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Evaluating the role of Adrenergic compounds on Synaptosomal Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase during Central Thyroid Hormone Homeostasis

Samita Kundu[1*], Sumedha Roy[2], Angshuman Biswas[3], Arun K. Ray[4]

[1*]Post-Graduate Department of Zoology, Vivekananda College, Thakurpukur, Kolkata, India

[2] Ex-Assistant Professor, The University of Burdwan, West Bengal, India.

[3]Department of Zoology, Sreegopal Banerjee College, Hooghly, West Bengal, India.

[4]Division of Molecular Medicine, Bose Institute, Kolkata, India

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Abstract: **Hypothyroidism is associated with significant symptoms and an adverse effect on the quality of life. There exists a central homeostatic mechanism in the adult mammalian brain to defer these adverse neuropsychological manifestations, for some days. Thyroid hormone is known to affect the activities of some enzymes involved in neurotransmission. In this study, a reflection of the physiological consequences of 'central thyroid hormone homeostasis' has been addressed with the study of the activities of the synaptosomal membrane Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase on the days of onset and termination of the homeostatic phenomenon. Moreover, certain adrenergic receptor (α_2- and β-) agonists and antagonists have profound influence on synaptosomal thyroid hormone levels during the days of initiation and termination of the 'central homeostasis'. This notion has further prompted us to investigate the effects of these compounds on Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase during the start and end days of this central homeostatic phenomenon. The altered activities of these enzymes on these two days point towards their dependency on cerebral thyroid hormone levels and thus the hormonal involvement in neurobiochemical events. The entire phenomenon of homeostasis thus appears to be a stress-mediated event involving an array of adrenergic non-genomic effects, further indicating a nongenomic role of thyroid hormone during homeostasis.**

Keywords: **Synaptosome, Na^+-K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase, thyroid hormone, homeostasis.**

I. INTRODUCTION

The adult mammalian brain can maintain a normal level of thyroid hormone (TH) up to a certain duration despite ensuing peripheral hypothyroidism. This novel homeostatic mechanism or the 'central thyroid hormone (TH) homeostasis' has the onset between the 1st and 2nd day of anti-thyroid drug treatment, continues for 16-18 days and ends between the 18th and 20th day [1]. This 'central homeostasis' serves to defend the adverse neuropsychological manifestations commonly associated with peripheral hypothyroidism, for a certain length of time. Chronic and acute hypothyroidism are associated with a spectrum of psychobehavioural disorders, involving derangements in neurotransmitter metabolism and function [2].

Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are two ATP-hydrolyzing enzymes which maintain the electrochemical gradient in the cells in an energy-dependent manner. Na^+-K^+-ATPase extrudes three Na^+ molecules in exchange for internalization of two K^+ molecules. Membrane Na^+-K^+-ATPase is linked with several aspects of neuronal activity that involve active ion transport across the cell membrane, maintenance of a polarized condition of neuron and also helps in the metabolic uptake and release of neurotransmitters [3]. The synaptosomal membrane Na^+-K^+-ATPase has also been identified as a thyroid hormone responsive physiological parameter in adult rat cerebral cortex, where stimulation of this enzyme activity has been accompanied with higher level of synaptosomal T3 in cerebro-cortical synaptosomal membrane during hypothyroid condition [4], [5].

Calcium (Ca^{2+}) regulates a diversity of physiological processes related to neurotransmission, so regulation of intracellular Ca^{2+} is significant. The Ca^{2+} homeostasis is achieved by the concerted operation of several Ca^{2+}-transporting systems located in the plasma membrane, endoplasmic reticulum and mitochondria [6], [7]. In general, ATPases are present to transport or extrude Ca^{2+}. The plasma membrane Ca^{2+}-stimulated Mg^{2+}-dependent ATPase (Ca^{2+}/Mg^{2+}-ATPase) is a Ca^{2+} pump that expels Ca^{2+} from the cells. This enzyme is characterized by a strict dependence on Mg^{2+} for activity, a high affinity for Ca^{2+} and relatively high affinities for both Mg^{2+} and ATP [8]. Interestingly, relationship exists between TH levels and $[Ca^{2+}]_i$ and stimulatory effects of TH on plasma membrane Ca^{2+}/Mg^{2+}-ATPase has been reported in erythrocytes, thymocytes, myocardium, skeletal muscle and also in adult rat cerebro-cortical synaptosomes [9], [10], [11], [12], [13]. A dose-dependent activation of the enzyme activity have been well correlated under *in vitro* conditions with a similar rise in intrasynaptosomal $[Ca^{2+}]_i$ in depolarized synaptosomes prepared from adult rat brain [14].

