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L-Triiodothyronine differentially and nongenomically regulates synaptosomal protein phosphorylation in adult rat brain cerebral cortex: Role of calcium and calmodulin

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ABSTRACT

Adult-onset thyroid disorders in humans impair several important central nervous system functions, causing various neuropsychiatric diseases. However, the mechanisms of thyroid hormone (TH) action in the mature mammalian brain remain unclear. Recent nongenomic actions of TH in adult brains are spotlighted. Many nongenomic mechanisms are modulated by phosphorylation–dephosphorylation of substrate proteins. In the present study, L-triiodothyronine (L-T3) demonstrated differential regulation of phosphorylation status of five different synaptosomal proteins (63, 53, 38, 23, and 16 kD) in both a Ca^{2+}/calmodulin (CaM)-dependent and -independent manner. L-T3 increased the level of phosphorylation of all these five proteins. Ca^{2+}/CaM further stimulated phosphorylation of 63- and 53-kD proteins by L-T3, which were inhibited both by EGTA (Ca^{2+}-chelator) or KN62 (Ca^{2+}/CaM kinase-II [CaMK-II] inhibitor), suggesting the role of CaMK-II. L-T3 increased the phosphorylation of 23- and 38-kD proteins; the effect was independent of EGTA or KN62. The presence of Ca^{2+} decreased L-T3-induced phosphorylation of 63-, 53- and 38-kD proteins. Surprisingly, L-T3-induced phosphorylation of 16-kD protein was not augmented further with Ca^{2+} or Ca^{2+}/CaM; instead, the presence of CaM abolished the L-T3-induced phosphorylation. EGTA or KN62 could not restore the effect of CaM-induced dephosphorylation of this protein. This study identified the role of Ca^{2+}/CaM in the regulation of L-T3-induced protein phosphorylation and supported a unique nongenomic mechanism of second messenger-mediated regulation of protein phosphorylation by TH in mature rat brain. This has profound implications for higher mental functions and strategies for novel therapeutics.

Introduction

Thyroid hormones (TH) contribute their vital actions all through our life. Adult-onset thyroid dysfunctions are well-known to impair various central nervous system (CNS) functions, resulting in several neuropsychiatric disorders such as cognitive dysfunction, declined learning and memory, intellectual deterioration, dementia, anxiety, depression, bipolar disorder, Alzheimer's disease and many more (Stern et al., 2004; Sampaolo et al., 2005; Venero et al., 2005; Montero-Pedrazuela et al., 2006; Stuerenburg et al., 2006; Rivas and Naranjo, 2007; Samuels et al., 2007; Yarbrough et al., 2007). In contrast to the developmental effects of TH, which are mediated through the activation of specific nuclear receptors for TH (NTR) leading to gene expression (Haas et al., 2004; Bernal, 2007), most of these CNS-related changes occurring during adult dysthyroidism are reversible with the proper adjustment of circulatory TH levels (Henley and Koehnle, 1997; Bunevicius and Prange, 2000; Constantinou et al., 2005; Montero-Pedrazuela et al., 2006). Recently, in vivo TH treatment has also been shown to reverse impairment in hippocampal neurogenesis linked with learning and memory during adult-onset hypothyroidism (Montero-Pedrazuela et al., 2006). In spite of these clinical evidences, the underlying molecular mechanism of TH action in the adult CNS remains substantially unexplored and unclear. Although NTRs are present in adult brain (Manzano et al., 2007), no suitable functional properties, except for a relatively few effects on CNS gene expression in adulthood (Haas et al., 2004; Constantinou et al., 2005; Bernal, 2007), could be attributed to them.

The nascent body of emerging evidence suggests that some of the effects of THs are mediated by direct and rapid nongenomic actions of the hormones indicating the existence of a unique nongenomic mechanism for THs in adult mammalian brain (Dratman and Gordon, 1996; Sarkar, 2002; Oetting and Yen, 2007). The distribution, concentration, binding, metabolism and metabolic actions of TH within the mature brain also imply its crucial role (Dratman and Gordon, 1996; Palha et al., 2002; Sarkar, 2002; Scanlan et al., 2004; Sarkar et al., 2006; Kundu et al., 2006). Recent ongoing search on biochemical characterization and unveiling of the important steps of the signaling pathways to understand the molecular basis of the nontranscriptional mechanism of action of TH that leads to physiologic functions are fascinating (Hiroi et al., 2006; Flamant et al., 2007). We have also shown varied levels of THs in adult rat brain cerebrocortical synaptosomes in different thyroid conditions (Sarkar

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and Ray, 1994). L-T3 also has been shown to regulate intracellular calcium (Ca^{2+}) levels in adult rat brain synaptosomes (Mason et al., 1990; Chakrabarti and Ray, 2000; Sarkar and Ray, 2003), in hypothyroid mouse cerebral cortex (Iqbal et al., 2002), and in single rat myocytes (Lomax et al., 1991) rapidly and nongenomically. One possible nongenomic action of THs includes modulation of GABA uptake (Mason et al., 1987a,b). Specific nongenomic effects of THs on chloride flux at the ionotropic brain receptor for the $GABA_A$ receptor are also of interest (Martin et al., 2004).

Many nongenomic mechanisms are synchronized by rapid post-transcriptional amendments, such as protein phosphorylation and dephosphorylation reactions, which act like a molecular switch to modulate intracellular signaling mechanisms. Protein phosphorylation–dephosphorylation mechanisms highly regulate numerous functions of the synaptic network via the actions of protein kinases and protein phosphatases. Abnormalities of these imperative regulatory signaling processes generate deleterious effects on the CNS. As a consequence, variety in unusual protein phosphorylation is the end result of many major neuropsychological dysfunctions leading to diseases. Adequate evidence indicates involvement of several second messenger molecules (such as cAMP, cGMP, NO, and Ca^{2+}) in the activation/deactivation of protein kinases/protein phosphatases through phosphorylation/dephosphorylation reactions (Patwardhan and Miller, 2007). Hypothyroidism has been associated with decreased levels of phosphorylated mitogen-activated protein kinases (MAPKs) in hippocampus from intact adult rat brain (Gerges and Alkadhi, 2004). Recently, we have also reported L-T3-stimulated increase in phosphorylation of a few synaptosomal proteins within minutes in a dose- and time-dependent manner and suggested activation of several kinase pathways in adult rat brain synaptosomes (Sarkar et al., 2006).

We recently reported that L-T3 enhanced phosphorylation levels of a few synaptosomal proteins (113 ± 1 kD, 63 ± 1 kD, 53 ± 1 kD and 38 ± 1 kD) in a dose- and time-dependent manner within minutes in adult rat brain cerebrocortical synaptosomes. We also demonstrated that L-T3 increased dose-dependent phosphorylation of the 112-kD protein at the seryl residues and the 95-kD protein at the tyrosyl residues, suggesting the involvement of several kinase pathways for the regulation of L-T3-induced protein phosphorylation in adult mammalian brain (Sarkar et al., 2006).

On the basis of these observations, the present study aims to identify specific signal transduction pathway(s) involved in the nongenomic TH effect in young adult rat brain. This investigation demonstrates differential regulatory properties of Ca^{2+} and calmodulin (CaM) on L-T3-mediated protein phosphorylation of phosphoprotein substrates in adult rat brain cerebrocortical synaptosomes.

