Thyroid hormones modulate GABA$_A$ receptor-mediated currents in hippocampal neurons

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ABSTRACT

Thyroid hormones (THs) play a crucial role in the maturation and functioning of mammalian central nervous system. Thyroxine (T4) and 3, 3′, 5-L-triiodothyronine (T3) are well known for their genomic effects, but recently attention has been focused on their non genomic actions as modulators of neuronal activity. In the present study we report that T4 and T3 reduce, in a non competitive manner, GABA-evoked currents in rat hippocampal cultures with IC$_{50}$s of 13 ± 4 μM and 12 ± 3 μM, respectively. The genomically inactive compound rev-T3 was also able to inhibit the currents elicited by GABA. Blocking PKC or PKA activity, chelating intracellular calcium, or antagonizing the integrin receptor αVβ3 with TETRAC did not affect THs modulation of GABA-evoked currents. THs affect also synaptic activity in hippocampal and cortical cultured neurons.

T3 and T4 reduced to approximately 50% the amplitude and frequency of spontaneous inhibitory synaptic currents (sIPSCs), without altering their decay kinetic. Tonic currents evoked by low GABA concentrations were also reduced by T3 ($40 \pm 5\%$, $n = 14$), but not by T4. Similarly, T3 decreased currents elicited by low concentrations of THIP, a low affinity GABAA receptor agonist that preferentially activates extrasynaptic receptors, whereas T4 was ineffective. Thus, our data demonstrate that T3 and T4 selectively affect GABAergic phasic and tonic neurotransmission. Since THs concentrations can be regulated at the level of the synapses these data suggest that the network activity of the whole brain could be differently modulated depending on the relative amount of these two hormones.

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1. Introduction

Thyroid hormones (THs) are critically involved in the development and function of central nervous system. In a classic view THs mediate their effect by controlling gene expression through the binding to nuclear receptors TRα and TRβ.

THs genomic effects are indeed considered fundamental for brain development but less important for adult brain function (Bernal, 2002, 2007). However, also in adult life thyroid dysfunction is often associated with neuropsychiatric disorders such as cognitive impairment, depression, and anxiety (Haggerty et al., 1990; Burmeister et al., 2001; Simon et al., 2002; Gulseren et al., 2006), that can be reversed after adjusting circulating THs levels to normal values.

The molecular mechanisms responsible for the mental disorders caused by THs imbalance are not completely understood yet.

The genomic mechanism has been complimented in the past decade by evidences of THs actions that do not involve nuclear receptors. Recent studies identify THs binding elements in the membrane (integrin α$_V$β$_3$ receptor) (Bergh et al., 2005) that mediate several of the short-term effects of the hormones. Among them are the activation of several protein kinases (MAPK, PKC, PKA, PLC and PLD), the modulation of plasma membrane ion channels (Na$^+$, K$^+$) and pumps (Ca^{2+}/ATPase, Na$^+$/ATPase) (Davis et al., 2005).

An important target for the non-genomic action of THs is also the GABAergic system (Wiens and Trudeau, 2006). There are reports on the THs modulation of GABA uptake (Mason et al., 1987) and on their effects at the level of GABA$_A$ receptor (Martin et al.,

1996, 2004). These works were performed on synaptosomes and heterologus cells expressing recombinant GABA$_A$ receptors but no study so far has investigated the short-term effect of THs on native GABA$_A$ receptors.

In the present work we have studied the effect of T3 and T4 on currents activated by exogenously applied GABA, and on GABAergic neurotransmission in hippocampal and cortical neurons in culture. Two types of GABAergic inhibitions has been identified: a phasic current due to synaptically released GABA and a tonic one mediated by extrasynaptic receptors (Farrant and Nusser, 2005) we analyzed THs modulation of phasic, i.e. sIPSCs, and of the tonic conductance.

2. Material and methods

2.1. Primary cultures of hippocampal and cortical neurons

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609).

Primary cultures were prepared from newborn Sprague-Dawley rats as previously described (Luo et al., 2002). Briefly, cells from hippocampi or cortex were dispersed with trypsin (0.24 mg/ml; Sigma Aldrich, Milan, Italy) and plated at a density of 0.8×10^6 cells/ml on 35 mm Falcon dishes coated with poly-L-lysine (10 μg/ml, Sigma Aldrich). Cells were plated in basal Eagle's Medium (BME; Celbio, Milan, Italy), supplemented with 10% fetal bovine serum (Celbio), 2 mM glutamine and 100 μg/ml gentamycin (Sigma Aldrich) and maintained at 37 °C in 5% CO$_2$. After 24 hours in vitro, the medium was replaced with 1:1 mixture of BME and Neurobasal medium (Celbio) containing 2% B27 supplement, 1% antibiotic, and 0.25% glutamine (Invitrogen). At 5 days in culture (DIC 5), cytosine arabinofuranoside (Ara-C) was added at final concentration of 1 μM. Thereafter, half of the medium was replaced twice a week with Neurobasal medium containing 2% B27 supplement, 1% antibiotic, and 0.25% glutamine. The experiments were performed on cells at 8-10 DIC.

2.2. Electrophysiological recordings

Recordings were performed at room temperature, under voltage-clamp in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Electrodes were pulled from borosilicate glass (Hidelberg, FRG) on a vertical puller (PB-7, Narishige) and had a resistance of 5–7 MOhm. Neurons were voltage clamped at -60 mV and access resistance was monitored throughout the recordings. Currents were amplified with an Axopatch 1D amplifier (Axon Instruments, Foster City. CA), filtered at 5 kHz, digitized at 10 kHz.