The current study attempts to evaluate the activity of Ca^{2+}/Mg^{2+}-ATPase and correlate it with the Na^+/K^+-ATPase activity in synaptosomes during the specific days of onset (Day '2') and (Day '20') termination of 'central thyroid hormone homeostasis'. TH produces effects similar to those of noradrenergic receptor [15]. There are reports of a complex inter-relationship between α and β- adrenergic receptor (AR) agonists and antagonists on thyroid status during Days '2' and '20' of central homeostasis [16]. With this view, attempts have also been made to assess the activities of Ca^{2+}/Mg^{2+}-ATPase and Na^+-K^+-ATPase when specific doses of AR agonists and antagonists were administered during the days of onset and termination of central thyroid hormone homeostasis.

In the present study, Section I was a brief introduction along with the related work on the roles of Ca^{2+}/Mg^{2+}-ATPase and Na^+-K^+-ATPase and their relationship with T3. Section II describes the methodology used for the preparation of synaptosomes and measurement of Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities. Section III provides the results with figures. Section IV is for discussion and Section V concludes the research findings promoting scope for future study.

II. MATERIALS AND METHODS

Animals and treatment:

Adult male albino rats of Sprague Dawley strain weighing 150-160 g were maintained in a temperature-controlled room (24 ± 1°C) with 12-hour light-dark cycle. Animals were fed *ad lib.* with standard rat diet and were given free access to drinking water. The experiment was carried out with approval from the local ethical committee in accordance with the guidelines of the Govt. of India for care and use of experimental animals (Bose Institute Registration No. 95/99/CPCSEA).

Animals were injected intraperitoneally with each of the following alpha (α) and beta (β) adrenergic receptor (AR) agonists and antagonists (purchased from Sigma Chemical Co., USA): (1) Clonidine hydrochloride (CLON): 0.1 mg/kg b.w.; (2) Yohimbine hydrochloride (YOH): 10 mg/kg b.w.; (3) (-) Isoproterenol hydrochloride (ISO): 10 mg/kg b.w.; and (4) DL-Propranolol hydrochloride (PROP): 10 mg/kg b.w. These drugs were injected either singly or in combination with an anti-thyroid drug PTU (6n-propyl-2-thiouracil) at a dose of 2 mg /100 g b.w. for 2 consecutive days (Days '0' and '1') and sacrificed on Day '2' (day of onset of central homeostasis for thyroid hormone). These AR drugs were also administered on Days '18' and '19' with PTU that was being given daily from Day '0' and sacrificed on Day '20' (day of termination of central TH homeostasis). Rats injected with vehicle (alkalinized normal saline) were kept as control for each group in parallel and sacrificed along with treated group. In experiments where PTU injections were combined with AR drugs, PTU was administered after 30 minutes of pre-treatment with single doses of these drugs [16]. Each group consisted of 12–15 individuals pooled from three separate experiments. All chemicals and reagents were of analytical grade and purchased from Sigma Chemical Co., USA.

Preparation of synaptosome:

Synaptosomes from adult rat brain were prepared using a 10% cerebro-cortical homogenate in cold 0.32M sucrose [17]. The homogenate was centrifuged at 2000 x *g* (Biofuge 28RS, Heraues Sepatech, Germany) at 4°C for 5 min. The supernatant was cautiously layered over 1.2M sucrose and centrifuged at 50,000 x *g* at 4°C for 50 min in an ultracentrifuge (Beckman-L7, USA). The fraction from the junction of 1.2M and 0.32M sucrose was carefully collected and diluted in 1:1.5 ratio with ice-cold double distilled water. It was again layered over 0.8M sucrose and centrifuged at 50,000 x *g* at 4°C for 30 min. The synaptosomal pellet thus obtained was washed, suspended in 0.32M sucrose and preserved at -80°C. The purity of the synaptosomes was also checked. The synaptosomes were ruptured hypo-osmotically with ice-cold double distilled water prior to further biochemical assays.