Materials and methods

Materials

Adenosine triphosphate disodium (ATP), 3,5,3′-L-triiodothyronine (L-T3), calmodulin (CaM), bovine serum albumin (BSA), 2-mercaptoethanol, ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), *N*-(2-hydroxyehtyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), sodium dodecyl sulfate (SDS), sucrose, magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), KN-62 and other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). X-ray film CL-Xposure was purchased from Fisher Scientific, Houston, Texas, USA. [γ-^{32}P]-ATP (10 Ci/mmol) was purchased from Perkin Elmer Life and Analytical Sciences, Boston, MA, USA. All other chemicals were of highest reagent grade.

Animals

Young adult male Sprague Dawley rats (~3 months old) were purchased from Harlan Sprague Dawley, Inc., Indianapolis, USA. The rats were housed at 25 ± 1 °C in 12 h dark–12 h light conditions (the light phase began at 8:00 AM) and fed ad libitum with standard rat diet and water. The animals were maintained in the Syracuse University animal facilities at Institute of Sensory Research according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the IACUC of Syracuse University which determined that the experiments appropriately minimized the number of animals used.

Preparation of synaptosomes

Animals were euthanized by quick decapitation, brains were removed and cerebral cortices were surgically dissected out in ice-cold condition. The synaptosomes from the cerebral cortices were prepared as described (Sarkar and Ray, 1992). Briefly, the cerebral cortices were homogenized in 10 volumes of 0.32 M sucrose solution using 8 strokes of a Teflon-glass homogenizer (15 s). The resulting homogenate was centrifuged at 1000 ×g for 10 min at 4 °C. The supernatant was collected and re-centrifuged as above. The supernatant obtained from the second centrifugation was layered over 1.2 M sucrose, and centrifuged at 34,000 ×g for 50 min at 4 °C. The fraction collected between the 0.32 M and 1.2 M sucrose layer was diluted at 1:1.5 with ice-cold bi-distilled water (to avoid shrinkage of the synaptosomes at 0.8 M sucrose concentration and to maintain isoosmotic conditions), further layered on 0.8 M sucrose, and centrifuged at 34,000 ×g for 30 min. The pellet thus obtained was washed, repelleted at 20,000 ×g for 20 min. The pellet was washed again by centrifuging at 20,000 ×g for 20 min. Finally the pellet was collected as purified synaptosomes, and was lysed by hypotonic shock, and assayed for the determination of protein concentration (Vera, 1988).

Protein phosphorylation reactions

Preparation of L-T3 dilutions

For all reactions, L-T3 was dissolved in a minimum volume of 0.1 M NaOH, diluted with reaction buffer containing 50 mM HEPES, 10 mM $MgCl_2$ at pH 7.4, and the final pH was adjusted to 7.4. Control solutions and all hormone dilutions were prepared with the same concentration of NaOH and reaction buffer, and were protected from light.

Dose–response effect of L-T3 on protein phosphorylation

Synaptosomal lysates were incubated at a final concentration of 0.33 mg protein/ml in a reaction mixture containing 50 mM HEPES, 10 mM $MgCl_2$, 0.1 mM EGTA at pH 7.4 in the presence and absence of various concentrations of L-T3 (3 nM to 1 μM). L-T3 was preincubated with the synaptosomal proteins without the ATP for 1 h at 0 °C to ensure maximal effectiveness as pre-determined in a study of synaptosomal membrane binding of ^{125}I-L-T3 (Sarkar and Ray, 1998). The reaction mixture then followed a second preincubation for 5 min at 30 °C for temperature equilibration. The reaction was initiated by adding 20 μM [γ-^{32}P]-ATP (3 μCi). After 1 min, reaction was terminated by adding 1/3 volume of the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer consisting of 125 mM Tris–HCl (pH 6.6), 3% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue at pH 7.

Ca^{2+}/CaM-dependent protein phosphorylation by L-T3

The synaptosomal lysates were incubated at a final concentration of 0.33 mg protein/ml in a reaction mixture containing 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 20 μM [γ-^{32}P]-ATP (3 μCi), 5 μM CaM, 0.5 mM $CaCl_2$, and 2.8 mM EGTA and 20–40 μM KN62 (specific blocker of Ca^{2+}/CaMK-II). L-T3 (10 nM) was preincubated with the synaptosomal protein to obtain maximum effect (Sarkar and Ray, 1998) without the ATP for 1 h at 0 °C followed by a second preincubation for 5 min at 30 °C for temperature equilibration. The reaction was initiated by

adding 20 μM [γ-^{32}P]-ATP (3 μCi). After 1 min the reaction was terminated by adding 1/3 volume of the SDS-PAGE sample buffer.

Analysis of proteins

SDS-PAGE and autoradiography

The samples were heat-denatured for 2 min at 100 °C, mixed, and cooled to room temperature. Proteins were separated by subjecting them to SDS-PAGE (8%) at a constant current (Laemmli, 1970). The gel was stained with silver nitrate, dried and exposed to X-ray film for autoradiography at −80 °C for 48–72 h. The optical densities (OD) of the protein bands in the gel and the phosphorylated bands in the autoradiogram were quantified using 1DSCAN Image PC software program (Scanalytics, Inc., Fairfax, VA, USA) and graphed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Arbitrary densitometric units were normalized in each lane by the ratio of phosphorylation to protein concentration.

Measurement of protein concentrations

Synaptosomal protein content was measured by a turbidimetric method using BSA as a standard (Vera, 1988).

Statistical analysis of data

The values for phosphorylation (integrated OD of an autoradiography band per measure of the corresponding protein in the lane) of three to four separate repetitions of each experiment were expressed as band density (phosphorylation/protein ratio) in arbitrary units. The band densities of the phosphorylated proteins were normalized by calculating the ratio with the corresponding proteins. Results were expressed as mean±SEM from three to four independent experiments. The normalized data were combined and subjected to a one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc comparison test, using SigmaStat software for Windows considering $p<0.05$ as the level of significance.

Results

L-T3 dose-dependently stimulated synaptosomal protein phosphorylation in vitro

Protein phosphorylation and dephosphorylation are important cellular mechanisms regulated by protein kinases and protein phosphatases. In vitro addition of L-T3 (3 nM to 1 μM) resulted in a dose-dependent increase in phosphorylation of several synaptosomal proteins. Particularly consistent with our previous report (Sarkar et al., 2006), three proteins with molecular weights of 38±1, 53±1, and 63±1 kD (determined by calibration using standard molecular weight markers) showed most prominent effects as visualized in the autoradiogram (Fig. 1A). Fig. 1A shows one representative autoradiogram (a). This increase in protein phosphorylation was determined by the amount of ^{32}P-incorporated into the proteins as indicated by the increase in band densities which were then quantified, and the data were normalized with the corresponding protein bands in silver-stained gels (b). The data of four to five different experiments were quantified, normalized, and presented as mean±SEM as shown in Fig. 1B as a function of graded concentrations of L-T3. Three synaptic proteins showed significant levels of dose-dependent phosphorylation induced by L-T3 (3 nM to 1 μM) added in vitro (63-kD protein: $F=22.7$, $p<0.0001$; 53-kD protein: $F=12.9$, $p<0.0001$; 38-kD protein: $F=8.04$, $p<0.0001$). The level of phosphorylation of these three proteins gradually increased from 3 nM to maximally up to 30 nM; it then decreased significantly at higher doses of L-T3 (100 nM to 1 μM), denoting a biphasic nature, a well-known phenomenon observed under the influence of TH in numerous tissues. Particularly, the level of phosphorylation of the 63-kD protein was significantly increased by ~2.2-fold ($p<0.001$), ~2.6-fold ($p<0.001$), and ~1.8-fold ($p<0.001$) at L-T3 concentrations of 3 nM, 10 nM, and 30 nM, respectively,