2.3. Solutions and drugs

The recording chamber was continuously perfused at 5 ml/min with an artificial extracellular solution composed of (mM): 145 NaCl, 5 KCl, 1 CaCl$_2$, 5 Hepes, 5 Glucose, 20 Sucrose, pH 7.4 with NaOH. Electrode intracellular solution contains (mM): 140 KCl, 3 MgCl$_2$, 5 EGTA, 5 Hepes, 2 ATP-Na, pH 7.3 with KOH. T3, T4, THIP, GABA, Bicuculline, BAPTA, chelerythrine, H-89 and TETRAC were purchased from Sigma-Aldrich (Milan, Italy). PS was from Steraloid (Newport, RI, USA). THs were dissolved in NaOH 0.1 N and kept in stocks at 10 mM. After dilution to the final concentration, an equal amount of HCl 0.1 N was added to balance the pH. GABA was dissolved from 10 mM frozen stocks in water. Drugs were applied directly by gravity through a Y-tube perfusion system (Murase et al., 1989). Drug application had a fast onset and achieved a complete local perfusion of the recorded cell.

BAPTA was dissolved in the intracellular solution and included in the patch pipette, cheleryhrine and H-89 were bath perfused, TERAC was incubated with the cells for 1 hour before the experiment and then bath perfused.

Spontaneous IPSCs (sIPSCs) were recorded in presence of 5 μM NBQX (Tocris) to block AMPA mediated events and 10 μM MK801 to block NMDA currents. GABA tonic currents were recorded in the continuous perfusion of GABA (50 nM) to standardize the ambient GABA surrounding the recorded neuron, similarly to Glykys and Mody (2006).

2.4. Data analysis

All data were expressed as the arithmetic mean ± standard error of mean (SEM) of n experiments. Unpaired or paired Student's t test were used to compare raw data of different experimental conditions unless otherwise specified.

The fitting of the concentration-response relationship was performed on the data normalized to the maximal response using the logistic equation $\%I\text{max} = 100/I\text{max}$ $(1 + (IC_{50}/[ANTAGO]n_h))$ where Imax is the maximal current elicited by the antagonist, IC_{50} is the agonist concentration eliciting the half-maximal response, n_h is the Hill coefficient.

IPSCs were analyzed using MiniAnalysis software (Synaptosoft, Decatur GA, USA). Peak amplitude and decay time (measured as the time to reach 33% of the peak amplitude) was detected for each event and the mean ± SEM was reported. For frequencies analysis interevent intervals between all IPSCs were measured and Kolmogorov-Smirnov (KS) test was performed to determine statistical significance after drug perfusion.

Tonic currents were measured as the difference in the mean baseline current (devoid of synaptic event) during and before application of bicuculline (40 μM). A minimum of 40 s of continuous recording was used for baseline current. Cells with unstable baseline were not included in the analysis. Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit 9 (Molecular Device, Sunnyvale, CA, USA), Origin 6.1 (Microcal, Northampton, MA, USA), MiniAnalysis and Microsoft Office software (Microsoft).

3. Results

3.1. THs inhibit GABA-evoked currents in hippocampal neurons in culture

Using the patch-clamp technique in the whole-cell configuration we tested T4 and T3 on GABA-evoked currents recorded in hippocampal neurons in culture. Both THs reduced currents in a dose dependent way (Fig. 1).

The maximal effect of T4 and T3 was measured at 50 μM and was $-47 \pm 5\%$ and $-67 \pm 4\%$ respectively and fully reversed after 60 s of washout. The concentration response curves showed approximate IC$_{50}$s of 13 ± 4 μM for T4 and of 12 ± 3 μM for T3. No direct channel gating by THs was observed at any of the concentrations tested. The genomically inactive compound rev-T3 also inhibited GABA-evoked currents of $20 \pm 5\%$ at 10 μM and of $57 \pm 4\%$ at 50 μM.
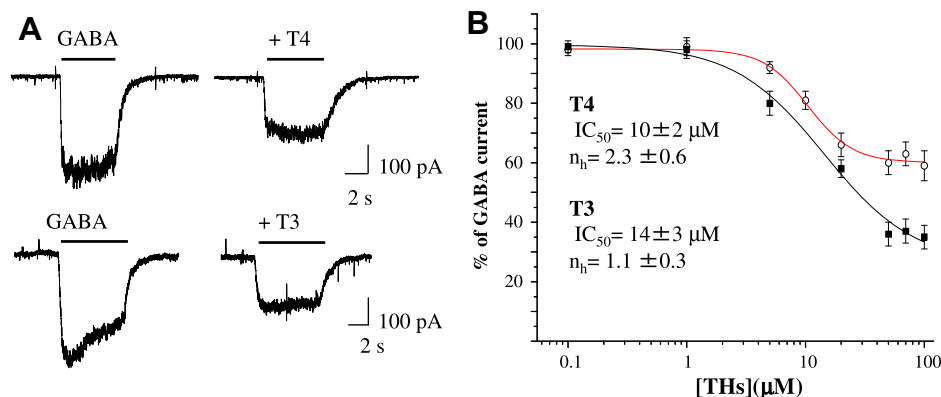
Fig. 1. THs inhibit GABA-evoked currents in cultured hippocampal neurons. A. Whole cell recordings from a cortical neuron in culture showing the current response to GABA 1 μM in control conditions and in the presence of T4 and T3, both at 20 μM. Holding potentials (Hp) = −60 mV. B. Concentration response curves of T3 (filled square) and T4 (empty square) with the indication of the potency (IC$_{50}$) and of the Hill Coefficient (n_H). Each data point is the mean ± SEM of at least 6 cells.