Assay of Na^+–K^+-ATPase (EC 3.6.1.3):

Synaptosomal membrane Na^+–K^+-ATPase activity was measured by incubating hypo-osmotically ruptured synaptosomes in reaction mixtures of i) 30 mM imidazole-HCl, 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$ and ii) 30 mM imidazole–HCl, 4 mM $MgCl_2$ and 1 mM ouabain at pH 7.4, for 60 min at 0°C [4], [18]. The reaction was started by addition of 4 mM Tris–ATP at 37°C and stopped after 10 min using 0.1 ml of 20% SDS. The inorganic phosphate (Pi) liberated was determined by reading the absorbance at 850 nm in a UV–Vis spectrophotometer (Model Shimadzu 2401 PC) [19]. The enzyme activity was expressed as µmols of Pi liberated per h per mg synaptosomal protein from a standard curve of potassium dihydrogen phosphate.

Assay of Ca^{2+}/Mg^{2+} ATPase activity (EC 3.6.1.3):

The synaptosomal Ca^{2+}/Mg^{2+} ATPase activity was determined using 25 mM Tris-HCl (pH 7.4), 50 mM KCl, 2mM $MgCl_2$, 1 mM ouabain and 2 µM $CaCl_2$ [14]. Separate blank incubation for each sample was performed in presence of 10 µM sodium orthovanadate with omission of Ca^{2+} and Mg^{2+} in the medium. [20]. The enzymatic reaction was initiated *in vitro* by addition of 4 mM Tris-ATP. It was incubated at 37°C for 20 minutes and terminated by 0.1 ml of 20% SDS. The enzyme activity was expressed as the rate of formation of inorganic phosphate (Pi) that was released as a product of ATP hydrolysis. The Pi content was estimated and read at 850 nm in a spectrophotometer (Model Shimadzu, UV-2401PC) [19]. The specific activity of Ca^{2+}/Mg^{2+} ATPase was calculated after subtracting the Pi content of the blank from total Pi content of the respective samples and expressed as µmole Pi per minute per mg protein.

Protein estimation:

Protein content of the samples was determined using Bovine Serum Albumin (BSA) as standard [21].

Statistical analysis:

Data are expressed as Mean ± SEM of the number of experiments indicated. Statistical significance between groups was analyzed by ANOVA followed by Newmann-Keuls post hoc test for comparison between multiple groups.

III. RESULTS

Synaptosomal Na^+–K^+-ATPase activity:

The synaptosomal Na^+–K^+-ATPase activity (µmol Pi formed/h/mg protein) (Fig.1) was maintained at a constant level in all the control animals (2.852 ± 0.303). A highly significant increase in the enzyme activity was observed in the PTU-treated animals on Day '2' (5.212 ± 0.276, $p<0.001$) and also on Day '20' (5.349 ± 0.284, $p<0.001$).

There was no significant change in the enzyme activity compared to control after either CLON (α_2-AR agonist) or CLON + PTU treatment for two days (3.443 ± 0.173 and 2.866 ± 0.229 respectively). However, treatment of the antagonist, YOH, alone for two days resulted in a significantly low enzyme activity of 1.789 ± 0.084 compared to control ($p<0.01$). But combined injections of YOH and PTU showed an increased activity to 3.193 ± 0.124 µmoles Pi/h/mg protein compared to only YOH ($p<0.001$).

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On administration of the β-AR agonist, ISO, for two days, there was a significant decline in the activity of Na^+-K^+-ATPase to 1.871 ± 0.084 µmoles Pi/h/mg protein from the control (2.852 ± 0.303; $p<0.01$). But co-administration of PTU with ISO increased the enzyme activity to 3.098 ± 0.119, similar to control, but significantly lower than only PTU-treated rats ($p<0.001$). The β-AR antagonist, PROP, either alone or in combination with PTU, did not significantly affect the enzyme activity compared to control (3.255 ± 0.389 and 3.663 ± 0.208 µmoles Pi/h/mg protein respectively).