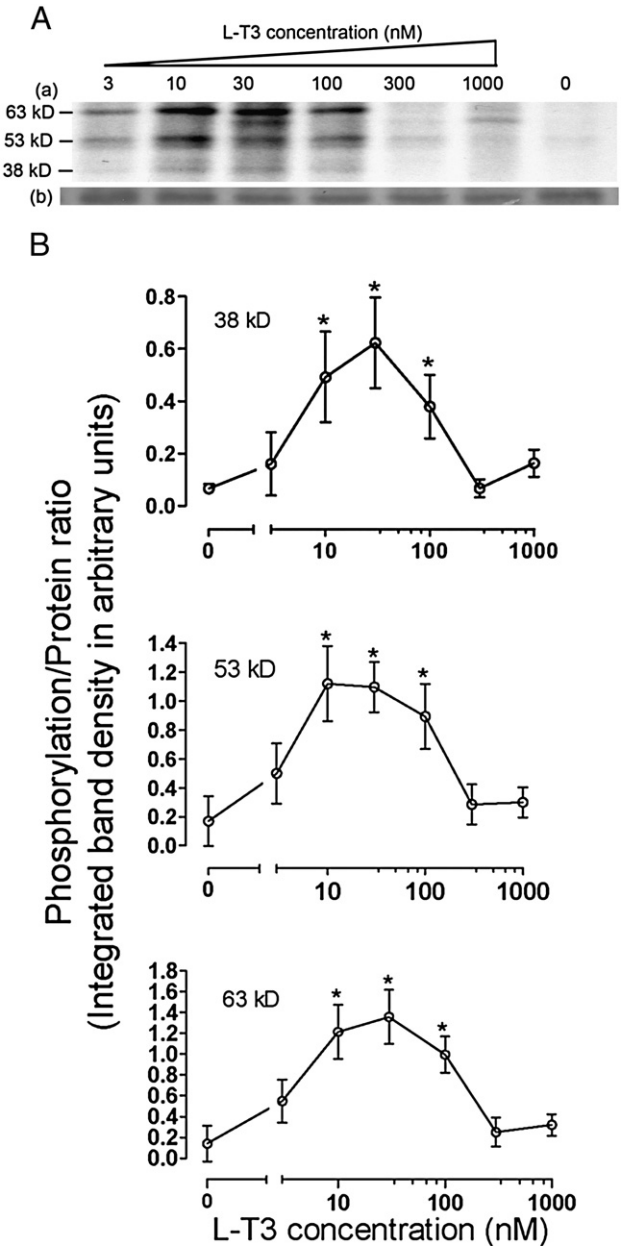

Fig. 1. A. L-T3 dose-dependently regulates phosphorylation of 63-, 53- and 38-kD proteins in a biphasic manner in vitro. (a) Representative autoradiogram of SDS-PAGE (8%) separation of proteins incorporating ^{32}P. The lanes were loaded with synaptosomal lysates obtained following preincubation at 0 °C for 1 h in the presence and absence of graded concentrations of L-T3 (0 nM to 1 μM) and 30 °C for 5 min for temperature equilibration, and then finally incubated with 20 μM of γ^{32}P-ATP (3 μCi) for 1 min at 30 °C. Incorporation of ^{32}P to the 63-, 53- and 38-kD proteins, followed by in vitro addition of increasing concentrations of L-T3, are shown. (b) Corresponding protein bands obtained from the silver-stained gel of the same autoradiogram to visualize equal loading of protein bands, which were further used to normalize protein phosphorylation data. B. The quantification of the dose-dependent L-T3-induced phosphorylation presented as a graph of ratio of the band densities of the phosphorylated proteins in the autoradiogram (a) and the corresponding protein in the silver-stained gel (b) as a function of the concentration of L-T3 (0 nM to 1 μM). The data presented are normalized results combined from three to four individual experiments (mean±SEM) for an indicated protein band (from top 38, 53, and 63 kD) and analyzed by one-way ANOVA, followed by Newman–Keuls test, and described in the Statistical analysis and Results sections. SEMs are represented by vertical bars in the graph considering $p<0.05$. * indicates level of significance $p<0.05$, compared with the basal level (control group) (t test).

compared with the control value with no L-T3 added. The level of phosphorylation of the 53-kD protein was also significantly increased by ~2.2-fold ($p<0.001$), ~2.2-fold ($p<0.001$), and ~1.8-fold ($p<0.001$) at L-T3 concentrations of 3 nM, 10 nM, and 30 nM, respectively, compared with the control value with no L-T3 added. The level of phosphorylation of the 38-kD protein was less prominent in the dose–response experiment compared with the other two proteins (63 kD and 53 kD) but was significantly increased by ~3-fold ($p<0.001$), ~4-fold ($p<0.001$), and ~2.3-fold ($p<0.001$) at L-T3 concentrations of 3 nM, 10 nM, and 30 nM, respectively, compared with the control value with no L-T3 added. No significant difference was noticed between the 10 nM and 30 nM groups for each protein.

L-T3-induced stimulation of phosphorylation of 63- and 53-kD proteins was regulated by Ca^{2+} and calmodulin

Various second messenger molecules, including Ca^{2+} together with CaM, activate several protein kinases such as CaMK-II and control protein phosphorylation mechanisms. The present experiment supported the role of Ca^{2+}/CaM in the L-T3-induced synaptosomal protein phosphorylation (Fig. 2). The 63- and 53-kD proteins significantly increased L-T3-induced phosphorylation in the presence of Ca^{2+}/CaM (Fig. 2). Fig. 2A demonstrates a representative autoradiogram (a) showing the levels of phosphorylation of the 63-, and 53-kD proteins following in vitro addition of 10 nM L-T3. This concentration of L-T3 (10 nM) was determined from the dose–response curve (Fig. 1) and was considered to be within the brain physiological concentration of L-T3 in adult rat synaptosomes as we previously reported (Sarkar and Ray, 1994). Corresponding silver-stained protein bands in Fig. 2A (b) demonstrated equal amounts of sample loading and were used to normalize phosphorylation induced by L-T3 (the ratio of the band densities obtained from the phosphorylated proteins and corresponding silver-stained protein bands). The normalized levels of protein phosphorylation (ratio of phosphorylation to protein concentration) of the 63-kD and 53-kD proteins at different treatment conditions are shown in Fig. 2B. The phosphorylations of 63-kD ($F=37.5$, $p<0.0001$) and 53-kD ($F=22.7$, $p<0.0001$) proteins were significantly stimulated by L-T3 (10 nM) alone by 2-fold and 1.3-fold, respectively, compared with control values. The presence of Ca^{2+} and CaM increased significantly the level of phosphorylation of these proteins (63 and 53 kD) by ~3-fold and ~1.6-fold compared with basal level control values, and by ~1.3-fold and ~1.2-fold compared with Ca^{2+}/CaM control values, respectively. Also, the addition of Ca^{2+} showed a slight decrease in the level of phosphorylation for both 63- and 53-kD proteins ($p<0.05$). The Ca^{2+}/CaM-induced increase in L-T3-effect was further inhibited significantly both by 2.8 mM EGTA (Ca^{2+} chelator) (lanes 7 and 10), and by KN62, a specific blocker of CaMK-II, (lane 8 = 20 μM KN62, lane 11 = 40 μM KN62) as shown in Fig. 2A. Lanes 9, 10, and 11 in Fig. 2A (a) show a representative autoradiogram from a second set of experiments with treatment conditions similar to those of lanes 6, 7, and 8 to observe the effect of KN62 on CaMK-II, except that the 40 μM KN62 was used (lane 11) to check the blocking activity of KN62 at a higher dose. The 40 μM concentration of KN62 completely blocked the effect of Ca^{2+}/CaM-dependent phosphorylation of 63- and 53-kD proteins induced by L-T3 (lane 11). This enhancement of Ca^{2+}/CaM-dependent protein phosphorylation by brain physiological concentrations of L-T3 (10 nM) (Sarkar and Ray, 1994) suggested the involvement of CaMK-II in regulating the phosphorylation of 63- and 53-kD proteins.

