TH. TH deficiency in both mother and foetus results in growth retardation as well as profound irreversible structural neurological deficits and mental retardation in the offspring [4, 5]. To be efficient on the normal brain development, T4 replacement has to be initiated shortly after birth, suggesting a "critical period" of TH actions for normal neural maturation [6]. In the early 1970s strong opinion prevailed that once the brain attains maturity it loses its sensitivity to TH, as it does not show any functional receptor-thyroid interaction. In fact, decrease in T3-binding has been shown in adult rat brain compared to developing brain [7, 8]. Extensive research, have revealed interesting findings like sequestration of T3, maintenance of T3 concentration, possible release of T3 as a neurotransmitter in nerve terminals [9, 10], identification of specific membrane binding sites of T3 in the synaptosomal fraction of adult rat brain [11, 12]. Moreover, studies using magnetic resonance spectroscopy and positron emission tomography have firmly indicated the adult brain as TH-responsive organ [13] and have provided a biological basis for the prevalent neurological and psychiatric signs observed during hypothyroidism [14]. These along with other evidences are sufficient to prove the involvement of TH in normal physiological functioning of the adult mammalian brain. But, it may be mentioned that the function of TH shows a paradigm shift from developmental to functional aspects in the adult brain. Adult-onset hypo- or hyper-thyroidism often impairs cognitive function and results in mood disturbances [15]. The neuropsychological symptoms commonly associated with hypothyroidism include inattentiveness, inability to concentrate, memory deficits, psychomotor slowing, depressive mood state, dementia, anxiety and delusions. Disturbances of cognitive and motor functions are also apparent in hyperthyroidism. Interestingly, these changes can be reversed by adjusting the circulatory levels of THs [16, 17].

II. Thyroid hormone and adult mammalian brain

(1) Transport and localization: The adult brain contains significant amount of both T4 and T3 with the molecular ratio T3/T4 considerably greater than that in the circulation [18]. Therefore, the uptake of T4 into brain from the circulating blood is the first step for all subsequent TH actions in the brain. The plasma T4 binding protein "transthyretin" (TTR) is a major binding protein in cerebrospinal fluid and facilitate the transport of T4 across the blood-brain barrier to the brain [19]. TH may then enter neurons directly from the interstitial fluid or indirectly from the glial cells. T4 uptake into neurons is a non-saturable process occurring by diffusion. In contrast, T3 uptake and entry into neurons is a carrier-mediated saturable process [20]. Now-a-days several molecular entities have also been identified as TH transporters [21]. Among them, members of the OATP (organic anion-transporting polypeptides) family like human OATP-F [22] and rat Oatp14 [23] are preferentially expressed in the brain. Monocarboxylate anion transporter protein (MCT8) has been detected in adult rat brain [24]. Autoradiographic studies have demonstrated selective

localization of [^{125}I]T3 in discrete neural systems after its intravenous administration to adult thyroidectomized rats. The radio-label is initially concentrated in nerve cell bodies in specific areas of grey matter and is subsequently transferred to related synapses by axonal transport [25].

(2) Deiodination : Most of the T3 is produced by enzymatic outer ring deiodination (ORD) of T4 in peripheral tissues. Alternatively, inner ring deiodination (IRD) of T4 yields the inactive metabolite, reverse T3 (rT3). Normally about one-third of T4 is converted by ORD to T3 which is further metabolized by IRD and rT3 largely by ORD, yielding in both cases the metabolite 3,3′-T2. Three enzymes catalyzing these deiodination reactions have been identified. The enzymes called iodothyronine deiodinases type I (DI), type II (DII) and type III (DIII) show distinct development- and tissue-specific pattern of expression. The deiodinases, act as 'guardians of the gate' of ligand-activated transcription modulation [26-28]. Up to 70% of T3 production originates via DI activity, mainly in the liver. DI is also expressed in the kidney, thyroid, pituitary and at lower levels in lung, intestine, muscle, spleen, [26]. 5'-deiodinase type II (DII) is extensively localized in the CNS, pituitary and brown adipose tissue (BAT) of rat [26-28]. DII is particularly important in the brain, producing more than 75% of local T3 [29, 30]. Moreover, DII activity is increased in hypothyroidism and decreased in hyperthyroidism [26, 28]. 5-deiodinase type III (DIII) mediates the degradation of TH since it has only IRD activity. DIII activity has been identified in placenta, neonatal skin, skeletal muscle and the CNS [28]. The brain is the predominant DIII-expressing tissue in adult animals, and may thus be the main site for the production of plasma rT3 from T4 and for clearance of plasma T3 by forming 3, 3′-T2 [27, 28, 30]. Therefore, this enzyme contributes to TH homeostasis by protecting tissues from an excess of TH [27, 28].