3.2. Mechanism of action of THs

To identify the type of mechanism responsible of the THs' effect we applied T4 and T3 (both at 20 μM) on currents evoked by increasing GABA concentrations. The dose response curves of GABA, GABA + T3 and GABA + T4 show similar EC_{50} (see Fig. 2) but differ in their maximal effect. This behavior is typical of compounds that act through a non competitive mechanism of action. Moreover the effect of THs was significantly decreased at high GABA concentrations: T3 reduced of $44 \pm 2\%$ the current elicited by GABA 0.5 μM and of $24 \pm 5\%$ that evoked by GABA 5 μM. Similarly, T4 reduction was of $32 \pm 5\%$ at GABA 0.5 μM and of $18 \pm 5\%$ at GABA 5 μM.
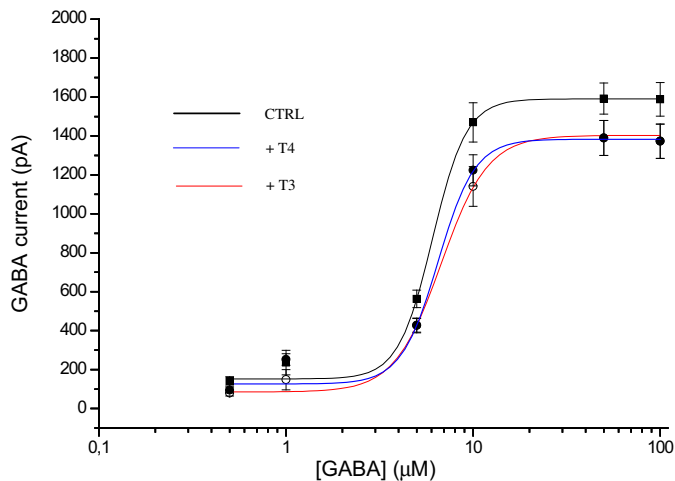
Previous works (Segal, 1990; Zamoner et al., 2005, 2006; Sarkar, 2008) identify intracellular Ca^{2+} concentrations and protein phosphorylation as important for the rapid effect of the hormones. To address this point we tested whether chelating intracellular calcium ions or blocking kinases activity (PKC or PKA) could affect THs modulation of GABA-evoked currents.

In the presence of the Ca^{2+} chelator BAPTA (10 mM), of the PKC blocker chelerythrine (5 μM) or of the PKA blocker H89 (2 μM) (see Methods section) the effect of T3 and T4 on GABA currents was unchanged (Fig. 3).
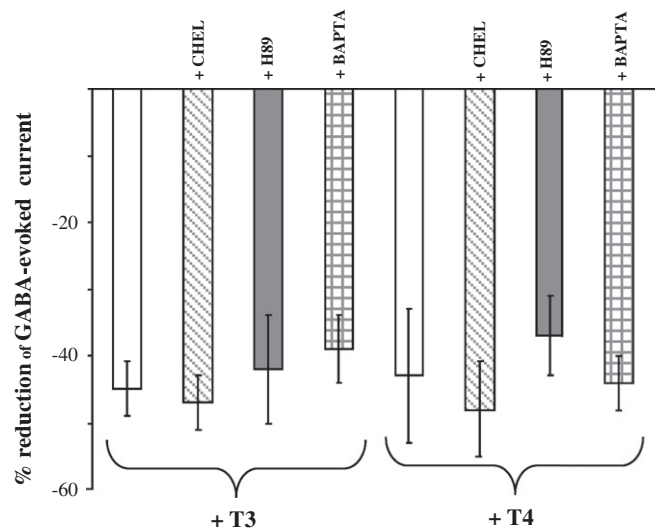
T4 and to a lesser extent T3 bind to the membrane receptor for the integrin αVβ3 (Davis et al., 2005). To check if this receptor is involved in the effect of THs we applied TETRAC, a selective antagonist of the αVβ3 receptor, and measured the modulation of T3 or T4. In these experiments the capability of THs of reducing GABA-evoked current was unmodified compared to control ($T3_{(TETRAC)} = -33 \pm 3\%$ $n = 5$, $T3_{(control)} = -30 \pm 5\%$ $n = 4$; $T4_{(TERAC)} = -25 \pm 5\%$ $n = 4$, $T4_{(control)} = -20 \pm 7\%$ $n = 4$).

Since the three-dimensional structure of T3 is similar to that of pregnenolone sulfate (PS; Martin et al., 2004), we tested if their action is mediated by the binding to the same site on the $GABA_A$ receptor.

We have used concentrations of T3 and PS that elicit the maximal response (i.e. 70 μM for T3 and 100 μM for PS) and measured their effect when applied alone or in combination (Fig. 4A). Our data show that their effect is additive (Fig. 4B) the peak current being

Fig. 3. THs effect does not depend on protein phosphorylation nor on intracellular Ca^{2+} concentration changes. Histogram showing THs effect (both at 20 μM) on currents evoked by GABA alone or in presence of the PKC blocker chelerithine (CHEL; 5 μM in the bath) of the PKA blocker H89 (2 μM in the bath) or the Ca^{2+} chelator BAPTA (10 mM in the patch pipette). Each bar is the mean ± SEM of 4–6 cells.