Inert action of Ca^{2+} and calmodulin upon the independent effect of L-T3 on the phosphorylation of 38- and 23-kD proteins

L-T3 only slightly enhanced the phosphorylation of the 38-kD protein ($p<0.05$, $F=3.74$) by ~1.2-fold in the presence of Ca^{2+}/CaM (Fig. 3A: lane 6) compared with Ca^{2+}/CaM control group (Fig. 3A: lane 3). Although addition of Ca^{2+} (lane 5) decreased the level of L-T3-induced phosphorylation of 38-kD protein (p = non-significant), it was significantly increased ($p<0.05$) in the presence of CaM (lane 6) compared with Ca^{2+} + L-T3 treatment (lane 5) and only L-T3 effect (lane 4). However, the presence of Ca^{2+} (lane 5) or the Ca^{2+}/CaM (lane 6) did not further affect the phosphorylation status of the 38-kD protein. No significant counteraction of L-T3-induced phosphorylation of the

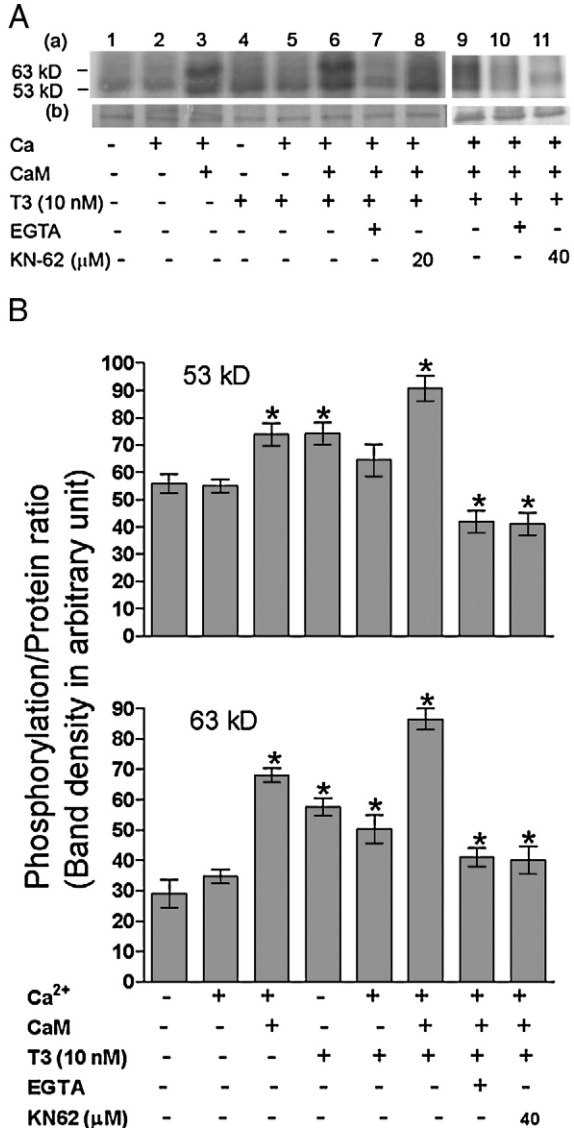

Fig. 2. A. L-T3-stimulated phosphorylation of 63- and 53-kD proteins is regulated by Ca^{2+}/CaM-dependent protein kinase II. (a) Representative autoradiogram of the 63- and 53-kD proteins, followed by various treatment conditions as described. The samples (lanes 1–11) were separated by 8% SDS-PAGE. In lane 8, the concentration of KN62 used was 20 μM. To observe a more pronounced effect of KN62, a separate experiment was conducted and a representative autoradiogram is shown (lanes 9, 10, and 11) with the same experimental condition as described for lanes 6, 7, and 8, except for lane 11 containing 40 μM KN62, which showed a very prominent effect (lane 11) by counteracting CaMK-II activity. (b) Corresponding protein bands from silver-stained gel used for normalization of the data and demonstration of comparable equal amounts of sample loading. B. The quantification of the L-T3 (10 nM)-induced phosphorylation presented as a graph of ratio of the band densities of the phosphorylated proteins in the autoradiogram (a) and the corresponding protein in the silver-stained gel (b) at different treatment conditions as indicated. The data presented are normalized results combined from three to four individual experiments (mean ± SEM) for an indicated protein band (from top 53 and 63 kD) and analyzed by one-way ANOVA, followed by Newman–Keuls test, and described in the Statistical analysis and Results sections. SEMs are represented by vertical bars in the graph considering $p<0.05$. * indicates levels of significance $p<0.05$, compared with the basal level (control group) (t test).

