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Rapid action of Triiodothyronine on Mitochondrial H^+, Ca^{2+} and Mg^{2+}-Dependent ion Transporters in Cortex, Hippocampus and Cerebellum of Restraint Mice

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Abstract

Thyroid hormones (TH) have a multitude of actions, mainly on development and differentiation during early life and play many vital roles in almost all tissues including neuronal tissues. TH rapidly alters the mitochondrial functions both by its genomic and direct actions on mitochondrial binding sites. The functional relationship between TH and mitochondrial ion transport during stress response has not yet been elucidated in mammals so far. Here, we report a rapid *in vivo* action of triiodothyronine (T_3) on mitochondrial ion transporter functions in the neuronal clusters of cortex, hippocampus and cerebellum of Swiss Albino mouse (*Mus musculus*) treated short-term with triiodothyronine (T_3; 200ng g^{-1}) for 30 min either in non-stressed or in restraint-stressed (30 min each day for 7 days). The mH^+-ATPase activity in the cortex decreased to significant levels after T_3 treatment in both non-stressed and restraint-stressed mice. On the contrary, the mH^+-ATPase activity in the hippocampus and cerebellum increased to significant levels after T_3 treatment in both non-stressed and restraint-stressed mice. The mCa^{2+}-ATPase activity in the cortex and cerebellum decreased to significant levels after T_3 treatment in both non-stressed and restraint-stressed mice. The mCa^{2+}-ATPase activity in the hippocampus that increased to significant levels after T_3 treatment, showed a reversal after restraint-stress in T_3-treated mice. The mitochondrial Mg^{2+}-ATPase activity in the cortex decreased to significant levels after T_3 treatment in restraint-stressed mice. On the contrary, T_3 treatment in restraint stressed mice increased to significant levels the mitochondrial Mg^{2+}-ATPase activity in the cerebellum. The mitochondrial Mg^{2+}-ATPase activity in the hippocampus, which increased to significant levels after T3 treatment in non-stressed mice, reversed its activity in T_3-treated restraint-stressed mice. Spatial and differential action of T_3 on the mitochondrial ion transporters has been found in the present study that corroborates with a rapid modulatory action of T_3 on the transport of H^+, Ca^{2+} and Mg^{2+} in the brain mitochondria of mice which appears to be sensitive to restraint stress.

Keywords: Brain, Mice, Mitochondrial Ca^{2+}, H^+, Mg^{2+} ATPase, Restraint Stress, Triiodothyronine

1. Introduction

Thyroid hormones (THs) are essential regulators of growth, development and normal body functions, and their release is coordinated by the hypothalamic-pituitary-thyroid (HPT) axis. The regulation of HPT axis has been shown as an acutely stress-responsive neuroendocrine system[1]. Thyroid hormones are known for their activity to

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maintain cellular basal metabolic rate and are considered as major regulators of energy metabolism, mitochondrial activity, oxygen consumption and active oxygen metabolism[2]. The combination of circulating levels of T_4, expression of deiodinases and thyroid hormone receptors (TRs) governs spatial and temporal TH signaling in brain[3]. Thyroid hormones possess both central effects that consist of a direct signaling on the central nervous system, and peripheral effects that correspond to direct effects in responsive tissues[4]. Triiodothyronine (T_3) controls energy expenditure via both central and peripheral pathways and acts predominantly through the nuclear receptors, and TH receptors alpha (THRA) and beta (THRB)[5]. Additional factors that impact TH action in the brain include metabolism, activation of thyroxine (T_4) to T_3 by the enzyme 5'-deiodinase Type 2 (Dio2), inactivation by the enzyme 5-deiodinase Type 3 (Dio3) to reverse T_3 (rT_3), which occurs in glial cells, and uptake by the monocarboxylate transporter 8 (Mct8) in neurons[6]. The transductional regulation of ion transport activity is a prominent mechanism by which hormone signals influence the neuronal communication and circuit function of the mammalian central nervous system[7]. Hormone-induced changes in the activity of these electrogenic proteins permit a rapid regulation of intrinsic neuronal excitability and the resultant reconfiguration of circuit outputs[8].

Stress as a disruption in the normal homeostatic function causes dysfunction of physiological processes[9]. Induction of physiological stress such as restraint stress imposes threat to physiological processes[10-12]. The stress response invites a series of physiological and behavioral modification that demands activation of hypothalamo-pituitary-adrenal (HPA) axis and the brain sympathetic-chromaffin (BSC) axis that helps the organism to cope with these challenges[13-14]. As a stress model, physical restraint stress causes stress-associated changes in the physiological, immunological, and neurobiological status of mammals[15]. Moreover, a number of studies have reported that immobilization or restraint stress is accompanied by disturbance in antioxidative capacity of the organism[16] and ion transport functions[12].