(3) Thyroid hormone receptor : Generally, TH enter the cell, proceed to the nucleus, and bind to the thyroid hormone receptors (TRs). TRs are DNA-binding transcription factors belonging to the nuclear receptor super-family. There are two isoforms of TR viz., TRα and TRβ. Of the two predominant TH, T3 binds to TR with greater affinity than T4. The human TRα gene, located on chromosome 17, encodes five protein products, TRα1, TRα2, TRα3 and the truncated products ΔTRα1 and ΔTRα2 through alternative splicing. Only TRα1 binds T3. The human TRβ gene located on chromosome 3 produces four T3 binding proteins, of which TRβ1, TRβ2 and TRβ3 bind to DNA and a truncated protein, ΔTRβ3 that binds T3 but not DNA. TRα2 do not bind T3 and thus, does not operate hormonal function [31]. Moreover, a transfection study also suggests its role as blocker of T3 effect mediated by TRα1 and TRβ1 [32]. Thus, the apparent unresponsiveness of adult brain to T3 for its nuclear-mediated function could be due to high levels of TRα2 isoform. Apart from nuclear TH receptor, the adult brain contains both cytosolic [33] and synaptosomal T3 binding sites preferentially localized on the synaptic membrane [11, 12]. These are of particular importance in adult since their binding capacity is higher than that found in developing brain. Their presence is consistent with selective accumulation of TH in synaptosomes after intravenous administration [34].

(4) Genomic and non-genomic action : Some actions of TH are independent of traditional nuclear TH receptors [35]. Recognition of the existence of such non-

nuclear or non-genomic TH actions has provided a complex picture of the roles of TH. However, "non-genomic" actions could modulate cell activities that are genomically driven by the hormone. Most importantly, time of onset of non-TR-mediated effects has been observed to require seconds to few hours, in contrast to the longer duration of TR-mediated effects. This is particularly significant in adult brain as TH has short term rapid non-genomic neurotransmitter-like action, distinct from the genomic actions regulating CNS development during the critical period of early life [9, 10]. Thyroxine-dependent modulation of actin polymerization in cultured astrocytes constitutes the best-documented example of non-genomic actions [36]. The influence of THs on G-protein synthesis, membrane receptor-G-coupling events, adenylyl cyclase activity, and phosphorylation events involved in transcriptional activities in the adult CNS are well established [37- 38]. Specific membrane binding of T3 leading to inhibition of Na^+-K^+-ATPase activity in cerebrocortical synaptosomes [12], T3 induced rapid influx of Ca^{2+} and rise of intrasynaptosomal free ionized Ca^{2+} level followed by nitric oxide synthase (NOS) activation in depolarization-induced adult rat cerebrocortical synaptosomes [39] seem to be possible non genomic response of TH in adult rat brain.

III. Synaptosomes as a novel biological model to study thyroid hormone action:
When brain tissue is homogenized in iso-osmotic aqueous sucrose under conditions of moderate shear force, the club-like central presynaptic nerve terminals are torn away from their axons and seal up to form detached particles, which are called as "synaptosome"[40]. Synaptosomes are important artificial models used for investigations of neurobiochemical events [41-42]. Cerebral cortex is the most important part of the central nervous system associated with motor function and intellectual performances. It is highly enriched with cholinergic neurons along with inputs from the noradrenergic and dopaminergic systems. Moreover, the highest numbers of TH receptors have been traced in the cerebral cortex of the rat brain. All these factors have made synaptosomes prepared from the cerebral cortex as the focus to study thyroid hormone-brain interaction.

(1) Thyroid hormone-synaptosome interaction: Some findings from this laboratory:
Studies have indicated the existence of two sets of synaptosomal T3 binding sites: one with high affinity low capacity having a mean Kd of 11.8 ± 5.3 pM and MBC (maximum binding capacity) of 3.73 ± 0.07 fmols per mg protein have high physiological significance. Another binding site, probably without any physiological significance, is of relatively low affinity high capacity having a mean Kd of 1.38 ± 0.50 nM and MBC of 348.50 ± 6.77 fmols per mg protein [11-12]. The Na^+-K^+-ATPase activity, functions as sodium pump by pumping sodium (Na^+) out of cells and potassium (K^+) in to maintain the membrane ionic gradients in neurons [43]. Maintenance of ionic gradients by plasma membrane Na^+-K^+-ATPase is one of the cellular processes by which THs regulate neuronal energy metabolism [44]. Experiments conducted *in vitro* with exogenous T3 revealed a graded increase in both the percentage of saturation of synaptosomal membrane specific T3 binding sites and the corresponding inhibition of synaptosomal Na^+-K^+-ATPase activity. These findings reflected a correlation between the binding of the hormone to the