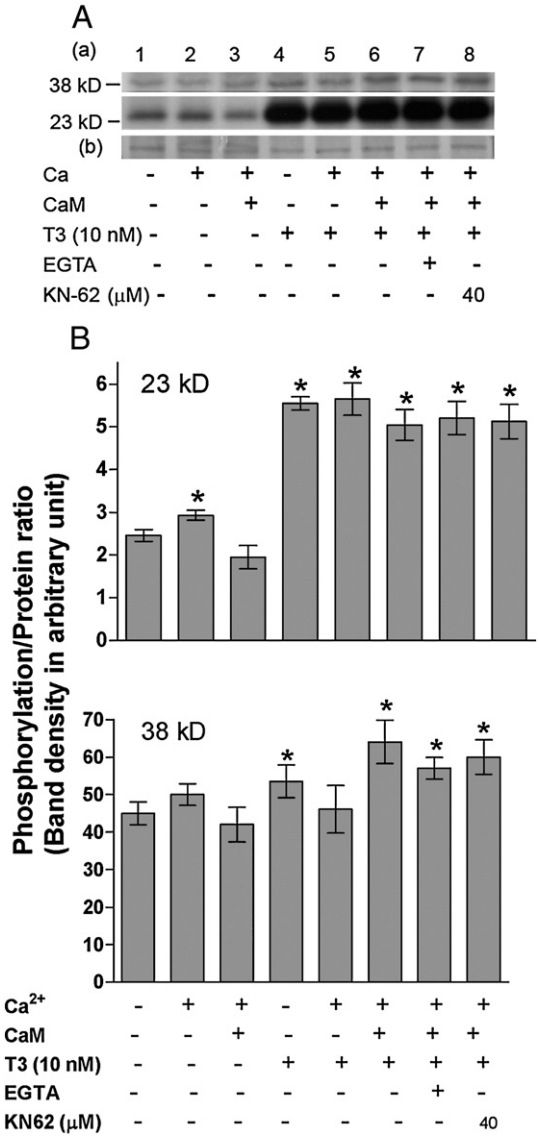

Fig. 3. A. Ca^{2+}/CaM do not modulate L-T3-stimulated phosphorylation of 23- and 38-kD proteins. (a) A representative autoradiogram of the 23- and 38-kD protein separated by SDS-PAGE showing independent stimulatory action of L-T3 (10 nM) upon the phosphorylation of the 23- and 38-kD proteins, which were not further influenced by inclusion of Ca^{2+} and CaM. Addition of EGTA or KN62 was also unable to block the action exerted by L-T3. B. The quantification of the L-T3 (10 nM)-induced phosphorylation presented as a graph of ratio of the band densities of the phosphorylated proteins in the autoradiogram (a) and the corresponding protein in the silver-stained gel (b) at different treatment conditions as indicated. The data presented are normalized results combined from three to four individual experiments (mean ± SEM) for an indicated protein band (from top 23 and 38 kD) and analyzed by one-way ANOVA, followed by Newman–Keuls test, and described in the Statistical analysis and Results sections. SEMs are represented by vertical bars in the graph considering $p<0.05$. * indicates levels of significance $p<0.05$, compared with the basal level (control group) (t test).

38-kD protein was noticed in the presence of either EGTA (lane 7) or KN62 (lane 8) and the phosphorylation levels were not altered. This further suggested no involvement of Ca^{2+}/CaM-dependent pathways mediated through CaMK-II. The autoradiogram (a) in Fig. 3A demonstrates the phosphorylation status of another protein with molecular weight of 23 kD. The phosphorylation level of 23-kD protein was highest among the proteins. In vitro addition of L-T3 (10 nM) significantly increased the phosphorylation level of 23-kD protein by ~2.2-fold (lane 4) ($F=38.6$, $p<0.0001$) compared with the basal level of control values (lane 1: without Ca^{2+}/CaM and L-T3). No noticeable changes were observed following the addition of Ca^{2+} (lane 5) and Ca^{2+}/CaM (lane 6). Specially of interest, EGTA (lane 7) or KN62 (lane 8) did not show any more or less influence on the L-T3-induced increase in the phosphorylation status of the 23-kD protein, suggesting a lack of significant regulation by CaMK-II. Fig. 3B describes the normalized data for the levels of phosphorylation (phosphorylation to protein concentration ratio) of the 38-kD and 23-kD proteins at different treatment conditions.

Calmodulin dephosphorylated 16-kD protein following L-T3-induction

In vitro addition of 10 nM L-T3 significantly increased the level of phosphorylation of 16-kD protein by ~8-fold (Fig. 4A: lane 4) ($F=116.6$, $p<0.0001$). The L-T3-induced phosphorylation of the 16-kD protein was not further activated in the presence of Ca^{2+} (lane 5). The presence of CaM remarkably abolished it (lane 6). Neither chelation of Ca^{2+} by EGTA (lane 7) nor blocking of CaMK-II by KN62 (lane 8) could further restore the level of phosphorylation. The effect of CaM was also noticeable at the basal level of phosphorylation (lane 3) compared with basal level of Ca^{2+}-stimulated phosphorylation (lane 2). Fig. 4B represents the normalized levels of protein phosphorylation (phosphorylation to protein concentration ratio) of the 16-kD protein at different treatment conditions.

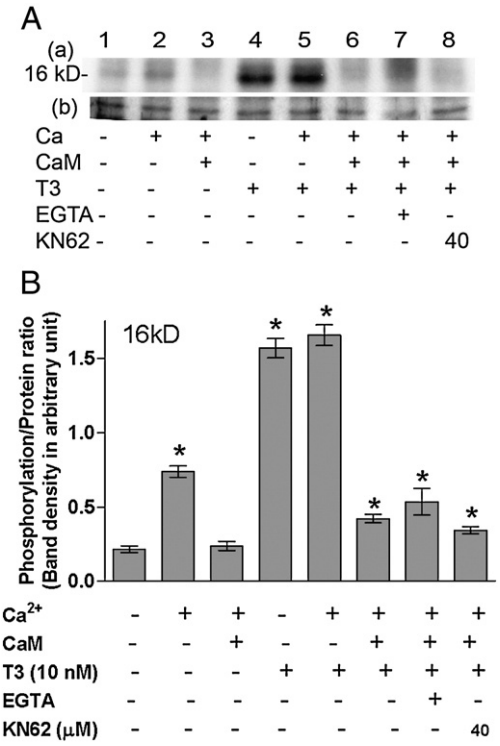

Fig. 4. A. Phosphorylation of 16-kD protein by L-T3 was conquered by the dephosphorylation activity of CaM. (a) A representative autoradiogram of the 16-kD protein separated by SDS-PAGE showing independent stimulatory action of L-T3 (10 nM) on the phosphorylation of the 16-kD protein (lane 4), which persisted even in the presence of Ca^{2+} (lane 5), but was dephosphorylated immediately following the addition of CaM (lane 6) in the presence of Ca^{2+}. This effect of CaM is also noticed at the basal level (lane 3) where Ca^{2+}-induced increase (lane 2) in phosphorylation was dominated by CaM. Addition of EGTA or KN62 was unable to block the action exerted by CaM. (b) Corresponding protein bands of silver-stained gel. B. The quantification of the L-T3 (10 nM)-induced phosphorylation and its dephosphorylation by CaM are presented as a graph of ratio of the band densities of the phosphorylated proteins in the autoradiogram (a) and the corresponding protein in the silver-stained gel (b) at different treatment conditions as indicated. The data presented are normalized results combined from three to four individual experiments (mean ± SEM) for an indicated protein band and analyzed by one-way ANOVA, followed by Newman–Keuls test, and described in the Statistical analysis and Results sections. SEMs are represented by vertical bars in the graph considering $p<0.05$. * indicates levels of significance $p<0.05$, compared with the basal level (control group) (t test).

Discussion

In spite of recent information, the mechanism of action of TH in the regulation of synaptic functions and subsequent signal transduction that systematize delicate regulation of protein phosphorylation–dephosphorylation mechanism in adult mammalian CNS is not clearly defined. The present observations clearly support the role of TH in regulating protein phosphorylation mechanisms in adult rat brain. This study demonstrates the differential effect of brain physiological concentrations of L-T3 (Sarkar and Ray, 1994) on protein phosphorylation in adult rat brain cerebrocortical synaptosomes, suggesting fundamental implications of TH in neuronal signaling in mature mammalian brain. Protein phosphorylation and dephosphorylation through activation/deactivation of protein kinases and protein phosphatases are major regulatory molecular mechanisms with supreme sensitivity for intracellular signal transduction, abnormalities of which may result in immense alterations in normal neuropsychological manifestations (Bibb and Nestler, 2005; Lau and Huganir, 2005; Shchemelinin et al., 2006).