decreased of $78 \pm 2\%$ after T3 + PS while it was reduced of $69 \pm 3\%$ in the presence of T3 alone and of $38 \pm 6\%$ after PS application. This result suggests that the site of action (within the receptor) of T3 and PS is different. Furthermore their modulation is also "qualitatively" different: PS decreased more the steady state current (ss-currents) than the peak current, while T3 reduced both peak and steady-state current (Fig. 4A and B). The peak/ss of GABA current was 1.12 ± 0.03 in the control, 2.30 ± 0.40 after PS, 1.21 ± 0.05 after T3 and 2.40 ± 0.50 after the coapplication of PS and T3.
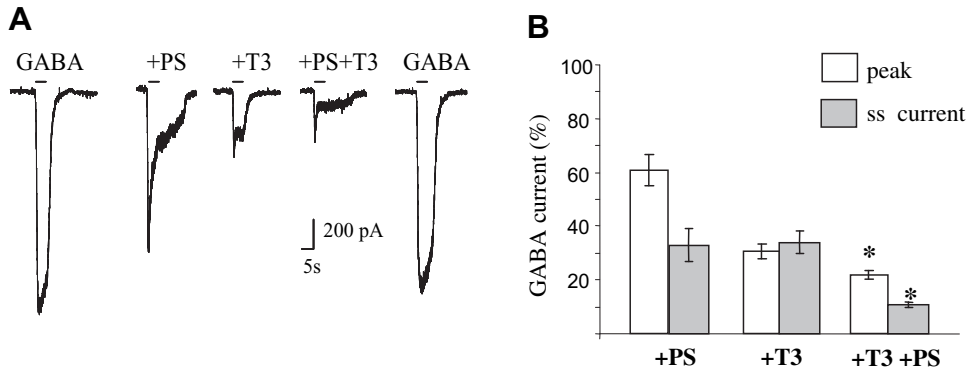
3.3. THs modulate GABAergic phasic and tonic currents

Two types of GABAergic currents have been identified: a phasic current mediated by the synaptic $GABA_A$ receptors and a tonic one mediated by extrasynaptic receptors (Farrant and Nusser, 2005).

We first investigated the effect of THs on sIPSCs recorded in hippocampal cultures at DIC 11–12 (Fig. 5). In all cells tested bath application of T3 (10 μM) reduced sIPSCs peak amplitude ($-32 \pm 5\%$; $p < 0.05$) without altering the decay kinetics (Table 1). Similarly, T4 (10 μM) decreased peak amplitude ($-40 \pm 8\%$; $p < 0.05$) with no changes in the decay phase of sIPSCs. Frequency of sIPSCs was significantly diminished by T3 in 4 out of 8 cells and by T4 in 5 out of 9 cells (KS test $p < 0.05$). The mean frequency values, calculated in the responsive cells, were reduced from 0.70 ± 0.38 Hz in control to 0.37 ± 0.17 Hz in the presence of T3 and from 0.57 ± 0.22 Hz to 0.20 ± 0.06 Hz in the presence of T4.

We performed similar experiments on cultured cortical neurons to disclose a possible cell selectivity in the action of the hormones. As in hippocampal neurons, in cortical cultures T3 and T4 reduced peak amplitude by $50 \pm 4\%$ and $58 \pm 5\%$ respectively leaving the decay times unchanged (Table 1).

IPSCs frequencies were significantly (KS test $p < 0.05$) reduced by T3 in 4 out of 5 cells and by T4 in 6 out of 7 cells. The mean frequency values, in the responsive cells, were reduced from 1.57 ± 0.58 Hz in control to 0.59 ± 0.27 Hz in the presence of T3 and from 1.06 ± 0.29 Hz to 0.45 ± 0.16 Hz after T4.

We then considered the effect of THs on the tonic current mediated by high affinity $GABA_A$ receptors. Tonic current, elicited by application of GABA 50 nM (Glykys and Mody, 2006) was

Fig. 2. THs antagonism is not competitive. GABA concentration-response curves in the presence or absence of T3 or T4. Cortical neurons at 8 DIC were perfused with increasing concentrations of GABA alone or with T3 (20 μM) or T4 (20 μM). GABA EC_{50} were 6.1 ± 0.4 μM in the absence of hormones, 6.5 ± 0.4 μM with T4 and 6.7 ± 0.9 μM with T3. Results are presented as % of the response to 100 μM GABA. Each bar is the mean ± SEM of 6–13 experiments.

Fig. 4. T3 effect is additive to that of PS. A. Representative experiment showing the modulation of GABA-evoked current by PS, T3 and the combination of the two. Currents were recorded from a cortical neuron in culture (Hp = −60 mV). B. Histogram showing the GABA current (%) peak or steady state (SS) after application of PS (100 μM), T3 (70 μM) and PS +T3. Each bar is the mean ± SEM of 5 experiments. (*$p < 0.05$ paired t test vs. T3 alone).

reduced of 40 ± 5% ($n = 14$, $p < 0.01$) by 20 μM T3 while it was not significantly changed after perfusion with 20 μM T4 ($n = 9$) (Fig. 6A–C).

In order to preferentially activate extrasynaptic GABA$_A$ receptors, we used low concentrations (2 μM) of THIP, a ligand with partial agonist behavior at most synaptic receptors and full agonist

properties at the extrasynaptic one containing δ subunit (Mortensen et al., 2010). Also in these experiments, T3 reduced THIP current (−43 ± 13%, $n = 6$), while T4 was ineffective ($n = 4$) (Fig. 6D).