synaptic membrane receptors and inhibition of synaptosomal Na^+-K^+-ATPase activity, showing that the enzyme is T3 responsive [12]. Acetylcholinesterase (AChE) plays an important role in brain cholinergic neurotransmission. It hydrolyses acetylcholine (ACh) released from pre-synaptic nerve terminals and thus terminates the action of this neurotransmitter. Although a complex relationship has been shown to exist between THs and cholinergic function during developmental stages and in adulthood [45-46], the definite role of TH on cholinergic function of adult brain cerebral cortex is still far from clear. T3 stimulated AChE activity in depolarization-induced intact synaptosomes suspended in calcium supplemented choline chloride buffer after T3 administration in a time (45-60 sec) and dose-dependent manner. Thus T3 was postulated to have a role in the Ca^{2+} dependent release/co release of ACh from intact synaptosomes concomitant with the acceleration of choline uptake mechanisms that has been reported to accompany elevation of AChE activity. Additionally electron microscopic structures showed condensation of the cytosolic content with increase in electron density, formation of intrasynaptosomal coarse vesicles and appearance of vesicular fusion like structures at the periphery in depolarization-induced T3-treated intact synaptosomes, further indicating the occurrence of release of neurotransmitters [47]. Administration of T3 also increased Mg^{2+}-ATPase activity maximally at 24 h in a dose dependent way. T3 stimulated ACh metabolism by increasing AChE activity as well as uptake of the released ACh through an increase in synaptosomal Mg^{2+}-ATPase activity. This indicated a positive impact of T3 on the cholinergic system in adult mammalian brain [48]. Calcium (Ca^{2+}) regulates a diversity of physiological processes, including neuronal excitability. Most of the intracellular Ca^{2+} remains bound at the endoplasmic reticulum, which act as a storehouse from where Ca^{2+} can be mobilized, when required, to increase the concentration of free Ca^{2+} in the cytoplasm. An increase in neurotransmitter release is accompanied by increased neuronal free $[Ca^{2+}]_i$ levels due to Ca^{2+} influx through voltage-sensitive Ca^{2+}-channels [49-50]. There have been reports of a link between TH and $[Ca^{2+}]_i$ [51]. T3 has been found to raise $[Ca^{2+}]_i$ during depolarized condition in a dose-dependent manner. The maximum rise in $[Ca^{2+}]_i$ within 5 sec of T3 application indicated an early event of Ca^{2+} accumulation that may be mediated through the altered activity of the voltage gated Ca^{2+} channel [39, 52]. TH is supposed to play a role on adult brain neuronal Ca^{2+}-mobilization through stimulation of the Ca^{2+}/Mg^{2+}-ATPase activity in nerve terminals. T3 also induced maximum activation of Ca^{2+}/Mg^{2+}-ATPase in depolarization-induced intact synaptosomes at 10sec after T3 application. T3-induced stimulation of Ca^{2+}/Mg^{2+}-ATPase activity is related to Ca^{2+} mobilization in nerve terminals of adult mammalian brain in rapid time dependent (<1 min) and dose dependent (within physiological range) manner [53]. The rise of intrasynaptosomal $[Ca^{2+}]_i$ level was again found to activate the constitutive nitric oxide synthase (NOS) enzyme, thereby producing NO which in turn is involved in the release of glutamate from adult brain synaptosomes. T3 mediated maximum NOS response seemed to be a resultant effect of maximum saturation of T3 binding sites on synaptosomal membrane at such T3 levels [39, 51, 53]. All these evidences indicated a relationship between TH, Ca^{2+} and NO in neurotransmission in adult mammalian brain.

(2) Thyroid hormone-synaptosome interaction: Recent findings

Concept of 'Central Thyroid Hormone Homeostasis': The influence of thyroid hormones in the normal functioning of the adult mammalian brain is diverse. The neurological and psychobehavioural abnormalities associated with adult-onset dysthyroidism are not a myth [15]. Nature has equipped the brain with a unique mechanism of autoregulation for its local T3 formation, to counteract the adverse neurobehavioural syndromes during altered peripheral levels of TH [18]. Such protective mechanism of the CNS for maintenance of T3 concentration so as to prevent the adverse neurobehavioural effects frequently associated with the hormonal imbalance is known as the 'central thyroid hormone homeostasis' [54]. This mechanism of central homeostasis is mainly mediated by the actions of the deiodinase enzymes, DII and DIII. While the activity of DII is upregulated during hypothyroidism, DIII activity is predominant under hyperthyroid conditions, to protect the brain from TH lack or excess [28, 55]. Thus, a coordinated regulation in the activities of DII and DIII is critical for the maintenance of the 'central thyroid hormone homeostasis' [28]. However, these defensive responses must have their limits. In prolonged hypothyroidism, near-exhaustion of the peripheral T4 stores would inevitably result in depletion of the brain THs [18] and the central homeostasis would cease to exist leading to development of clinical abnormalities. Although this concept of 'central homeostasis' has long been known, its onset, duration and termination were unknown till date. Thus, search for the time of onset of this homeostasis, its tolerance limit and termination became very much desirable.

(a) Evaluating the time of onset and termination of central thyroid hormone homeostasis (Table 1-2; Fig. 1-5) :

Groups	Total T4 (µg/dl)	Total T3 (ng/ml)	Synaptosomal T3 (ng/mg protein)
Control	7.425 ± 0.24	2.600 ± 0.19	2.282 ± 0.149
Day '2'	8.737 ± 0.53	1.711 ± 0.12	3.182 ± 0.020
Day '4'	7.292 ± 0.43	1.845 ± 0.06	3.828 ± 0.234
Day '6'	7.031 ± 0.53	1.621 ± 0.32	3.105 ± 0.174
Day '8'	3.676 ± 0.17	1.224 ± 0.27	3.106 ± 0.192
Day '10'	3.445 ± 0.24	1.191 ± 0.20	3.175 ± 0.109
Day '12'	2.394 ± 0.01	1.068 ± 0.04	3.178 ± 0.311
Day '14'	2.273 ± 0.04	0.949 ± 0.04	3.139 ± 0.149
Day '16'	0.778 ± 0.02	0.552 ± 0.16	2.861 ± 0.065
Day '18'	0.922 ± 0.06	0.615 ± 0.07	2.798 ± 0.186
Day '20'	1.314 ± 0.19	0.720 ± 0.02	1.782 ± 0.035
Day '24'	0.608 ± 0.13	0.725 ± 0.01	1.655 ± 0.179
Day '30'	0.883 ± 0.01	0.516 ± 0.06	1.391 ± 0.171