As in our previous study (Sarkar et al., 2006), L-T3 dose-dependently increased the levels of phosphorylation of the three proteins (Fig. 1). This observation predominantly reflected protein kinase activities and highlighted the role of second messengers, such as Ca^{2+}. Our earlier findings supported the involvement of Ca^{2+}. This, in fact, showed that a range of brain physiological concentrations of L-T3 (Sarkar and Ray, 1994), added in vitro, raised intracellular Ca^{2+} levels within adult rat brain cerebrocortical synaptosomes as measured in euthyroid and propylthiouracil (PTU)-induced hypothyroid rats (Sarkar and Ray, 2003). TH-mediated Ca^{2+}-entry was also described in adult rat brain (Mason et al., 1990; Chakrabarti and Ray, 2000), and in hypothyroid mouse cerebral cortex (Iqbal et al., 2002). Increased intracellular Ca^{2+} levels lead to various Ca^{2+}-dependent activation of several intracellular proteins and membrane proteins, including various serine-threonine protein kinases. Many intracellular effects of Ca^{2+} are mediated by CaMKs, particularly the isotype CaMK-II. The regulation of phosphorylation of several Ca^{2+}-dependent proteins in neuronal signal transduction is well documented (Yamauchi, 2005). Hence, the effect of Ca^{2+} and/or CaM on L-T3-induced protein phosphorylation was expected.

The present study described a rapid and nongenomic effect of L-T3 on protein phosphorylation in the adult rat brain cerebrocortical synaptosomal lysates, which was monitored in a Ca^{2+}/CaM-dependent and -independent manner. The inclusion of Ca^{2+} and CaM further showed significant augmentation and regulation of the phosphorylation status of these proteins in the presence of L-T3 (Fig. 2). This effect of L-T3 was blocked by chelating Ca^{2+}-ions by EGTA, suggesting that Ca^{2+} is required in this phosphorylation. Moreover, KN62, a specific blocker of CaMK-II, significantly abolished the level of this phosphorylation, particularly of the 63-kD and 53-kD proteins, suggesting a possible role of CaMK-II in this L-T3-stimulated phosphorylation. Hypothyroidism had been shown to impair long-term potentiation and cognition in adult rat brain with a significant decrease in the levels of phosphorylated CaMK-II, total CaMK-II, and basal levels of CaM within the CA1 region of the hippocampus. L-T4 replacement therapy restored normalcy (Alzoubi et al., 2005). The potential involvement of CaM and Ca^{2+}-dependent adenylate cyclase (AC) and guanine nucleotide regulatory unit had been suggested in non-neural tissues, such as in rat thymocyte plasma membranes as a nongenomic action of L-T3 (Segal, 1990). The role of CaMK-II was also highlighted as a key regulator for the activation of second messenger-independent protein kinases, such as mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK), which used small-molecular-weight guanine nucleotide-binding proteins (Ras, Raf etc.) through the epidermal growth factor receptor/Ras/Raf pathway in thyroid epithelial cells (Montiel et al., 2007). Activation of Ca^{2+}-stimulated AC and CaMK-II had been implicated in the regulation of neuroplasticity and in memory and learning processes in mammalian CNS (Benfenati, 2007). No evidence of rapid and nongenomic regulation of L-T3-stimulated protein phosphorylation in a Ca^{2+}/CaM-dependent way had been reported before in adult rat brain cerebrocortical synaptosomes.

This study also showed that L-T3 independently increased the phosphorylation status of the 23-kD and 38-kD proteins as shown in Fig. 3. L-T3 showed the most pronounced effect on the 23-kD protein phosphorylation in synaptosomal lysates in vitro. This effect of L-T3 was not modulated by either EGTA or KN62, suggesting the involvement of different kinase pathway(s) other than regulated by Ca^{2+} and/or CaM. Involvement of other second messenger-mediated protein kinases may also be possible. The present study already showed that L-T3-stimulated protein phosphorylation might occur through the activation of CaMK-II as observed in the 63- and 53-kD proteins (Fig. 2). The activation of CaMK-II may further trigger MAPK/ERK cascade mechanisms via EGFR/Ras/Raf signal transduction pathways (Montiel et al., 2007). L-T3-induced actin gene expression was also shown to be blocked transcriptionally by the inhibition of protein kinase A (PKA) but not by protein kinase C (PKC) or tyrosine kinase in developing rat cerebra, whereas the expression of tubulin gene was regulated posttranslationally (Sarkar et al., 1999). PKA are activated by cAMP, which is formed by the catalytic activity of AC. Multiple forms of mammalian AC isotypes exist in the mammalian nervous system. They are classified in terms of variance in amino acid sequences and individual differential management by intracellular Ca^{2+}, CaM, and protein phosphorylation. Accordingly, some AC isotypes are Ca^{2+}/CaM-stimulated (AC-I, AC-II and AC-VIII); others are Ca^{2+}-insensitive (AC-II) (Sunahara and Taussig, 2002). It may be possible that increase in intracellular Ca^{2+} by L-T3 activated AC directly or indirectly through the activation of PKC to produce cAMP, which, in turn, activated PKAs, leading to regulation of subsequent substrate protein phosphorylation.

The present study also indicated a remarkably independent effect of L-T3 on one more protein with a molecular weight of 16 kD. The phosphorylation status of the 16-kD protein was significantly increased in the presence of L-T3 alone. The addition of Ca^{2+} could not alter the L-T3-effect any further. However, surprisingly enough, as soon as CaM was added to the reaction media containing both L-T3 and Ca^{2+}, the protein was drastically dephosphorylated (Fig. 4: lane 6). The effect of dephosphorylation ability of CaM was also evident where addition of CaM (Fig. 4: lane 3) caused suppression of the basal level of Ca^{2+}-stimulated phosphorylation (Fig. 4: lane 2) and returned it to the control value (Fig. 4: lane 1). Neither EGTA nor KN62 could restore the L-T3-stimulated phosphorylation level. CaM-induced retention of the dephosphorylation state prevailed in the presence of Ca^{2+} and L-T3 (Fig. 4: lanes 7 and 8). This suggested the dominating power of CaM-dependent phosphatase or calcineurin activity over certain L-T3 action. Calcineurin is a Ca^{2+}/CaM-dependent protein phosphatase 2B. PKA also was shown to activate protein phosphatase 2A (Ahn et al., 2007). The nongenomic rapid action of TH was shown to be mediated via second messenger-independent protein serine-threonine kinases. Decreased levels of phosphorylated MAPK were noticed in hippocampus from intact adult hypothyroid rat brain (Gerges and Alkadhi, 2004). In 293T cells, THs nongenomically promoted MAPK-mediated phosphorylation of nuclear TH receptor α-1 isoform (Lin et al., 1999; Shih et al., 2001). TH-induced MAPK phosphorylation in a variety of intact cell lines was shown to be mediated via a G-protein (Lin et al., 2003; Tang et al., 2004). A direct nongenomic effect of decarboxylated TH derivatives (iodothyronamines) was demonstrated. 3-Iodothyronine (T_1AM) was described as an endogenous and rapid-acting derivative of TH and was shown to be associated with G_s-protein coupled-trace amine receptor 1 in human embryonic kidney (HEK) cells (Scanlan et al., 2004; Zucchi et al., 2006). T_1AM also increased hemodynamic effects and exerted dose-dependent negative ionotropic effects in rats in vivo and in cardiomyocytes by regulating tyrosine kinase. Thus, it was speculated that T_1AM might induce dephosphorylation by activating phosphotyrosine phosphatase. These cardiac

effects of T_1AM were not affected by PKA, PKC, CaMK-II, phosphoinositide 3-kinase (PI3K), MAPK-2, and MAPK kinase (Chiellini et al., 2007). TH caused nongenomic activation of PI3K/protein kinase Akt pathway in human cardiovascular endothelial cell culture by increasing association of TH nuclear receptor, TRα1, with the p85α subunit of PI3K, leading to the phosphorylation and activation of Akt and endothelial nitric oxide synthases (eNOS) (Hiroi et al., 2006). Despite these emerging reports of nongenomic actions of TH in non-neural tissues, knowledge regarding the mechanism of TH action in mature mammalian brain remains controversial and unclear.