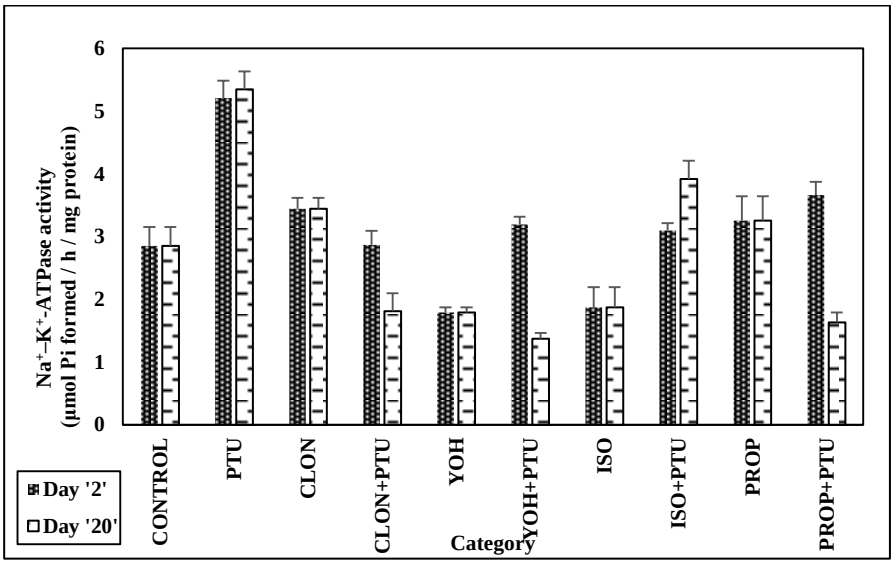

Figure 1: Synaptosomal membrane Na^+-K^+-ATPase (µmoles Pi formed/h/mg protein) activity of adult male Sprague Dawley rats during onset (Day '2') and termination (Day '20') of 'central thyroid hormone homeostasis'.

Synaptosomal Ca^{2+}/Mg^{2+}-ATPase activity:

Control male rats injected with vehicle either for '2' or '20' consecutive days showed a synaptosomal Ca^{2+}/Mg^{2+}-ATPase activity of 0.700 ± 0.02 µmoles Pi formed/h/mg synaptosomal protein (Fig. 2). PTU treatment for two consecutive days resulted in a significant decline in synaptosomal Ca^{2+}/Mg^{2+}-ATPase activity to 0.622 ± 0.01 µmoles Pi/h/mg protein ($p<0.05$). But PTU treatment for twenty consecutive days increased the enzyme activity to 0.926 ± 0.09 µmoles Pi/h/mg protein ($p<0.05$).

The α_2-AR receptor agonist CLON when injected alone for 2 days showed a significant decline in Ca^{2+}/Mg^{2+}-ATPase activity to 0.473 ± 0.05 µmoles Pi/h/mg protein in comparison to the control enzyme activity ($p<0.01$). When treated along with PTU for the first (Days '0' and '1') and last (Days '18' and '19') two days, the enzyme activity increased to 0.614 ± 0.03 and 0.576 ± 0.02 µmoles Pi/h/mg protein respectively with respect to only CLON ($p<0.01$), but remained significantly less than control ($p<0.05$). Injection of the α_2-AR antagonist YOH resulted in a Ca^{2+}/Mg^{2+}-ATPase activity of 0.263 ± 0.04 µmoles Pi/h/mg protein, which was significantly lower than the control value ($p<0.001$). This value rose to 0.790 ± 0.02 and 0.691 ± 0.05 µmoles Pi/h/mg protein respectively ($p<0.001$) similar to control, when YOH was injected with PTU either for the first or last two days.

The β-AR receptor agonist ISO, when injected alone for two days had a Ca^{2+}/Mg^{2+}-ATPase activity of 0.419 ± 0.01 µmoles Pi/h/mg protein, significantly lower than the control activity ($p<0.01$). ISO injections with PTU for the first two and last two days resulted in an increase in enzyme activity to 0.976 ± 0.04 and 1.032 ± 0.08 µmoles Pi/h/mg protein respectively ($p<0.001$ with respect to both ISO alone and control). PROP (β-AR antagonist) injections for two days resulted in a level of enzyme activity of 0.289 ± 0.05 µmoles Pi/h/mg protein ($p<0.001$ compared to control). Injections of both PROP and PTU for two days increased the Ca^{2+}/Mg^{2+}-ATPase activity to 0.653 ± 0.05 µmoles Pi/h/mg protein, identical with control. The enzyme activity showed a significantly higher value than control (0.906 ± 0.06, $p<0.05$) when PROP was administered on the 18^{th} and 19^{th} days to rats already treated with PTU from the '0' day.