Table 1: Serum total T4/T3 levels and synaptosomal T3 content of Control and PTU-treated adult male Sprague Dawley rats.

Data are presented as Mean ± SEM of 12-15 individual animals in each group pooled from three sets of experiments. The control animals as sacrificed on different days in parallel with the treatment groups did not show any significant variation in the hormone level. Therefore, the control data were pooled in a single group and presented in the table.

	CONTROL	**PTU 2**	**PTU 20**
DII activity (fmoles T3/min/mg protein)	19.7 ± 1.8	31.0 ± 3.1	9.50 ± 0.9
cAMP content (pmoles/mg protein)	0.282 ± 0.014	0.367 ± 0.018	0.236 ± 0.012
Na$^+$-K$^+$-ATPase activity (μmoles Pi formed/h/ mg protein)	2.851 ± 0.303	5.212 ± 0.276	5.349 ± 0.284
AChE activity (nmoles thiocholine/min/ mg protein)	62.666 ± 1.160	51.999 ± 2.275	48.885 ± 0.085
[Ca^{2+}]$_i$ concentration (nM)	200.75 ± 12.31	107.11 ± 21.66	104.95 ± 16.19
Ca^{2+}/Mg^{2+}ATPase activity (μmoles Pi/h/mg protein)	0.700 ± 0.02	0.622 ± 0.01	0.926 ± 0.09

Table 2: Various neurobiochemical parameters related to operation of 'central thyroid hormone homeostasis' of adult male Sprague Dawley rats treated with PTU during onset and termination of 'central homeostasis for thyroid hormone' (Day '2' and Day '20' respectively).

Data are presented as Mean ± SEM of 12-15 individual animals in each group pooled from three sets of experiments.
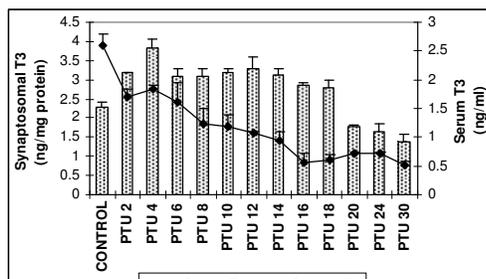

Fig. 1: Synaptosomal total T3 content (ng/mg protein) and serum total T3 (ng/ml) level of Control and PTU-treated adult male Sprague Dawley rats.
Daily intra-peritoneal injections of PTU (2 mg / 100g b.w.) were given to the animals from '0' day (day of first injection) to '30' day. Parallel control animals were injected with an equal volume of vehicle. Animals were sacrificed and cerebral cortex collected on alternate days (after 24 h of the last injection) for preparation of synaptosome. Blood samples for T3 ELISA were collected from the retro-orbital sinus before sacrifice of the animals. Data are presented as Mean ± SEM of 12-15 individual animals in each group, pooled from three sets of experiments. The vertical line denotes standard error. The control animals, as sacrificed on different days in parallel with the treatment group, did not show any significant variation in the hormone content. Therefore, the control data were pooled in a single group and shown in the figure.
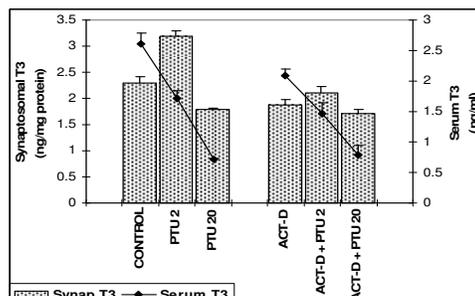
Fig. 2: Synaptosomal total T3 content (ng/mg protein) and serum total T3 (ng/ml) level of adult male Sprague Dawley rats treated with PTU along with ActinomycinD during onset and termination of 'central homeostasis for thyroid hormone' (Day '2' and Day '20' respectively).
Daily intra-peritoneal injections of actinomycin D (ACT-D; 20μg/100g b.w) were given to the animals either singly or in combination with PTU (2 mg/100g b.w.) for the first (Day '0' and '1') and last (Day '18' and '19') two consecutive days preceding the onset (Day '2') and termination (Day '20'), respectively, of central thyroid hormone homeostasis. Parallel control animals were injected with equal volume of vehicle. Animals were sacrificed and cerebral cortex was collected on Day '2' and Day '20' for preparation of synaptosome for T3 ELISA. Blood samples for total T3 ELISA were collected from the retro-orbital sinus before sacrifice of the animals. Data are presented as Mean ± SEM of 12-15 individual animals in each group, pooled from three sets of experiments. The vertical line denotes standard error.