Current elicited by high concentrations of THIP (10 μM), that recruit extrasynaptic and synaptic receptors, is significantly reduced by both hormones (T4: −24 ± 5%, $n = 8$; T3: −56 ± 3%, $n = 6$) (Fig. 6D).
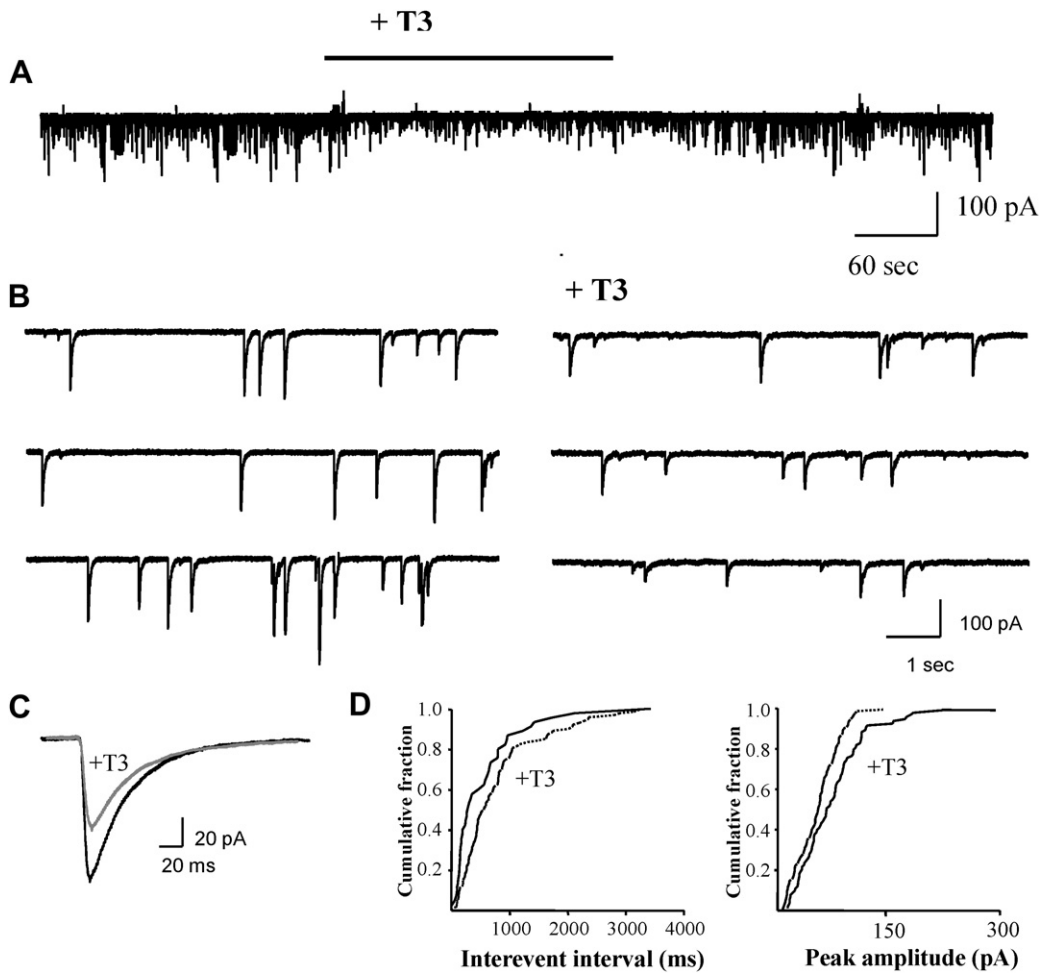
Fig. 5. THs inhibit spontaneous Inhibitory Postsynaptic Currents. A. Typical recording of sIPSCs from a hippocampal neuron, in the presence of NBQX 5 μM and MK801 10 μM, under control conditions and after bath application of T3 10 μM. The bar depicts the time length of the hormone application. B. sIPSCs as in A at a different time scale in control conditions (left trace) and after perfusion of T3 (right trace). C. Averaged sIPSCs in control and after T3 (gray) from the same neuron as in A. The decay time constant in the two paradigms is unchanged (decay time$_{control}$ = 39 ms, decay time$_{T3}$ = 38 ms). D. Cumulative probability distribution of the inter-event intervals (left) and of the peak (right) of sIPSCs in control and after T3 perfusion from the same neuron as in B. ($p < 0.01$, KS test).

Table 1
Summary of THs effects on GABA sIPSCs recorded from hippocampal and cortical cultures.

	Peak (pA)	0.33 Decay (ms)
sIPSCs hippocampal		
CTRL	75 ± 14	36 ± 3
+T3	$45 \pm 7^*$	36 ± 3
CTRL	74 ± 24	36 ± 4
+T4	$37 \pm 10^*$	35 ± 5
sIPSCs cortical		
CTRL	84 ± 14	30 ± 3
+T3	$42 \pm 13^*$	26 ± 6
CTRL	75 ± 15	25 ± 2
+T4	$44 \pm 7^*$	$32 \pm 3^*$

The values of sIPSCs peak amplitude and decay are the mean $\pm$ SEM of 8–9 cells. T3 and T4 were perfused at 10 μM (*$p < 0.05$ paired t test vs control)

4. Discussion

Thyroid dysfunction in adults is often associated with psychiatric manifestations. Several neurotransmitter systems could be affected by THs imbalance either through a slow genomic (e.g. changes in the expression of receptors) or non-genomic mechanisms of action (e.g. fast changes in the biochemical or biophysical properties of the receptors) (Davis et al., 2005).

Our group has recently focused on the rapid nongenomic effects of thyroid hormones on neurotransmission in hippocampus. We have shown that THs modulate excitatory neurotransmission through a non-transcriptional mechanism at the level of glutamate ionotropic receptors (Losi et al., 2008).