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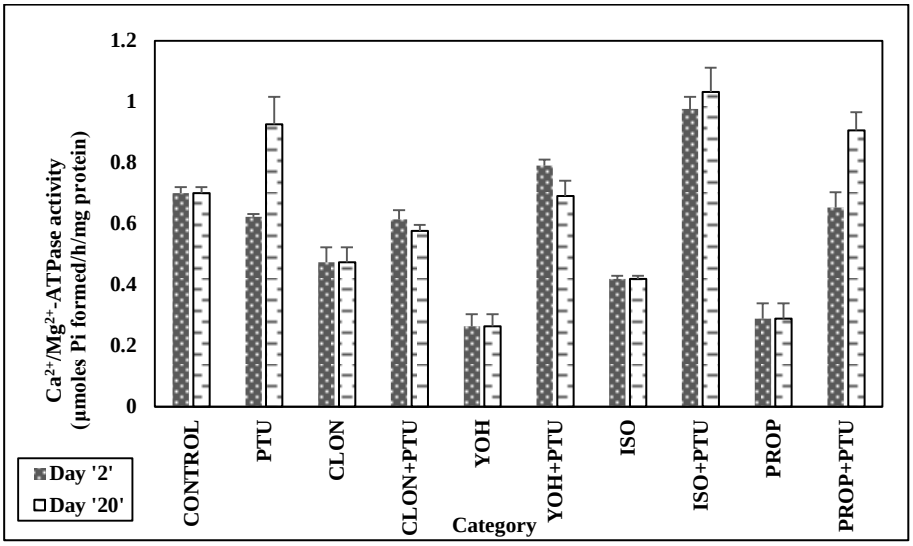

Figure 2: Synaptosomal Ca^{2+}/Mg^{2+}-ATPase (µmoles Pi formed/h/mg protein) activity of adult male Sprague Dawley rats during onset (Day '2') and termination (Day '20') of 'central thyroid hormone homeostasis'.

IV. DISCUSSION

Na^{+}-K^{+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase are enzymes known to maintain intracellular gradients of ions that are essential for signal transduction and neuronal transmission. K^{+}, Na^{+} and Ca^{2+} play important roles in neuronal signaling due to conduction of electrical activity of neurons [22]. Since K^{+}, Na^{+} and Ca^{2+} play important roles in developing electrochemical gradients and in neuronal signaling, the altered activities of Na^{+}-K^{+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase may have a significant impact on brain function [23].

A condition of 'central thyroid hormone homeostatic mechanism' is known to operate for some time in adult mammalian brain, despite profound peripheral hypothyroidism [1]. The membrane bound enzyme Na^{+}-K^{+}-ATPase also showed increased activity on the second and twentieth days after PTU treatment, which coincided with the respective days of onset and termination of the 'central homeostasis' [1], that was due to initiation of a stress due to sudden increase or decrease in brain T3 level [24], [25].

Both CLON and CLON + PTU treatment before Day '2' did not change the Na^{+}-K^{+}-ATPase activity compared to control, consistent with an unperturbed level of synaptosomal T3. But during Day '20', the enzyme activity of both CLON and PTU-treated rats was significantly lower in spite of a higher synaptosomal T3 content and continuation of the homeostatic phase. YOH inhibited enzyme activity compared to control rats as the activity of Na^{+}-K^{+}-ATPase is inhibited by agents that activate cAMP [26], [27]. But YOH + PTU rats on Day '2' had a 2.5-fold higher enzyme activity in comparison to only YOH-treated rats. It must be mentioned here that YOH + PTU animals had a higher level of brain T3 than only PTU-treated animals [16] and this extra T3 might have been successful in raising the enzyme activity similar to that observed with only PTU treated rats. But the value did not reach up to the PTU level due to a simultaneous effect of YOH. Similarly, on Day '20' when the synaptosomal T3 content of YOH + PTU rats reached the control level [16], the enzyme activity also declined to the only YOH level. This confirmed that the higher enzyme activity can be directly attributed to the presence of T3. It can be mentioned here that YOH is a G_i blocker or more precisely inhibits some kind of α_2-AR-mediated stress. The release of such stress by YOH on Day '20' might have decreased the Na^{+}-K^{+}-ATPase activity. Injections of ISO, which is a cAMP-dependent PKA activator, decreased the Na^{+}-K^{+}-ATPase activity. Hormonal regulation of Na^{+}-K^{+}-ATPase can occur via phosphorylation of Ser-943 on its α-subunit. ISO significantly increase the level of phosphorylation of the α-subunit and this phosphorylation is accompanied by significant inhibition of the enzyme activity [27]. ISO + PTU injected rats on Day '2', when compared to ISO-treated rats had increased activity of Na^{+}-K^{+}-ATPase by 1.8 folds, similar to only PTU-treated rats. This increase might be attributed to the presence of higher brain T3 compared to PTU alone [16]. Here also the value did not reach up to the PTU level due to the inhibitory effect of ISO. But ISO + PTU injections during the termination of 'central homeostasis' did not decrease the enzyme activity, although the brain T3 level was lowered to the control level [16]. One possibility may be that since ISO is a G_S activator, the stress remains to increase the enzyme activity. PROP alone counteracted the ISO-mediated inhibition of Na^{+}-K^{+}-ATPase activity, since it is a blocker of cAMP generation. Contrary to ISO + PTU, PROP + PTU treatment on Day 2' did not