TH-induced protein phosphorylation reactions are too fast to be accounted for in genomic action. Overall, the present study has documented, for the first time, the rapid and nongenomic action of TH-induced protein phosphorylation in mature mammalian brain and its differential regulation by Ca^{2+} and CaM. Regulation of intracellular Ca^{2+} and subsequent protein phosphorylation are crucial biological markers that have been shown to be the essence of preservation of various brain and cognitive functions during maintenance of various psychiatric disorders (Monnet, 2005). This study is further important in understanding the nature of phosphorylation/dephosphorylation and identifying and characterizing the specific proteins involved in neuronal signal transduction in mature mammalian brain, particularly in relation to insights about a novel nongenomic mechanism of action of TH. Hence, the present study leans more to constructing a nongenomic membrane-related action of TH, mediated possibly through second messenger(s) in the mature mammalian synaptosome. Understanding the basic molecular mechanism of action of TH in adult mammalian CNS has major implications on the higher mental functions and on the regulation of several neuropsychiatric disorders developed during thyroid dysfunctions in adult humans. This fundamental knowledge may lead to novel strategies to treat adult-onset thyroid disorders, even and possibly the prevention of thyroid-related neuropsychological diseases.

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References

Ahn, J.H., McAvoy, T., Rakhilin, S.V., Nishi, A., Greengard, P., Nairn, A.C., 2007. Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56delta subunit. Proceedings of the National Academy of Sciences U.S.A. 104 (8), 2979–2984.

Alzoubi, K.H., Gerges, N.Z., Alkadhi, K.A., 2005. Levothyroxin restores hypothyroidism-induced impairment of LTP of hippocampal CA1: electrophysiological and molecular studies. Experimental Neurology 195 (2), 330–341.

Benfenati, F., 2007. Synaptic plasticity and the neurobiology of learning and memory. Acta Biomedica 78 (Suppl 1), 58–66.

Bernal, J., 2007. Thyroid hormone receptors in brain development and function. Nature Clinical Practice Endocrinology Metabolism 3 (3), 249–259.

Bibb, J.A., Nestler, E.J., 2005. Serine and threonine phosphorylation, In: Siegelm, G.J., Albers, R.W., Brady, B., Price, D.L. (Eds.), Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 7th ed. Elsevier Inc., Massachusetts, pp. 391–414.

Bunevicius, R., Prange, A.J., 2000. Mental improvement after replacement therapy with thyroxine plus triiodothyronine: relationship to cause of hypothyroidism. International Journal of Neuropsychopharmacology 3 (2), 167–174.

Chakrabarti, N., Ray, A.K., 2000. Rise of intrasynaptosomal Ca^{2+} level and activation of nitric oxide synthase in adult rat cerebral cortex pretreated with 3-5-3′-L-triiodothyronine. Neuropsychopharmacology 22 (1), 36–41.

Chiellini, G., Frascarelli, S., Ghelardoni, S., Carnicelli, V., Tobias, S.C., DeBarber, A., Brogioni, S., Ronca-Testoni, S., Cerbai, E., Grandy, D.K., Scanlan, T.S., Zucchi, R., 2007. Cardiac effects of 3-iodothyronamine: a new aminergic system modulating cardiac function. Federation of American Societies for Experimental Biology Journal 21 (7), 1597–1608.

Constantinou, C., Margarity, M., Valcana, T., 2005. Region-specific effects of hypothyroidism on the relative expression of thyroid hormone receptors in adult rat brain. Molecular and Cellular Biochemistry 278 (1-2), 93–100.

Dratman, M.B., Gordon, J.T., 1996. Thyroid hormones as neurotransmitters. Thyroid 6, 639–647.

Flamant, F., Gauthier, K., Samarut, J., 2007. Thyroid hormones signaling is getting more complex: STORMs are coming. Molecular Endocrinology 21 (2), 321–333.

Gerges, N.Z., Alkadhi, K.A., 2004. Hypothyroidism impairs late LTP in CA1 region but not in dentate gyrus of the intact rat hippocampus: MAPK involvement. Hippocampus 14 (1), 40–45.

Haas, M.J., Mreyoud, A., Fishman, M., Mooradian, A.D., 2004. Microarray analysis of thyroid hormone-induced changes in mRNA expression in the adult rat brain. Neuroscience Letters 365 (1), 14–18.

Henley, W.N., Koehnle, T.J., 1997. Thyroid hormones and the treatment of depression: an examination of basic hormonal actions in the mature mammalian brain. Synapse 27 (1), 36–44.

Hiroi, Y., Kim, H.H., Ying, H., Furuya, F., Huang, Z., Simoncini, T., Noma, K., Ueki, K., Nguyen, N.H., Scanlan, T.S., Moskowitz, M.A., Cheng, S.Y., Liao, J.K., 2006. Rapid nongenomic actions of thyroid hormone. Proceedings of the National Academy of Sciences U.S.A. 103 (38), 14104–14109.

Iqbal, Z., Koenig, H., Trout, J.J., 2002. Triiodothyronine (T3) stimulates calcium influx and membrane transport processes in nerve cells and terminals of hypothyroid mouse cortex. Federation Proceedings 43, 735.

Kundu, S., Pramanik, M., Roy, S., De, J., Biswas, A., Ray, A.K., 2006. Maintenance of brain thyroid hormone level during peripheral hypothyroid condition in adult rat. Life Sciences 79 (15), 1450–1455.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lau, F., Huganir, R., 2005. Tyrosine phosphorylation, In: Siegel, G.J., Albers, R.W., Brady, B., Price, D.L. (Eds.), Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 7th ed. Elsevier Inc., Massachusetts, pp. 415–434.

Lin, H.Y., Shih, A., Davis, F.B., Davis, P.J., 1999. Thyroid hormone promotes the phosphorylation of STAT3 and potentiates the action of epidermal growth factor in cultured cells. Biochemical Journal, 338 (Pt 2), 427–432.

Lin, H.Y., Zhang, S., West, B.L., Tang, H.Y., Passaretti, T., Davis, F.B., Davis, P.J., 2003. Identification of the putative MAP kinase docking site in the thyroid hormone receptor-beta1 DNA-binding domain: functional consequences of mutations at the docking site. Biochemistry, 42 (24), 7571–7579.

Lomax, R.B., Cobbold, P.H., Allshire, A.P., Cuthbertson, K.S., Robertson, W.R., 1991. Triiodothyronine increases intra-cellular calcium levels in single rat myocytes. Journal of Molecular Endocrinology 7 (1), 77–79.

Manzano, J., Bernal, J., Morte, B., 2007. Influence of thyroid hormones on maturation of rat cerebellar astrocytes. International Journal of Developmental Neuroscience 25 (3), 171–179.

Martin, J.V., Padron, J.M., Newman, M.A., Chapell, R., Leidenheimer, N.J., Burke, L.A., 2004. Inhibition of the activity of the native gamma-aminobutyric acid A receptor by metabolites of thyroid hormones: correlations with molecular modeling studies. Brain Research 1004 (1-2), 98–107.

Mason, G.A., Walker, C.H., Prange, A.J., 1987a. Modulation of gamma-aminobutyric acid uptake of rat brain synaptosomes by thyroid hormones. Neuropsychopharmacology 1, 63–70.