4.1. THs reduce GABA-evoked currents

Here we present new evidence on THs modulation of GABA$_A$ receptor in hippocampal neurons in culture. Our data show that T3 and T4 reduce GABA-evoked currents with potencies in the micromolar range (12–13 μM). In a previous work Martin et al. (1996) reported an inhibitory effect of THs on GABA-stimulated Cl$^-$ flux in synaptosomes and a decrease of GABA-evoked current in recombinant receptors in HEK293 cells. THs' effect (IC$_{50}$ in the low micromolar range) was present either in $\alpha 1\beta 2\gamma 2$ or $\alpha 1\beta 2$ but not in $\alpha 6\beta 2\gamma 2$ GABA$_A$ receptors. Chapell et al. (1998) showed also in Xenopus oocytes a similar subunit specificity, moreover a direct channel gating evoked by THs was described in all the subunit combination tested. In our experiments we could not observe any direct effect of THs. This can be explained by the fact that in neurons the amount of receptors expressed is considerably lower than in oocytes, and in these cells THs- induced current, smaller than the GABA evoked one, might be difficult to detect.

The concentrations of THs able to modulate GABA$_A$ receptor function used in our experiments and in previous ones (Chapell et al., 1998; Martin et al., 2004) could be considered out of physiological range. It has been shown that average THs concentrations in rat whole brain are in the low nanomolar range (Morreale de
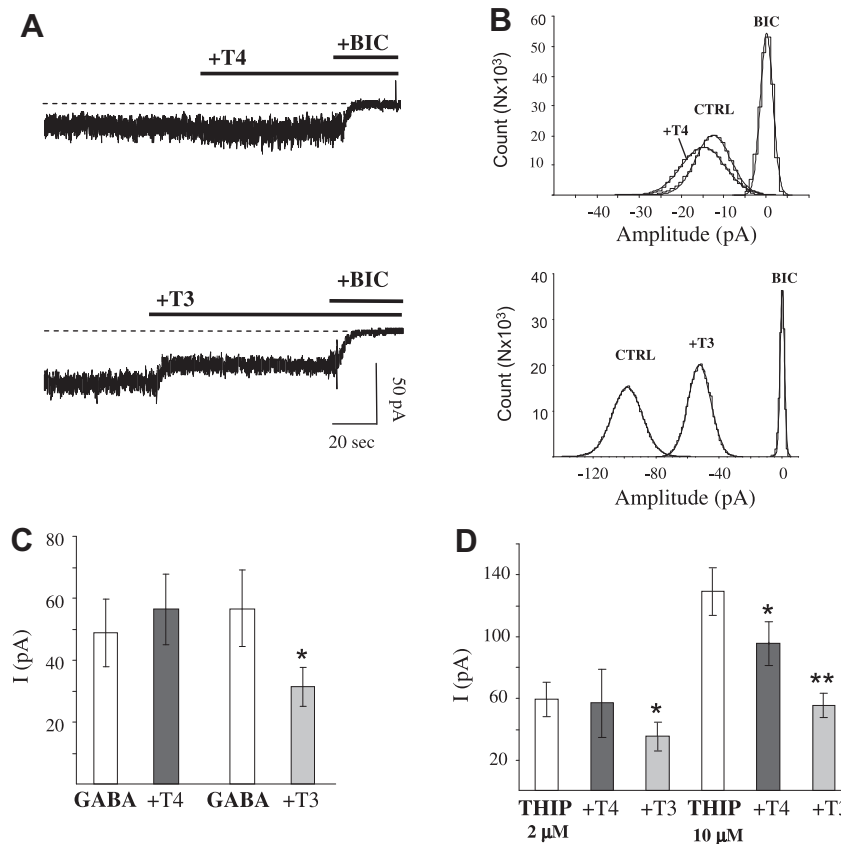
Fig. 6. T3 and T4 differently modulate tonic current. A. Current traces from hippocampal neuron in culture showing the effect of T4 and T3 (both at 20 μM) on the bicuculline (BIC; 40 μM)-sensitive current elicited by GABA 50 nM. B. Amplitude distributions of current traces shown in the panel A. Mean baseline current during the application of BIC was adjusted to 0. Distributions were drawn from current segments immediately preceding THs application for CTRL and before application of BIC for T3 and T4 (see Methods). C-D Histograms showing the mean tonic current sustained by GABA (50 nM) or by THIP (2 or 10 μM) without and with THs. Each bar is the mean $\pm$ SEM of 8–16 cells. (*$p < 0.05$ **$p < 0.01$, paired t test).

Escobar et al., 1994). However, hormones concentrations in presynaptic terminals are uncertain and the dynamic of THs levels in this compartment are poorly understood. Furthermore, it was reported that THs could accumulate in nerve endings (Dratman et al., 1976; Dratman and Crutchfield, 1978; Kastellakis and Valcana, 1989) suggesting that their concentrations at the GABA$_A$ receptor site could be higher than the whole brain levels.

Mason et al. (1993) demonstrated that T3 could be released from synaptosomes after depolarization with a Ca^{2+} dependent mechanism, supporting the hypothesis of a role for T3 as neurotransmitter or neuromodulator, as firstly developed by Dratman in 1974.

As for several steroids synthesized in the brain (neurosteroids) interacting directly with neurotransmitter receptors (Belelli and Lambert, 2005; Puia et al., 1990, 2003), also THs concentrations are locally regulated by the activity of the synthetic and metabolic enzymes (Courtin et al., 2005). Indeed the expression and activitiy of deiodinase type II (DII) and III (DIII) are different in the diverse brain areas (Pinna et al., 2002) and change after pharmacological treatments (Eravci et al., 2000) and in physiological or in pathophysiological situations (Broedel et al., 2003).