A number of factors including individual sensitivity and the type of stressor have been shown to influence the brain functions[17]. Restraint stress, a modified form of immobilization stress, is a validated experimental stressor that can induce both physical and psychological effects at the same time[18]. Frontal cortex and hippocampus are brain regions sensitive to stress-induced damage[19]. Stress stimulates hypothalamus to release corticotropin-releasing hormone (CRH) into the portal vein. CRH induces anterior pituitary to release adrenocorticotrophic hormone (ACTH), which in turn affects the adrenal cortex and increases synthesis and release of corticosteroids[20]. Corticosteroids include glucocorticoids, which regulate glucose metabolism, and mineralocorticoids, which regulate water balance and blood pressure. In humans, the major glucocorticoid is cortisol and in rodents including mice it is corticosterone. Corticosterone delivered into the blood causes diverse stress responses in tissues, and modulates the functions of hypothalamus and pituitary for negative feedback on CRH and ACTH secretion[20-21]. A rise in corticosterone has been found in mice kept for restraint-stress (Peter et al., unpublished). One of the most vulnerable targets of stress is hippocampus, because it abundantly expresses both glucocorticoid and mineralocorticoid receptors[22]. Stress that changes hippocampal neural activity and synaptic plasticity activates hippocampal glucocorticoid receptor (GR) and decreases neuronal cell survival and neurogenesis[23].

Mitochondria, because of their known physiological function, are the target of most studies on the calorigenic effects of TH. Mitochondria, in fact, provide about the 90% of the cellular energy supply, and they are also the headquarters for a multitude of biochemical pathways related to metabolism[24]. Indeed, besides ATP synthesis, mitochondria are the site of other important biochemical events such as oxidation of fatty acids, production of free radical, heme synthesis, the metabolism of some amino acids, iron metabolism, and calcium homeostasis[20]. In addition, mitochondria contribute to many processes central to both cellular function and dysfunction, including calcium signaling, cell growth and differentiation, cell-cycle control, and cell death[25]. Mitochondria utilize metabolic substrates to generate ATP via ATPase complex, which is coupled to oxygen consumption via the proton electrochemical gradient existing across the inner mitochondrial membrane[26].

ATPases are membrane pumps widely used as indicators of osmoregulatory indices in many animals including mammals[27-28,12]. Mitochondrial Ca^{2+} accumulation has a role in satisfying energy demands by increasing the ATP production through activation of mitochondrial enzymes, and modulation of the dynamics of calcium signals in cell functions[29]. Intracellular Ca^{2+} is a fundamental biochemical messenger that controls

numerous processes in neurons including transmitter/peptide release, ion channel activity, gene expression, and aerobic metabolism[30]. A property of Ca^{2+}-induced Ca^{2+}- release by the mitochondria exists in a variety of vertebrate and invertebrate neuronal types[31]. This process is initiated when Ca^{2+} derived from voltage-gated Ca^{2+} channels diffuses into the mitochondria through the inner mitochondrial Ca^{2+} uniporter[32]. Subsequently, Ca^{2+} is slowly released from the mitochondria into the cytosol by a Na^{+}/Ca^{2+} and/or H^{+}/Ca^{2+} exchanger[33].

Studies on ADP (Mg^{2+})-dependent de-activation and ATP (Mg^{2+})-dependent activation of mitochondrial ATPase revealed that the slow active/inactive transition is primarily controlled by the ATP/ADP ratio[34-35]. Likewise, the function of mitochondrial H^{+}-ATPase is mainly to synthesize ATP and transport H^{+}. The ATP is synthesized from ADP and Pi by H^{+} ATPase using the energy released by H^{+} flowing back to mitochondrial matrix. It couples ATP synthesis at three locations on the mitochondrial respiratory chain. At each location, one ATP molecule can be synthesized by every two electrons. Mitochondria transport Ca^{2+} via Ca^{2+}-ATPase while the final synthesis of ATP must be involved in the H^{+}-ATP, which suggests that inhibition of ATP synthesis may be associated with changes in mitochondrial Ca^{2+}-ATPase and H^{+}-ATPase activities[36].

The functional relationship between TH and brain mitochondrial ion transporter activity and its probable role during restraint stress has not been elucidated in mammals. The present study, therefore, examined the dose-dependent response of short-term *in vivo* T_3 treatment (30 min) on the mitochondrial H^{+}, Ca^{2+} and Mg^{2+} transporter activities in the cortex, hippocampus and cerebellum of mice kept either in non-stressed or restraint-stress condition.

2. Materials and Methods

2.1 Animal Holding Conditions

Twelve weeks-old healthy adult female Swiss albino mice (*Mus musculus*) born and reared in the in-house animal facility (University of Kerala), were used as the test species. Animals were kept in groups of four each in polypropylene cages (Size: 29 x 22 x 14 cm) with stainless steel–wire mesh top. All animals were maintained under a 12 h L:D cycle at 24 ± 4° C room temperature and 70 ± 10% relative humidity with minimum noise levels and limited handling. Animals were allowed *ad libitum* access to standard pellet feed (Sri Sai Durga Feeds and Products, Bangalore, India) as maintenance diet and purified tap water. Cage bedding was changed once every two days. The experiment was carried out under the same environmental conditions as those in the animal house, with the animals being brought to the experimental room in their home cages. The experiments were approved by the Institutional Animal Ethics Committee (IAEC) of the university IAEC-KU-31/2011-12-ZOO-MCSP (2).