Mason, G.A., Walker, C.H., Prange Jr., A.J., Bondy, S.C., 1987b. GABA uptake is inhibited by thyroid hormones: implications for depression. Psychoneuroendocrinology 12, 53–59.

Mason, G.A., Walker, C.H., Prange Jr., A.J., 1990. Depolarization-dependent ^{45}Ca uptake by synaptosomes of rat cerebral cortex is enhanced by L-triiodothyronine. Neuropsychopharmacology 3, 291–295.

Monnet, F.P., 2005. Sigma-1 receptor as regulator of neuronal intracellular Ca^{2+}: clinical and therapeutic relevance. Biology of the Cell 97 (12), 873–883.

Montero-Pedrazuela, A., Venero, C., Lavado-Autric, R., Fernandez-Lamo, I., Garcia-Verdugo, J.M., Bernal, J., Guadano-Ferraz, A., 2006. Modulation of adult hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior. Molecular Psychiatry 11 (4), 361–371.

Montiel, M., Quesada, J., Jimenez, E., 2007. Activation of calcium-dependent kinases and epidermal growth factor receptor regulate muscarinic acetylcholine receptor-mediated MAPK/ERK activation in thyroid epithelial cells. Cellular Signaling 19 (10), 2138–2146.

Oetting, A., Yen, P.M., 2007. New insights into thyroid hormone action. Best practice & research. Clinical Endocrinology & Metabolism 21 (2), 193–208.

Palha, J.A., Nissanov, J., Fernandes, R., Sousa, J.C., Bertrand, L., Dratman, M.B., Morreale, d.E., Gottesman, M., Saraiva, M.J., 2002. Thyroid hormone distribution in the mouse brain: the role of transthyretin. Neuroscience 113 (4), 837–847.

Patwardhan, P., Miller, W.T., 2007. Processive phosphorylation: mechanism and biological importance. Cellular Signaling 19 (11), 2218–2226.

Rivas, M., Naranjo, J.R., 2007. Thyroid hormones, learning and memory. Genes, Brain and Behavior 6 (Suppl 1), 40–44.

Sampaolo, S., Campos-Barros, A., Mazziotti, G., Carlomagno, S., Sannino, V., Amato, G., Carella, C., Di Iorio, G., 2005. Increased cerebrospinal fluid levels of 3,3′,5′-triiodothyronine in patients with Alzheimer's disease. The Journal of Clinical Endocrinology and Metabolism 90 (1), 198–202.

Samuels, M.H., Schuff, K.G., Carlson, N.E., Carello, P., Janowsky, J.S., 2007. Health status, mood, and cognition in experimentally induced subclinical hypothyroidism. The Journal of Clinical Endocrinology and Metabolism 92 (7), 2545–2551.

Sarkar, P.K., 2002. In quest of thyroid hormone function in mature mammalian brain. Indian Journal of Experimental Biology 40 (8), 865–873.

Sarkar, P.K., Durga, N.D., Morris, J.J., Martin, J.V., 2006. In vitro thyroid hormone rapidly modulates protein phosphorylation in cerebrocortical synaptosomes from adult rat brain. Neuroscience 137 (1), 125–132.

Sarkar, P.K., Ray, A.K., 1992. A simple biochemical approach to differentiate synaptosomes and non-synaptic mitochondria from rat brain. Methods and Findings in Experimental and Clinical Pharmacology, 14, 493–497.

Sarkar, P.K., Ray, A.K., 1994. Synaptosomal T_3 content in cerebral cortex of adult rat in different thyroidal states. Neuropsychopharmacology 11, 151–155.

Sarkar, P.K., Ray, A.K., 1998. Specific binding of L-triiodothyronine modulates Na^+–K^+-ATPase activity in adult rat cerebrocortical synaptosomes. NeuroReport 9 (6), 1149–1152.

Sarkar, P.K., Ray, A.K., 2003. Calcium mobilization within hypothyroid adult rat brain synaptosomes. Hormone and Metabolic Research 35 (9), 562–564.
Sarkar, S., Biswas, S.C., Chatterjee, O., Sarkar, P.K., 1999. Protein kinase A linked phosphorylation mediates triiodothyronine induced actin gene expression in developing brain. Molecular Brain Research 67 (1), 158–164.
Scanlan, T.S., Suchland, K.L., Hart, M.E., Chiellini, G., Huang, Y., Kruzich, P.J., Frascarelli, S., Crossley, D.A., Bunzow, J.R., Ronca-Testoni, S., Lin, E.T., Hatton, D., Zucchi, R., Grandy, D.K., 2004. 3-Iodothyronamine is an endogenous and rapid-acting derivative of thyroid hormone. Nature Medicine 10 (6), 638–642.
Segal, J., 1990. Calcium is the first messenger for the action of thyroid hormone at the level of the plasma membrane: first evidence for an acute effect of thyroid hormone on calcium uptake in the heart. Endocrinology 126 (5), 2693–2702.
Shchemelinin, I., Sefc, L., Necas, E., 2006. Protein kinases, their function and implication in cancer and other diseases. Folia Biologica (Praha) 52 (3), 81–100.
Shih, A., Lin, H.Y., Davis, F.B., Davis, P.J., 2001. Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. Biochemistry 40 (9), 2870–2878.
Stern, R.A., Davis, J.D., Rogers, B.L., Smith, K.E., Harrington, C.J., Ott, B.R., Jackson, I.M., Prange Jr., A.J., 2004. Preliminary study of the relationship between thyroid status and cognitive and neuropsychiatric functioning in euthyroid patients with Alzheimer dementia. Cognitive and Behavioral Neurology 17 (4), 219–223.
Stuerenburg, H.J., Arlt, S., Mueller-Thomsen, T., 2006. Free thyroxine, cognitive decline and depression in Alzheimer's disease. Neuro Endocrinology Letters 27 (4), 535–537.
Sunahara, R.K., Taussig, R., 2002. Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. Molecular Intervention 2 (3), 168–184.
Tang, H.Y., Lin, H.Y., Zhang, S., Davis, F.B., Davis, P.J., 2004. Thyroid hormone causes mitogen-activated protein kinase-dependent phosphorylation of the nuclear estrogen receptor. Endocrinology 145 (7), 3265–3272.
Venero, C., Guadano-Ferraz, A., Herrero, A.I., Nordstrom, K., Manzano, J., de Escobar, G.M., Bernal, J., Vennstrom, B., 2005. Anxiety, memory impairment, and locomotor dysfunction caused by a mutant thyroid hormone receptor alpha1 can be ameliorated by T3 treatment. Genes and Development 19 (18), 2152–2163.
Vera, J.C., 1988. Measurement of microgram quantities of protein by a generally applicable turbidimetric procedure. Analytical Biochemistry 174 (1), 187–196.
Yamauchi, T., 2005. Neuronal Ca^{2+}/calmodulin-dependent protein kinase II—discovery, progress in a quarter of a century, and perspective: implication for learning and memory. Biological and Pharmaceutical Bulletin 28 (8), 1342–1354.
Yarbrough, G.G., Kamath, J., Winokur, A., Prange Jr., A.J., 2007. Thyrotropin-releasing hormone (TRH) in the neuroaxis: therapeutic effects reflect physiological functions and molecular actions. Medical Hypotheses 69 (6), 1249–1256.
Zucchi, R., Chiellini, G., Scanlan, T.S., Grandy, D.K., 2006. Trace amine-associated receptors and their ligands. British Journal of Pharmacology 149 (8), 967–978.