show any change in enzyme activity despite presence of higher synaptosomal T3. Again, a decline in the enzyme activity was found on Day '20' after PROP + PTU treatment. This can also prove that the G_S blocker PROP also blocks the stress, unlike the G_S activator ISO.

Ca^{2+}/Mg^{2+}-ATPase that maintains $[Ca^{2+}]_i$ homeostasis in nerve terminals has been identified as a thyroid hormone-sensitive parameter. 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 [14]. In the present experiment, the decline in Ca^{2+}/Mg^{2+}-ATPase activity on the day of onset of 'central TH homeostasis' (Day '2') was consistent [16] 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 however be correlated with increased Na^+-K^+-ATPase activity. Moreover, Ca^{2+}/Mg^{2+}-ATPase activity may increase due to compensatory mechanisms.

In all cases of agonist and antagonist treatment there has been significant decrease in the activity of the enzyme Ca^{2+}/Mg^{2+}-ATPase. A plausible explanation can be given by the very short term (only for two days) treatment of these aminergic receptor drugs. Indeed, some drugs like clonidine, propranolol can decrease the enzyme activity due to their downregulating effect on cAMP level [16]. Synaptosomal Ca^{2+}/Mg^{2+}-ATPase activity is also known to be positively modulated by a cAMP-dependent phosphorylation reaction [28]. But the decreased activity of yohimbine and isoproterenol cannot be given any justifiable explanation at present. Acute treatment with the α_2-AR agonist CLON is reported to cause a decreased catalytic activity of the Ca^{2+}/Mg^{2+}-ATPase [29], [30], [31]. This has been found in the present experiment where CLON itself severely declined the enzyme activity. But PTU treatment with CLON raised the enzyme activity both on Days '2' and '20' that can be explained by the higher level of T3 and intrasynaptosomal $[Ca^{2+}]_i$ [16]. The response of YOH along with PTU on a comparatively higher Ca^{2+}/Mg^{2+}-ATPase activity than only YOH treatment on Day '2', could also be attributed to the higher synaptosomal T3 content as was observed [16]. But the reason behind decline on Day '20' needs further study. ISO + PTU injections both during the onset and termination of 'central TH homeostasis' showed higher Ca^{2+}/Mg^{2+}-ATPase activity coincident with a higher level of brain T3 and high intrasynaptosomal $[Ca^{2+}]_i$ [16]. PROP + PTU injections both during the onset and termination of 'central TH homeostasis' showed unchanged or higher Ca^{2+}/Mg^{2+}-ATPase activity coincident with a higher level of brain T3 [16].

V. CONCLUSION

There has been a close involvement of adrenergic receptor agonists and antagonists on synaptosomal thyroid hormone content. Moreover, a correlation also exists between T3 and intrasynaptosomal $[Ca^{2+}]_i$ on the days of onset and termination of 'central thyroid hormone homeostasis' [16]. This study also explores an involvement of these AR compounds on the activities of two key enzymes Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase on the two specific days. The altered activities of both Na^+-K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase with administration of AR compounds on Days '2' and '20' indicated alterations on neurotransmission. In fact, increased Na^+-K^+-ATPase activity leading to decreased acetylcholinesterase (AChE) activity was also observed indicating alteration in neurotransmission [1]. It has also been proposed that T3 plays a role on adult brain neuronal Ca^{2+}–mobilization through stimulation of the Ca^{2+}/Mg^{2+}-ATPase activity in nerve endings [14]. So, it can be speculated here that loss of 'central homeostasis' on Day '20' was accompanied by total disruption in the neurobiochemical events. However, there remains certain aspects which needs further study, specifically as to the scenario after some days of termination of 'central homeostasis'.

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