2.2 Experimental Protocol

2.2.1 Dose-Dependent Effect of T_3 in mice

The dose-responsive *in vivo* actions of T_3 on mitochondrial ion transporter activities were studied in the cortex, hippocampus and cerebellum of mice to understand the short-term actions of T_3. Mice were kept as four groups of four each. The first group received *intraperitoneal* injection (*ip*) of 0.9% NaCl in 100 µL as vehicle, and served as sham control. The remaining three groups of mice were administered *intraperitoneal* injection of varied doses (200, 400, 600 ng g^{-1}) of T_3, and the duration of treatment was 30 min.

2.2.2 Action of T_3 in Restraint-Stressed Mice

A selected dose of T_3 (200 ng g^{-1}) was employed in another set of experiment that tested the impact of restraint stress on brain ion transport functions in T_3-treated mice. For that purpose, two subsets of mice groups comprising 4 in each group were held as non-stressed mice and restraint-stressed mice, respectively. The first group in non-stressed subset that served as sham controls received saline (0.9% NaCl) as vehicle. Mice in the second group were given *ip* injection of a selected dose of T_3 (200 ng g^{-1}). Each group of mice in the stressed subset comprised 2 groups that were previously exposed to the psychosocial stress in the form of intermittent restraint stress of 30 min each day for seven days as reported earlier. They were then given saline injection (control) and T_3, respectively, for 30 min.

2.3 Sampling and Analysis

The mice were anaesthetized with Nembutal (Sisco Research Laboratories, Mumbai, India) and they were swabbed with 70% alcohol to wet the fur. The abdominal skin was removed. A midline abdominal incision was

made and abdominal aorta and vena cava were exposed. A #23 needle was inserted into the aorta and arterial blood was collected with the help of a canula. Then, the mice were sacrificed under euthanasia and the brain was dissected out and was separated into cortex, hippocampus and cerebellum. The isolated tissues were kept in brain-mitochondrial extraction (BME) buffer containing 0.25 mM sucrose, 10 mM HEPES, 0.5 mM EDTA, and 0.5 mM EGTA ((pH 7.4) and stored at -80°C for further analysis.

2.3.1 Isolation of brain mitochondria

Mitochondria were isolated from the cortex, hippocampus and cerebellum of mice brain as described by Peter et al[37]. Briefly, each segment of brain was kept in ice-cold brain mitochondrial extraction (BME) buffer (pH 7.4) containing 0.25 mM sucrose, 10 mM HEPES, 0.5 mM EDTA, and 0.5 mM EGTA. The brain tissue was chopped and homogenized using glass homogenizer fitted with teflon pestle giving 8-10 strokes as described previously[37]. Homogenates were centrifuged in Eppendorf 5430R at 2000 x g for 3 min at 4°C to separate the membrane constituents from mitochondria and synapses. The supernatant was then centrifuged at 12,000 x g for 8 min at 4°C. The pellets were then washed in the isolation buffer with BSA and centrifuged at 12,000 x g for 10 min. The pellets were then re-suspended in a 0.25 M sucrose solution and centrifuged again for 10 min. These final pellets were then suspended in the sucrose medium, which served as the enzyme source.

2.3.2 Quantification of Mitochondrial H^+, Ca^{2+} and Mg^{2+}-dependent ATPase Transporters

Frozen tissues were thawed and a 10% homogenate was prepared in BME buffer (pH 7.4) using a glass homogenizer fitted with teflon pestle as described previously[27]. The mitochondrial fraction prepared was used for quantifying the activity of ion-transport systems such as bafilomycin-sensitive H^+-dependent ATPase, vanadate-sensitive Ca^{2+}-dependent ATPase and oligomycin-sensitive Mg^{2+}-dependent ATPase. The purity of the mitochondria was validated by assaying cytochrome c oxidase and succinate dehydrogenase activities which represented intact mitochondria.

2.3.3 Bafilomycin-Sensitive H^+- Dependent ATPase Transporter Activity

Bafilomycin-sensitive H^+-dependent ATPase transporter activity in the mitochondrial fraction of neuronal tissues was quantified adopting the method of Peter et al.[27] and modified for microplate assay[38]. Briefly, sodium deoxycholate (0.1 mg protein^{-1}) was routinely added to optimize substrate accessibility. Samples in duplicates containing (1.0 µg protein) were added to a 96-well microplate containing 100 mM NaCl, 30 mM imidazole (pH 7.4), 0.1 mM EDTA, 5 mM $MgCl_2$ and 0.14 mM ouabain. Bafilomycin A (Sigma-Aldrich) was used as the inhibitor. After vortexing, the assay mixture was incubated at 37°C for 15 min. The reaction was initiated by the addition of 0.3 mM ATP and was terminated with addition of 8.6% TCA. The liberated inorganic phosphate was measured against phosphate standard at 700 nm in Synergy HT Biotek Microplate Reader.