PTU treatment is already known as the usual way for imposition of hypothyroidism in experimental animals [56, 57]. Adult male Sprague Dawley rats (150-160g) were injected intra-peritoneally with 2mg/100g body weight of 6n-propyl-2-thiouracil
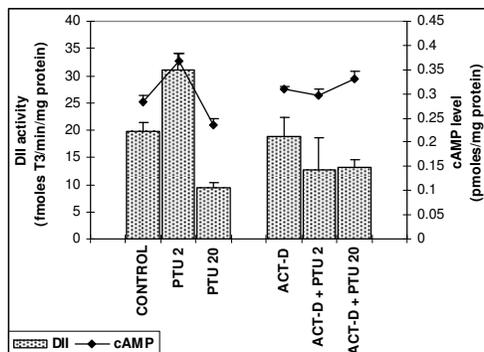
Fig. 3: Cerebro-cortical 3,5,3'-iodothyronine deiodinase type II (DII) activity (fmoles T3 formed/min/mg protein) of adult male Sprague Dawley rats treated with PTU along with Actinomycin D during onset and termination of 'central homeostasis for thyroid hormone' (Day '2' and Day '20' respectively). Daily intra-peritoneal injections of actinomycin D (ACT-D; 20µg/100g b.w) were given to the animals either singly or in combination with PTU (2 mg/100g b.w.) for the first (Day '0' and '1') and last (Day '18' and '19') two consecutive days preceding the onset (Day '2') and termination (Day '20'), respectively, of central thyroid hormone homeostasis. Parallel control animals were injected with equal volume of vehicle. Animals were sacrificed and cerebral cortex was collected on Day '2' and Day '20' for estimation of DII activity and cAMP level. Data are presented as Mean ± SEM of 12-15 individual animals in each group, pooled from three sets of experiments. The vertical line denotes standard error.
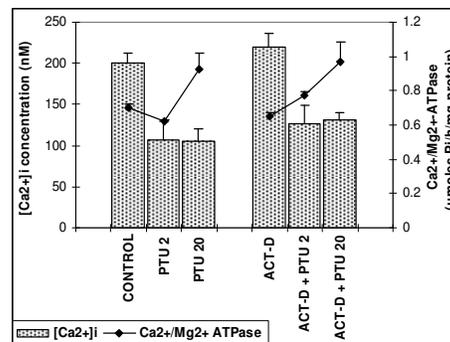

Fig. 4: Synaptosomal membrane Na$^+$-K$^+$-ATPase (µmoles Pi formed/h/mg protein) and Acetylcholinesterase (AChE, nmoles thiocholine formed/min/mg protein) activities of adult male Sprague Dawley rats treated with PTU along with Actinomycin D during onset and termination of 'central homeostasis for thyroid hormone' (Day '2' and Day '20' respectively). Daily intra-peritoneal injections of actinomycin D (ACT-D; 20µg/100g b.w) were given to the animals either singly or in combination with PTU (2 mg/100g b.w.) for the first (Day '0' and '1') and last (Day '18' and '19') two consecutive days preceding the onset (Day '2') and termination (Day '20'), respectively, of central thyroid hormone homeostasis. Parallel control animals were injected with equal volume of vehicle. Animals were sacrificed and cerebral cortex was collected on Day '2' and Day '20' for preparation of synaptosome for enzyme assay. Data are presented as Mean ± SEM of 12-15 individual animals in each group, pooled from three sets of experiments. The vertical line denotes standard error.

(PTU) for 30 consecutive days and sacrificed on every alternate day. We found an initial increase in serum total T4 on the 2nd day of PTU treatment. This response was typical of initiation of peripheral hypothyroidism involving hypothalamo-pituitary-thyroid axis [54, 56]. The level thereafter gradually declined from 4th day onwards. Unlike T4, the results indicated a triphasic pattern of decline in serum total T3 levels. The first phase began from the 2nd day of PTU treatment and continued up to the 6th day; the second phase lasted till the 14th day and the last phase continued till the 30th day. Histological evidences also were typical of peripheral hypothyroidism. Intact thyroid follicles with well-containing colloidal materials surrounded by epithelial cells in untreated animals were gradually replaced with diminishing colloidal space and infiltration of epithelial cells (hyperplasia and hypertrophy) as hypothyroidism progressed [57-58]. During peripheral hypothyroidism, as simulated in our experiment, cerebrocortical synaptosomal total TH levels were in direct contrast to the serum hormone titre. T4 level remained undetectable, as reported by Sarkar and Ray [54, 59]. This has been attributed to a rapid conversion of brain T4 to T3 by the PTU-insensitive DII enzyme [18, 29]. Moreover, T4 is converted to T3 by DII localized primarily in the glial cells adjoining neurons and the T3 thus formed is transported to the neurons via carrier-dependent processes across the neuronal cell membrane [20]. Therefore, due to high fractional rate of T4 deiodination, the chance of detecting T4 in the neuronal synaptosome remains minimal. We observed an