4.2. Which is the mechanism of THs action?

Some of the non genomic actions of THs depend on protein phosphorylation and intracellular Ca^{2+} concentration (Zamoner et al., 2005, 2006; Sarkar, 2008): for this reason in our experiments we analyzed THs' effect in the presence of chelerityne, H89 or BAPTA. The modulation of GABA-evoked currents was unchanged after these treatments suggesting that THs do not act through PKC or PKA activity or by affecting Ca^{2+} intracellular concentration.

Recently a cell surface receptor for THs, i.e. the integrin αVβ3, has been linked to several non genomic effects of THs (see Introduction). We used TETRAC, a deaminated analogue of T4, as a probe to screen for the participation of the integrin receptor in the action of THs. The presence of the antagonist of αVβ3 did not abolish the hormones effect ruling out the possible involvement of the integrin receptor in THs activity.

Is there a binding site for THs in the GABA$_A$R?

The analysis of the dose response curves of GABA in the presence of THs is consistent with a non competitive nature of the hormones antagonism. A similar type of antagonism for THs was found in Xenopus oocytes and in cortical synaptosomes by Chapell et al. (1998) and by Martin et al. (2004). The fact that THs effect was significantly reduced in the presence of high GABA concentrations though suggests that an allosteric interaction between the putative THs and the agonist site should exist.

THs do not bind to benzodiazepine (BZ) recognition site because flumazenil, an antagonist at this site, does not block their actions (Martin et al., 1996); additionally, the modulation of THs, similarly to that of neurosteroids (Puia et al., 1990), does not require the presence of γ subunit, that is essential for BZ activity.

PS is a neurosteroid that share with THs a lot of similarity either in the structure (Martin et al., 2004) or in the type of modulation (Majewska and Schwartz, 1987; Majewska et al., 1988). Discrete sites for neurosteroids binding have been identified in the GABA$_A$ receptor, among them one or two for inhibitory sulfate steroids such as PS (Hosie et al., 2006, 2007, 2009). Our work provides indirect evidence that the binding site of T3 and PS is different because at concentrations that elicit a maximal response their effect is additive (see Fig. 3).

The peak plateau ratio of the GABA-evoked current was increased by PS, indicating an increase in the rate of desensitization of the receptor, as previously shown by Shen et al. (2000). The same ratio did not change after T3 application suggesting that its effect was not due to an increase in receptor desensitization.

The activity of THs in heterologous systems, and the selectivity for certain subunit compositions (Chapell et al., 1998; Martin et al., 1996) prompted us to surmise that THs should directly interact with the receptor and that specific residues should confer to the protein the sensitivity for THs.

4.3. THs decrease amplitude and frequency of synaptic currents

We then investigated THs activity on currents produced by the spontaneous release of GABA at synaptic sites (sIPSCs). T3 and T4 reduced of about 50% the amplitude of sIPSCs (Table 1) in all neurons we recorded from, while a significative reduction in frequency was detected only in a subset of neurons. The reason for this difference could stem in the very different mechanisms responsible for the THs effect on amplitude and frequency of the synaptic events.

The reduction of current amplitude could be explained by a postsynaptic mechanism, i.e. an effect at the level of the GABA$_A$ receptor, as already proved by previous experiments in which the agonist was exogenously applied. The decrease of sIPSCs amplitude by THs is further supporting the finding of their non competitive mechanism of action since also at high concentrations of agonist, such as those found in the synaptic cleft following physic GABA release, the modulation of THs is still present. The reduction in the frequency of the events could instead be due to either a post- or pre-synaptic mechanism. A presynaptic action requires the presence in the presynaptic terminal of auto- or hetero-receptors regulating neurotransmitter release that are sensitive to THs modulation.

The lack of THs' effect on the frequency of events we detected in some cells could be indeed due to a lack of the receptor regulating neurotransmitter release in the presynaptic terminal. Furthermore THs could also change the release of GABA by acting at the level of cytoskeleton proteins (Farwell et al., 2005; Zamoner et al., 2006). Further experiments are needed to demonstrate these hypothesis.

The frequency of sIPSCs was decreased only in 50% of the hippocampal neurons while in cortical cultures the effect was present in 80% of the neurons we recorded from. This discrepancy could be ascribed to the low basal synaptic activity in the hippocampal cultures that hampered the detection of the effect in some cells. Another explanation for this reduced effect is a different sensitivity of THs for the two types of neuronal cultures as Martin et al. showed in neurosynaptosomes (1996).

A decrease in sEPSCs (spontaneous excitatory postsynaptic currents) frequency mediated by NMDA in hippocampal slice was also detected after perfusion with T3, but not with T4 (Losi et al., 2008).

Similarly to THs, PS decreases the frequency of sIPSCs via pre- and post-synaptic mechanisms even though the two effects were obtained with different concentrations of the neurosteroid (Mtchedlishvili and Kapur, 2003).

Hypo thyroidal rats exhibit an increase in the neurotransmitter release that is reversed by T3 administration (Vara et al., 2002). Even though these authors claimed that the effect was mainly genomic, i.e. an increase in the expression of proteins involved in the synaptic release (sinapsin I and synaptogamin I), the non-genomic action at the level of the GABA$_A$ receptor could not be excluded.