2.3.4 Vanadate-Sensitive Ca^{2+}- Dependent ATPase Transporter Activity

Vanadate-sensitive Ca^{2+}-dependent ATPase transporter activity in neuronal mitochondria was quantified adopting the method of Peter et al[27] and modified for microplate assay[37,38]. Samples were prepared as described above. Samples in duplicate containing (1.0 µg protein) were added to a 96-well microplate containing 60 mM imidazole (pH 7.4), 0.2 mM EGTA, and 75 mM KCl. 0.013 mM $CaCl_2$ was used as the promoter and 0.009 mM sodium orthovanadate (Sigma-Aldrich) was used as the inhibitor. The change in absorbance between promoter and inhibitor assays was calculated, and regression analysis was employed to derive the rate of activity of mCa^{2+}-ATPase and expressed in µmoles Pi liberated per hour for mg protein. The change in absorbance between promoter and inhibitor assays was calculated and regression analysis was employed to derive the rate of activity of mCa^{2+}-ATPase and expressed in µmoles Pi liberated per hour for mg protein.

2.3.5 Oligomycin-Sensitive Mg^{2+}-Dependent ATPase Transporter Activity

The oligomycin-sensitive Mg^{2+}-dependent ATPase transporter activity that corresponded to F0F1 ATPase in the neuronal tissue homogenates was quantified adopting the method of Peter et al.[27] and modified for microplate assay[38]. Samples were prepared as described above. Samples in duplicates containing (1.0 µg protein) were added to a 96-well microplate containing 60 mM imidazole (pH 7.4), 10 mM $MgCl_2$, 0.2 mM EDTA and 75 mM KCl. Oligomycin (Sigma-Aldrich) was used as

the inhibitor. The liberated inorganic phosphate was measured against phosphate standard at 700 nm in Synergy HT Biotek Microplate Reader. The change in absorbance between promoter and inhibitor assays was calculated and regression analysis was employed to derive the rate of activity of mMg^{2+}-ATPase and expressed in µmoles Pi liberated per hour for mg protein.

2.4 Statistical Analysis

Statistical difference among groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between groups was analyzed with the help of Graphpad Software (Graphpad Instat-3, San Diego) and the level of significance was accepted if $P < 0.05$.

3. Results

3.1 Short-Term in Vivo Action of T_3 on Mitochondrial H^{+-}ATPase

The mitochondrial H^+-ATPase activity in the cortex showed significant rise ($P<0.01$) after low (200 ng g^{-1}) and high (600 ng g^{-1}) doses of T_3 treatment for 30 min (Figure 1A). On the contrary, medium (400 ng g^{-1}) dose of T_3 treatment decreased the mH^+-ATPase activity to significant levels in the cortex and hippocampus ($P<0.01$, $P<0.05$) (Figure 1A). The mH^+-ATPase activity in the cortex decreased to significant levels ($P<0.05$) after T_3 treatment in both non-stressed and restraint-stressed mice (Figure 1B). On the contrary, the mH^+-ATPase activity in the hippocampus and cerebellum increased to significant levels ($P<0.05$, $P<0.001$) after T_3 treatment in both non-stressed and restraint-stressed mice (Figure 1C, 1D).