increase in the synaptosomal total T3 content on the 2nd day of PTU treatment compared to the control value, which reached a peak on the 4th day and maintained a higher level up to the 18th day. This study id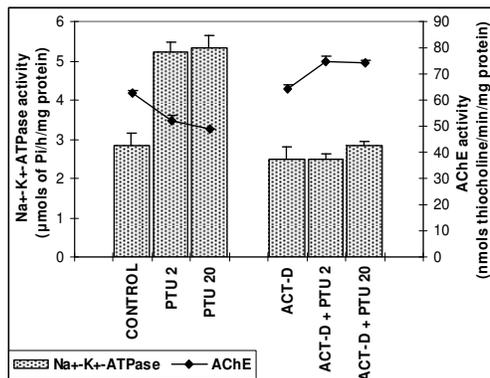entified that at sometime between the 1st and 2nd day lies the starting point of the very essential central auto-regulatory mechanism during peripheral hypothyroidism. This unique homeostatic phenomenon lasted for 16-18/20 days, after which the synaptosomal total T3 level declines consequently with the failure of the homeostatic mechanism [54, 58]. The deiodinase type II (DII) activity responsible for the cerebral conversion of T4 to T3, showed a high degree of correlation with synaptosomal T3 content (correlation coefficient r = 0.9857) [58]. DII activity increased on the day of onset (Day '2') of the 'central thyroid hormone homeostasis' concomitant to a rise in the synaptosomal T3 level. Such increased DII activity persisted till Day '18', the day up to which the brain T3 level is maintained. Thus, higher DII activity indicated an adaptive increase in response to PTU-induced

Fig. 5: Synaptosomal Ca^{2+}/Mg^{2+}-ATPase activity (µmoles Pi formed/h/mg protein) and intrasynaptosomal Calcium concentration ($[Ca^{2+}]_i$, nM) of adult male Sprague Dawley rats treated with PTU along with Actinomycin D during onset and termination of 'central homeostasis for thyroid hormone' (Day '2' and Day '20' respectively).

Daily intra-peritoneal injections of actinomycin D (ACT-D; 20µg/100g b.w) were given to the animals either singly or in combination with PTU (2 mg/100g b.w.) for the first (Day '0' and '1') and last (Day '18' and '19') two consecutive days preceding the onset (Day '2') and termination (Day '20'), respectively, of central thyroid hormone homeostasis. Parallel control animals were injected with equal volume of vehicle. Animals were sacrificed and cerebral cortex collected on Day '2' and Day '20' for preparation of synaptosome. Data are presented as Mean ± SEM of 12-15 individual animals in each group, pooled from three sets of experiments. The vertical line denotes standard error.

hypothyroidism to maintain the neuronal T3 level within a narrow limit. Again, the fall in DII activity after twenty days of continuous PTU injection (Day '20') is supportive of the decreased synaptosomal T3 content and disruption of the 'central thyroid hormone homeostasis' [58, 60]. The main signal for the central maintenance of T3 by increased DII activity is low serum total T4 content. In the absence or low concentration of T4, p29 (the substrate binding subunit of DII) is slowly endocytosed. Presence of T4 accelerates the rate of DII inactivation by polymerization of actin fibres [36, 61-62]. In the CNS, the DIII gene is highly T3 responsive, and its focal localization within the hippocampus and cerebral cortex suggests an important role for central T3 homeostasis. This DIII activity is decreased during peripheral hypothyroidism [28]. Again, DIII mRNA remained detectable in hypothyroid rat brain [63]. Therefore, another possibility is that the decreased DIII activity coupled with an increased DII responsiveness could mediate the central homeostasis [58]. DII is a cAMP-inducible enzyme [64]. It has been observed that cAMP stimulation led to an increase in the mass of the holoenzyme. Cyclic AMP stimulation also leads to the translocation of p29 to the plasma membrane coincident with the appearance of deiodinating activity [64]. There has been a significant rise in the cerebrocortical cAMP level on the day of onset of the 'central thyroid hormone