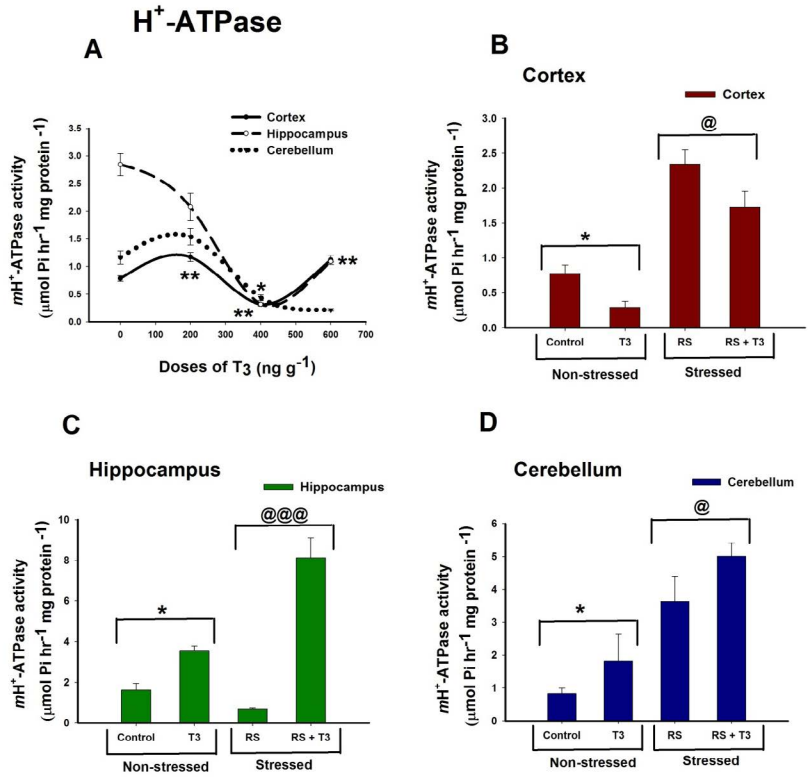

Figure 1. Rapid action of T_3 (200, 400 and 600 ng g^{-1}) treatment for 30 min on mitochondrial H^+-ATPase (mH^+-ATPase) activity in cortex, hippocampus and cerebellum of non-stressed mice (A). Action of selected dose of T_3 (200 ng g^{-1}) on mH^+-ATPase activity of mice kept at restraint stress for 30 min for consecutive 7 days (B–D). Each point/bar is mean ± SE of triplicates drawn for four mice. Significance between non-stressed control mice and T_3-treated mice were represented as * ($P<0.05$). Significance of difference between restraint-stressed (RS) mice and T_3-treated restraint-stressed mice are represented as @ ($P<0.05$) and @@@ ($P<0.001$).

3.2 Short-Term In Vivo Action of T_3 on Mitochondrial Ca^{2+}-ATPase

The mitochondrial Ca^{2+}-ATPase activity in the cortex, hippocampus and cerebellum remained unaffected after treatment of varied doses of T_3 (200, 400, 600 ng g^{-1}) for 30 min in mice (Figure 2A). The mCa^{2+}-ATPase activity in the cortex and cerebellum decreased to significant levels (P<0.01, P<0.05) after T_3 treatment in both non-stressed and restraint-stressed mice (Figure 2B, 2D). The mitochondrial Ca^{2+}-ATPase activity in the hippocampus that increased (P <0.001) to significant levels after T_3 treatment, showed a reversal after restraint-stress in T_3-treated mice (Figure 2C).

3.3 Short-Term In Vivo Action of T^3 on Mitochondrial Mg^{2+}-ATPase

The mitochondrial Mg^{2+}-ATPase activity in hippocampus showed significant decrease (P< 0.01) after medium (400 ng g^{-1}) and high (600 ng g^{-1}) doses of T_3 treatment for 30 min (Figure 3A). Low (200 ng g^{-1}) dose of T_3 treatment increased (P<0.001) the mMg^{2+}-TPase activity in cerebellum to significant levels, whereas its activity decreased to significant levels (P<0.01) after medium (400 ng g^{-1}) dose of T_3 (Figure 3A). The mMg^{2+}-ATPase activity in the cortex remained unaffected after varied doses of T_3 treatment (Figure 3A). The mitochondrial Mg^{2+}-ATPase activity in the cortex decreased to significant levels (P<0.01) after T_3 treatment in restraint-stress mice

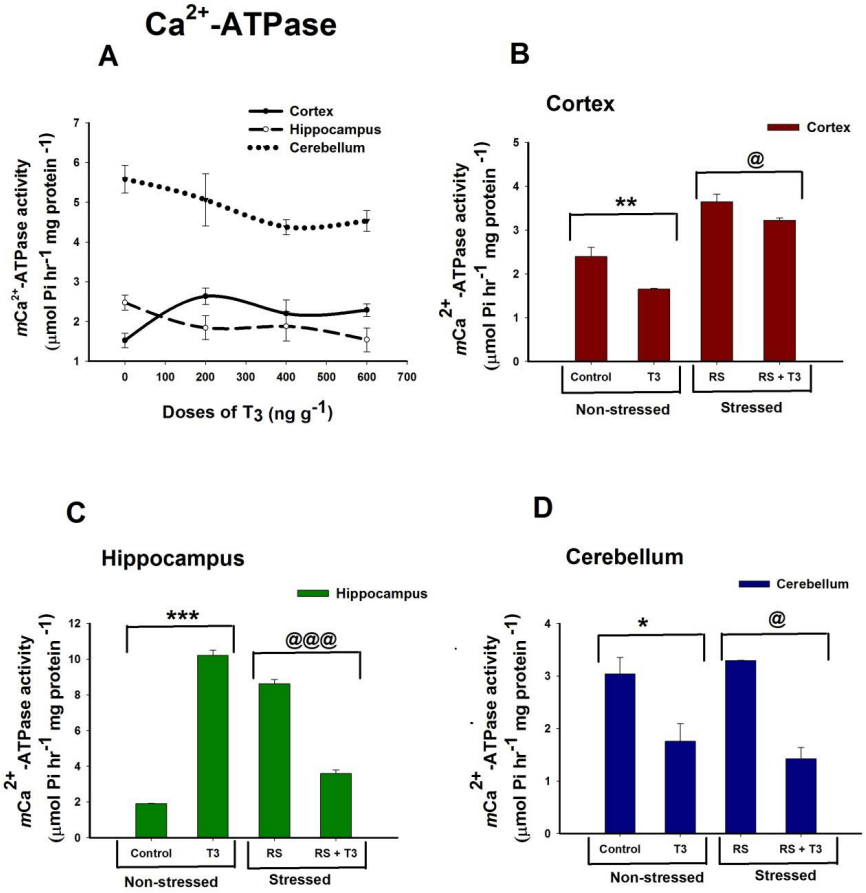

Figure 2. Rapid action of T_3 (200, 400 and 600 ng g^{-1}) treatment for 30 min on mitochondrial Ca^{2+}-ATPase (mCa^{2+}-ATPase) activity in cortex, hippocampus and cerebellum of non-stressed mice (A). Action of a selected dose of T_3 (200 ng g^{-1}) on mCa^{2+}-ATPase activity of mice kept at restraint stress for 30 min for consecutive 7 days (B–D). Each point/bar is mean ± SE of triplicates drawn for four mice. Significance between non-stressed control mice and T_3-treated mice are represented as * (P<0.05), ** (P<0.01) and *** (P<0.001). Significance of difference between restraint-stressed (RS) mice and T_3-treated restraint-stressed mice are represented as @ (P<0.05) and @@@ (P<0.001).

(Figure 3B). On the contrary, T_3 treatment in restraint-stressed mice increased (P<0.05) the mitochondrial Mg^{2+}-ATPase activity in the cerebellum to significant levels (Figure 3D). The mitochondrial Mg^{2+}-ATPase activity in the hippocampus increased to significant levels (P<0.001) after T_3 treatment in non-stressed mice but showed a decrease (P<0.001) after T_3 treatment in restraint stressed mice (Figure 3C).

4. Discussion

Thyroid hormones are critical for the regulation of development of CNS. As the active form of TH, T_3 is crucial for the neuronal development, differentiation of astrocytes and oligodendrocytes, and also for microglial development[39]. The non-genomic or genomic molecular mechanisms of TH support the functions of ion pumps