homeostasis' which can explain the increase in the deiodinating activity of DII and subsequent rise in brain T3 level. Similarly, the fall in cAMP level on Day '20' has been accompanied by a corresponding lower DII activity and T3 level. Thus the level of cAMP had a high correlation with the deiodinating capacity (correlation coefficient r = 0.9394) [58, 60]. Increased activity of Na^+-K^+-ATPase on the 2nd and 20th days after PTU treatment has been observed, which coincided with the respective days of onset and termination of the central homeostasis [54, 58]. This might be due to initiation of a stress phenomenon originating from sudden increase or decrease in brain T3 level [65]. The maintenance of a higher Na^+-K^+-ATPase activity along with a high T3 level during the homeostatic phase could be an indication of a neuronal homeostasis to maintain a stable neurophysiological function. This can be further justified by the decline in the enzyme activity after twenty days of PTU treatment with a fall in synaptosomal T3 content. The fact could be correlated with disruption of the 'central thyroid hormone homeostatic mechanism' [54, 58]. In our study, synaptosomal AChE activity decreased from the 2nd day of PTU injection accompanied with elevated brain T3 level [54, 58]. The high local synaptosomal level of T3 could be indicative of a depression in the AChE activity, exerting a feedback inhibition or toxic-inhibitory effect [48]. Moreover, Na^+-K^+-ATPase activity is negatively correlated with the AChE activity [66]. The decrease in AChE activity accompanied with a parallel decline in the synaptosomal T3 level after long twenty days of treatment could be correlated with decreased neurotransmitter receptor binding in cortex that may, however, imply some alterations in dendritic morphology of neurons [67]. Mental depression may arise when the homeostasis is lost as it is known that hypoactivity of cholinergic neurotransmission results in psychobehavioural changes [68]. In this study, the intrasynaptosomal $[Ca^{2+}]_i$ concentration was determined on the days of onset and termination of the 'central homeostasis for thyroid hormone' (Days '2' and '20' respectively). A fall in the level of $[Ca^{2+}]_i$ has been noted on both days [58]. On Day '2' the synaptosomal T3 concentration was high, which probably impeded the rise in $[Ca^{2+}]_i$. Chakrabarti and Ray (2000) also observed an attenuation of $[Ca^{2+}]_i$ with higher doses of T3, albeit under *in vitro* conditions. A lower $[Ca^{2+}]_i$ has also been observed on Day '20' when T3 was also low [58]. It has been reported above, that neuronal excitation and neurotransmitter release is accompanied by increases in intraterminal $[Ca^{2+}]_i$. During these two days of onset and termination of 'central thyroid hormone homeostasis', the Na^+-K^+-ATPase activity was elevated and AChE activity was suppressed. Literature suggested that $[Ca^{2+}]_i$ is associated with the release of endogenous ACh [69]. Moreover, Ca^{2+} is known to inhibit Na^+-K^+-ATPase activity [70], so the elevated Na^+-K^+-ATPase activity might be due to low $[Ca^{2+}]_i$. All these indicated a depressed state of neurotransmission. In the present experiment, the decline in Ca^{2+}/Mg^{2+}-ATPase activity on the day of onset of central thyroid hormone homeostasis (Day '2') was consistent with maintenance of lower intrasynaptosomal $[Ca^{2+}]_i$. But the rise in the enzyme activity on Day '20' deviate from the observation of a lower $[Ca^{2+}]_i$. It can again be speculated here that loss of central homeostasis has been accompanied by total disruption in the neurobiochemical events.

(b) Mechanism of homeostasis: Possible involvement of nuclear-mediated phenomenon (Table 3; Fig. 2-5):

	ACT-D	ACT-D + PTU 2	ACT-D + PTU 20
Serum T4 (µg/dl)	6.023 ± 0.15	6.583 ± 0.13	1.206 ± 0.02
Serum T3 (ng/ml)	2.096 ± 0.10	1.459 ± 0.19	0.779 ± 0.16
Synaptosomal T3 (ng/mg protein)	1.871 ± 0.114	2.107 ± 0.127	1.707 ± 0.089
DII activity (fmoles T3/min/mg protein)	18.9 ± 3.4	12.8 ± 5.7	13.1 ± 1.5
cAMP content (pmoles/mg protein)	0.311 ± 0.003	0.297 ± 0.014	0.330 ± 0.016
Na^+-K^+-ATPase activity (µmoles Pi formed/h/mg protein)	2.506 ± 0.292	2.488 ± 0.139	2.841 ± 0.114
AChE activity (nmoles thiocholine/min/ mg protein)	63.955 ± 1.598	74.719 ± 2.286	73.997 ± 1.507
$[Ca^{2+}]_i$ concentration (nM)	219.37 ± 16.84	125.79 ± 23.19	131.47 ± 7.950
Ca^{2+}/Mg^{2+}ATPase activity (µmoles Pi/h/mg protein)	0.650 ± 0.02	0.777 ± 0.02	0.966 ± 0.12

Table 3: Various neurobiochemical parameters related to operation of central thyroid hormone homeostasis of adult male Sprague Dawley rats treated with PTU alone or along with Actinomycin D (ACT-D) during onset and termination of 'central thyroid hormone homeostasis' (Day '2' and Day '20' respectively).

Data are presented as Mean ± SEM of 12-15 individual animals in each group, pooled from three sets of experiments.

During our investigation to understand the mechanism behind homeostasis, we injected the transcriptional (mRNA synthesis) blocker, actinomycin-D (ACT-D) at a dose of 20 µg/100g b.w along with PTU to the animals during the days of onset (Day '2') and termination (Day '20') of the homeostatic mechanism. There was no significant rise in synaptosomal T3 content after PTU injections of either ACT-D pre-treated rats on the 2nd day. One of the causes could be due to inhibition of protein biosynthesis for TSH from pituitary and related activation of thyroid gland for T3 release. Moreover, serum levels of T4 and T3 were also found to be low in this study. This eliminated the possible initiation of a homeostatic mechanism by inducing a rise in synaptosomal T3. On the 20th day, the synaptosomal T3 content in ACT-D + PTU-treated rats was the same as that observed after only PTU injection [60]. Treatment with ACT-D did not elicit any significant decrease in the DII activity at any time. It, however, did block the PTU-induced increase in DII activity observed on the day of initiation of central homeostasis (Day '2'), indicating that the increase require mRNA synthesis. Thus, the PTU-induced increase in DII activity was not due to activation of preexisting enzyme, but was due to an increase in expression of the gene, leading to subsequent enhanced synthesis of the protein. At the same time, an unchanged activity of the enzyme with respect to control could be due to our choice of the dose of the blocker that it did not disturb the control system, but it really blocked any stimulated gene expression. A further justification of the unchanged DII activity in all ACT-D-treated rats with respect to control animals can be had from the evaluation of the cortical cAMP level, which was maintained at the control value in all the ACT-D-treated groups [60]. The characteristic rise in the synaptosomal membrane Na^+-K^+-ATPase activity observed after PTU treatment on the 2nd and 20th days pertaining to the respective days of onset and termination of central thyroid hormone homeostasis was not observed after combined treatment with ACT-D and PTU. On the contrary, it resulted in significant decline in the enzyme activity, although the synaptosomal T3 level was unaffected. It has been proposed that hormones that

activate the cAMP-dependent protein kinase A (PKA) [71] down regulate the Na^+-K^+-ATPase activity. During administration of ACT-D with PTU, there was unchanged level of cAMP, that might have been a causative factor in the inhibition of Na^+-K^+-ATPase activity. Since, the higher level of brain T3 after only PTU-treatment for two consecutive days that induced a corresponding rise in the enzyme activity has already been attributed to initiation of some kind of stress [54, 58], treatment with the ACT-D might also have inhibited the initiation of the stress process leading to diminished Na^+-K^+-ATPase activity. Again, the unchanged or lower enzyme activity compared to control on the day of termination of central homeostasis can also be explained by blockade of the stress factor. Higher values of AChE activity in rats treated with ACT-D + PTU were observed both during initiation and termination of central homeostasis. This has been supposed to be due to decreased Na^+-K^+-ATPase activity during these days, as an inverse relation between the two enzyme activities is already known [66]. Weidoff *et al*. [72] observed that degradation of the acetylcholine receptor (AChR) was slower in ACT-D treated muscle culture cells resulting to a two-fold increase in receptor half-life; and any change in the rate of AChE secretion was accompanied by an identical change in the rate of AChR incorporation into the plasma membrane [73]. PTU-treatment along with ACT-D, both during the days of onset and termination of the 'central thyroid hormone homeostasis', resulted in significant decreases in the level of intrasynaptosomal calcium ($[Ca^{2+}]_i$) concentration. These drugs alone had no effect on $[Ca^{2+}]_i$ and this proves that the lower level can be attributed to the effect of PTU treatment. In case of ACT-D treatment, no perturbation in the level of synaptosomal T3 was observed, which could be the reason for an unchanged Ca^{2+}/Mg^{2+}-ATPase activity. But the enzyme showed an elevation in the 20th day, when termination of homeostasis has been predicted. This observation was similar to that observed when PTU was administered alone for twenty days.

Conclusion

Thyroid hormone is very much tricky in the sense that it effects almost all organs and affects perhaps a good number of biochemical parameters. Among other organs, brain was once supposed to be the most TH-inert organ, about half a century before. Thanks to the human endeavour for development of science, with creation of many micro-techniques in molecular biology. With such techniques and constant search for TH actions, the brain is now visualized as one of the most complicated TH-responsive organ. The existence of a 'central thyroid hormone homeostatic mechanism' under conditions of peripheral hypothyroidism to preserve brain T3, has long been presumed in the adult mammalian CNS, but not sequenced. Our work represented a pioneering attempt to define the chronology of the central thyroid hormone homeostasis by identifying its onset between 1st and 2nd day of an anti-thyroid drug treatment, its continuation for 16-18 days and its end-point between 18th and 20th day, after which the homeostasis faltered [54, 58].. Thus, despite an induction of profound peripheral hypothyroidism, a condition a central TH homeostatic mechanism has been indeed created for sometime. Under conditions of reduced substrate (T4) availability during hypothyroidism, the DII activity is stimulated accompanied with a parallel increase in cAMP content. The maintenance

of thyroid hormone concentrations in the adult mammalian CNS appears to be a novel and complicated phenomenon involving a host of factors ranging from mRNA and protein synthesis-mediated genomic mechanisms to cell signaling involving the G-protein coupled receptors. Neurotransmission is definitely altered as observed by increased Na^+-K^+-ATPase activity leading to decreased AChE activity, $[Ca^{2+}]_i$ accumulation and Ca^{2+}/Mg^{2+}-ATPase activity. Moreover, termination of the homeostasis was accompanied by total failure of the neurotransmission process. These neuronal modulators working synchronously during the homeostatic phase along with TH itself, may serve to counteract the various altered psychobehavioural responses of animals exposed to hypothyroid condition. The present review may be regarded as a foundation upon which much remains still to be done to get a clearly defined insight into the neuroendocrinology of TH during clinical hypothyroidism. This may be a key factor to unravel the mysteries of psychobehavioural and intellectual disturbances during altered thyroid states. Unraveling the mysteries of TH in brain function perhaps needs for creation of another "Aladdin with his magic lamp".

Acknowledgement

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