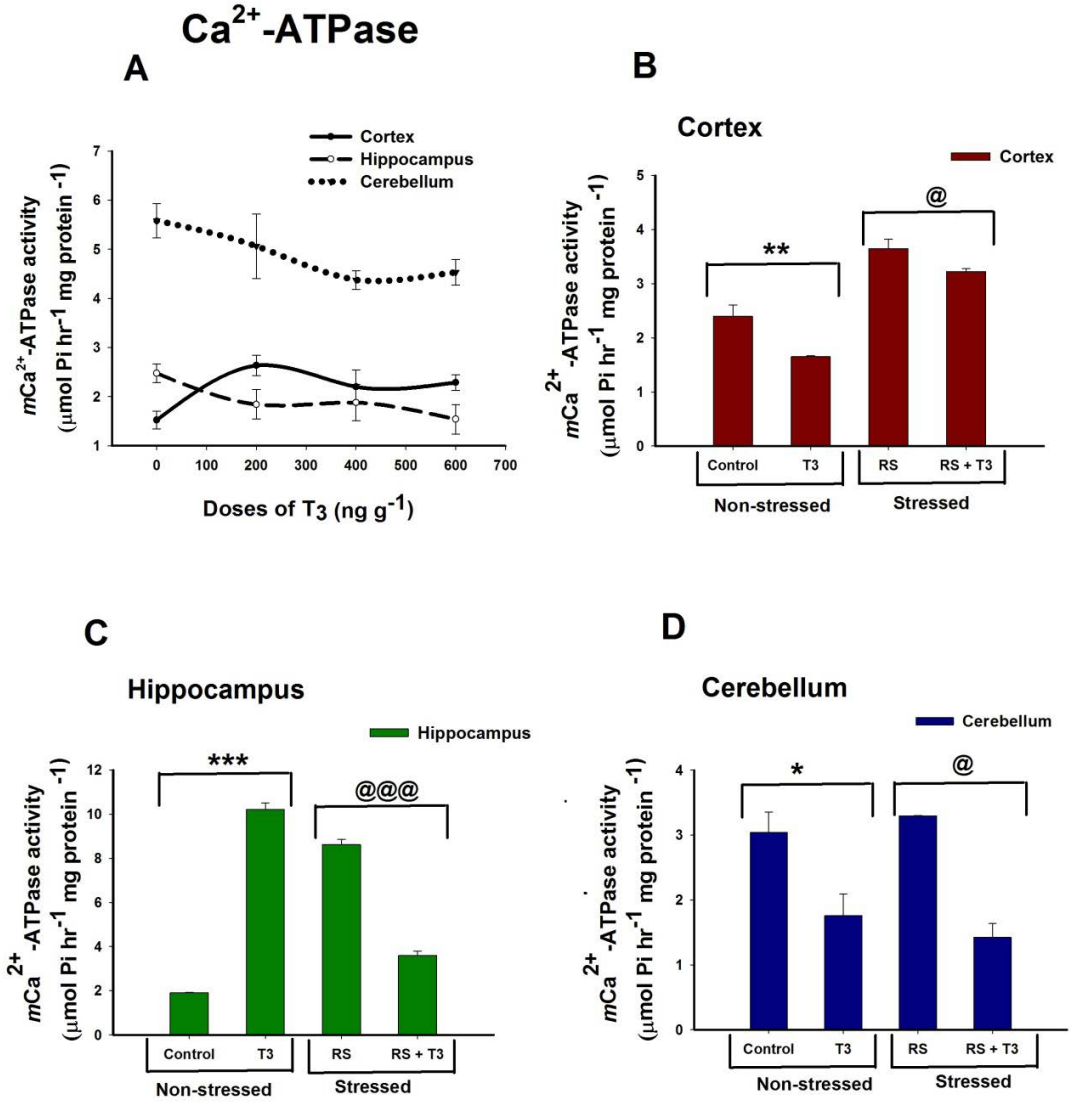

Figure 3. Rapid action of T_3 (200, 400 and 600 ng g^{-1}) treatment for 30min on mitochondrial Mg^{2+}-ATPase (mMg^{2+}-ATPase) activity in cortex, hippocampus and cerebellum of non-stressed mice (A). Action of a selected dose of T_3 (200 ng g^{-1}) on mMg^{2+}-ATPase activity of mice kept at restraint stress for 30 min for consecutive 7 days (B–D). Each point/bar is mean ± SE of triplicates drawn for four mice. Significance between non-stressed control mice and T_3-treated mice are represented as *** (P<0.001). Significance of difference between restraint-stressed (RS) mice and T_3-treated restraint-stressed mice are represented as @ (P<0.05), @@ (P<0.01) and @@@ (P<0.001).

and channels that are important to normal excitable cell function.

Transport of H^+ is an integral function of the mitochondrial ADP/ATP carrier (AAC), a major transport protein of the inner mitochondrial membrane[40]. Moreover, it exchanges mitochondrial ATP for cytosolic ADP and controls cellular production of ATP. The H^+ leak and mitochondrial uncoupling could be dynamically controlled by cellular ATP demand and the rate of ADP/ATP exchange[41]. Short-term T_3 can increase the mitochondrial H^+-ATPase activity in the hippocampus and cerebellum of both non-stressed and restraint-stressed mice, implying the role of T_3 in mitochondrial H^+ transport. THs are known for rapid action on ionotropic glutamatergic receptors in hippocampal neurons that could modulate brain function in physiological and pathological states[42-43]. T_3 has been shown to stimulate Na^+/H^+ exchanger activity in excitable cells and to enhance recovery of intracellular pH after an acid load[44]. The mechanism of increased exchanger activity includes TH-dependent activation of mitogen-activated protein kinase (MAPK)[45]. It is likely that during stress, accumulation of H^+ ions as a result of acidosis could enhance the Na^+/H^+ exchanger as well as the mitochondrial H^+-transport system. On the contrary, the lowered mitochondrial H^+-ATPase activity in the cortex after T_3 treatment in both non-stressed and restraint-stressed mice might account for a modulatory action of neuronal clusters involving serotonergic, dopaminergic, cholinergic and GABAergic neurons as studies have shown that TH specifically modulates the function of GABAergic neurons in both *in vivo* and *in vitro* in rats[46-47]. A reciprocal regulation of the thyroid and GABA systems exists in vertebrates[48].

In the present study, the mitochondrial Ca^{2+}-ATPase activity in cortex and cerebellum decreased after T_3 treatment in both non-stressed and restraint-stressed mice. This indicates that despite the stressed state T_3 can lower Ca^{2+}-dependent transport activity. On the contrary, in hippocampus, mitochondrial Ca^{2+}-ATPase activity that showed a rise after T_3 in non-stressed mice decreased in restraint mice, point to sensitivity of Ca^{2+} transport to T_3. Hippocampal neurons mainly release glutamate or gamma-aminobutyric acid[49]. Glutamate is the major excitatory neurotransmitter in the nervous system. As an amino acid and neurotransmitter, glutamate has a large array of normal physiological functions. GABA is the chief inhibitory neurotransmitter in the brain, and the major difference between glutamate and GABA is that the latter is synthesized from the former by the enzyme L-glutamic acid decarboxylase[50]. This elevated mitochondrial Ca^{2+} transport in hippocampus after T_3 treatment in non-stressed mice might be due to the excitatory action of glutamergic neurons. On the contrary, T_3 might have excited the GABAergic neurons during restraint stress that in turn lowered the mitochondrial Ca^{2+}-ATPase activity. Spontaneous Ca^{2+} discharges from presynaptic stores could trigger spontaneous synaptic release of glutamate in hippocampal area[51]. Studies in individual axonal varicosities of hippocampal mossy fibers showed that activation of $GABA_A$ receptors reduces presynaptic Ca^{2+} entry and elevates the Ca^{2+} background level[52]. Many studies have shown that T_3 or, in some cases, T_4 stimulates these ion pumps including Na^+/H^+ exchanger[44], Na^+/K^+-ATPase[53], Ca^{2+}-ATPases[54] and channels such as inward rectifier K^+ channel[55] and the Na^+ current[56-57].

Moreover, T_3 might have stimulated the serotonergic, dopaminergic, cholinergic and GABAergic neurons that constitute the cortex. It is likely that the excitatory action of these neurons might have lowered the mCa^{2+}-ATPase activity after T_3 treatment in both non-stressed and restraint-stressed mice brain. Likewise, the neuromodulatory role of glutamergic and GABAergic neurons in the cerebellum might lower the mitochondrial Ca^{2+}-ATPase activity observed after T_3 treatment in both non-stressed and restraint-stressed mice. Studies in *C. elegans* showed that neuromodulators, such as serotonin, can change Ca^{2+} signals and depolarization amplitudes in opposite directions, simultaneously, within a single neuron, thus altering neuronal excitability and synaptic strengths[58]. Dopamine release can be triggered in very low extracellular Ca^{2+} concentrations[59-60]. Studies have shown that TH increases the Ca^{2+}-ATPase activity of the sarcoplasmic reticulum in skeletal muscle, thereby increasing the energy-turnover associated with Ca^{2+}-cycling during contraction and rest[61]. Ca^{2+}-ATPase in plasma membranes from a variety of tissues has been shown stimulated by the non-genomic calmodulin-dependent action of TH that directly acts on cell membrane and independent of the cell nucleus[54]. In non-excitable and excitable tissues, ambient TH may set basal activity of Ca^{2+}-ATPase or magnitude of the enzymatic response to calmodulin Ca^{2+}[54]. Moreover, under physiological conditions, ionotropic glutamate receptors mediate the processes of excitatory neurotransmission and synaptic plasticity. During stressed conditions, sustained pathological release of glutamate from neurons

and glial cells causes prolonged activation of these receptors, resulting in massive depolarization and Ca^{2+} overload. High levels of Ca^{2+} activate many degradative processes depending on the metabolic status, causing neural cell death[62]. Mendes-de-Aguiar et al.[63] reported that T_3 would eliminate the "gliotoxic" effect of glutamate on cultured cerebellar astrocytes from newborn rats by its genomic action including GLT1 and GLAST proteins. Likewise, T_3 can protect rat hippocampal neurons against glutamate toxicity by a non-genomic mechanism[43] and T3 even exerts its regulation of glutamergic neurons by both genomic and non-genomic mechanisms[64].

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. In the present study, short-term T_3 treatment could decrease Mg^{2+}-ATPase activity in the cortex and hippocampus of restraint-stressed mice and increase the activity in the cerebellum. It appears that a spatial regulation of cholinergic neurons by lowering Ca^{2+} mobilization could account for this action of T_3 during restraint-stress. Moreover, the highest numbers of TH receptors have been traced in the cerebral cortex[65]. Studies in mammalian brain have shown that T_3 stimulates acetylcholine metabolism by increasing AChE activity as well as uptake of the released ACh through an increase in synaptosomal Mg^{2+}-ATPase activity. A positive impact of T_3 on the cholinergic system in mammalian brain is thus known[66]. TH has been shown to possess a vital role in brain neuronal Ca^{2+}-mobilization by stimulating $Ca^{2+/}Mg^{2+}$-ATPase activity in nerve terminals as a T_3-induced stimulation of Ca^{2+}/Mg^{2+}-ATPase activity in relation to Ca^{2+} mobilization is known in nerve terminals[67]. The mitochondrial ATP synthase is composed of two separable components: F1 (factor 1) and Fo (factor that confers sensitivity to oligomycin). The ATP synthase is a reversible molecular motor comprised of two parts: a proton turbine (within Fo) and a molecular machine (F1) that uses rotational energy to form ATP from ADP and phosphate. The proton turbine is powered by the flow of protons down a potential gradient across the mitochondrial membrane created by the electron transport chain during respiration. The rotor of the turbine is within the F1 and when it rotates, drives the synthesis of ATP[26]. An intriguing feature of the Fo-F1 complex is a slow active/inactive transition of their ATPase activity, which is under a complex control of adenine nucleotides, Pi and Mg^{2+}[34]. It is evident that ADP bound at some high-affinity nucleotide-specific site of Fl is a prerequisite for the Mg^{2+}-induced de-activation and anion induced activation of ATPase[34].

It appears that besides having both non-genomic and genomic actions, TH could repair certain neuronal dysfunctions during stress, particularly by modifying the intracellular H^+ accumulation by stimulating Na^+/H^+ exchanger, lowering Ca^{2+} by activating Ca^{2+}-ATPase and Mg^{2+}-ATPase activity through the differential and spatial regulation of neuronal clusters distributed in the varied neuronal regions. Overall, a neuroprotective and an integrative role of T_3 in modulating the neuronal ion transport could be seen during restraint stress in mice brain.

5. Acknowledgments

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