In a recent work Westerholz et al. (2010) showed that in hippocampal cultures, chronically treated with nanomolar concentrations of T3, spontaneous activity is increased, compared to the control, via nuclear receptor activation. They also tested the acute effect of THs but no changes in frequency were detected probably due to the low concentrations used.

4.4. THs differently modulate tonic currents

Tonic inhibition plays a crucial role in regulating neuronal excitability because it sets the threshold for the generation of action potential and also allows the integration of excitatory signals. Extrasynaptic GABA$_A$ receptors possess higher affinity for GABA and slower rate of desensitization than synaptic ones (Brickley et al., 1999) and most of them contain δ subunit (Farrant and Nusser, 2005). THIP is a partial agonist at most synaptic type receptors containing $\alpha_x\beta_x\gamma$ while is a full agonist at receptors containing δ subunit (Adkins et al., 2001; Brown et al., 2002; Belelli et al., 2004; Drasbek and Jensen, 2006). For this reason the use of low concentrations of THIP should recruit extrasynaptic GABA$_A$ receptors containing δ subunit. Our data show that T3 decreases the currents evoked by low concentrations of GABA and those evoked by THIP at both concentrations used.

Conversely, T4 is inactive on currents evoked by GABA 50 nM and THIP 2 μM, while it reduces the current evoked by THIP 10 μM probably by acting on synaptic receptors.

Thus T3 and T4 have a diverse activity on GABAergic inhibition: both hormones are active at the synaptic level but only T3 modulates extrasynaptic GABA$_A$ receptors.

A difference in the effect of T3 and T4 in modulating synaptic activity was also highlighted by Caria et al. (2009) that showed that in CA1 neurons in slices from euthyroidal rats T3 and T4 have opposite activity: T3 potentiates while T4 decreases the firing rate evoked by norepinephrine.

4.5. What is the physiological significance of the diverse behavior of T3 and T4?

It has been shown that in several pathophysiological situations changes in tonic current could occur (Curia et al., 2009; Maguire et al., 2005; Scimeni et al., 2005; Zhang et al., 2007). Blocking extrasynaptic GABA$_A$R in specific brain areas can have profound effect on neuronal excitability (Semyanov et al., 2003). For this reason by acting on tonic currents T3 could provide a stronger inhibition than T4 that only regulate synaptic transmission.

In the brain T3 is mainly derived from the metabolism of T4, so an increase or a decrease in the activity of the metabolizing enzyme DII, consequent to pathophysiological situations or to pharmacological treatments (Broedel et al., 2003; Eravci et al., 2000), could alter the relative ratio of the two hormones and the type of inhibition of the GABAergic transmission.

Moreover it is possible that other metabolites of T3 and T4, such as rT3 or T1AM (Caria et al., 2009; Gompf et al., 2010) could play a role in the modulation of GABAergic neurotransmission.

5. Conclusions

Our data show that THs can directly modulate inhibitory neurotransmission in hippocampal and cortical neurons acting as negative allosteric modulators of GABA$_A$ receptor.

GABAergic system regulates the network activity of the whole brain and is under the control of different endogenous substances (such as neurosteroids, endozepines). Our data suggest that also thyroid hormones can participate in this modulation and that changes in their levels, also in very limited regions of the brain, could produce profound alterations in the whole brain activity. In conclusion, we believe that the modulation of GABAergic transmission by THs derives from the co-partnership of non genomic and genomic effects, ensuring the first a fine and timing regulation of brain activity, the latter more profound and stable changes.

References

Adkins, C.E., Pillai, G.V., Kerby, J., Bonnert, T.P., Haldon, C., McKernan, R.M., Gonzalez, J.E., Oades, K., Whiting, P.J., Simpson, P.B., 2001. Alpha4beta3delta GABA(A) receptors characterized by fluorescence resonance energy transfer-derived measurements of membrane potential. J. Biol. Chem. 276, 38934–38939.

Belelli, D., Lambert, J.J., 2005. Neurosteroids: endogenous regulators of the GABA(A) receptor. Nat. Rev. Neurosci. 6, 565–575.

Belelli, D., Pedenm, D.R., Rosahl, T.W., Wafford, K.A., Lambert, J.J., 2004. Extra-synaptic GABA(A) receptors of thalamocortical neurons: a molecular target for hypnotics. J. Neurosci. 25, 11513–11520.

Bergh, J.J., Lin, H.Y., Lansing, L., Mohamed, S.N., Davis, F.B., Mousa, S., Davis, P.J., 2005. Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology 146, 2864–2871.

Bernal, J., 2002. Action of thyroid hormone in brain. J. Endocrinol. Invest. 25, 268–288.

Bernal, J., 2007. Thyroid hormone receptors in brain development and function. Nat. Clin. Pract. Endocrinol. Metab. 3, 249–259.

Brickley, S.G., Cull-Candy, S.G., Farrant, M., 1999. Single-channel properties of synaptic and extrasynaptic GABAA receptors suggest differential targeting of receptor subtypes. J. Neurosci. 15, 2960–2973.

Broedel, O., Eravci, M., Fuxius, S., Smolarz, T., Jeitner, A., Grau, H., Stoltenburg-Didinger, G., Plueckhan, H., Meinhold, H., Baumgartner, A., 2003. Effect of hyper and hypothyroidism on thyroid hormone concentrations in regions of the rat brain. Am. J. Physiol. Endocrinol. Metab. 285, E470–E480.

Brown, N., Kerby, J., Bonnert, T.P., Whiting, P.J., Wafford, K.A., 2002. Pharmacological characterization of a novel cell line expressing human alpha(4)beta(3)delta GABA(A) receptors. Br. J. Pharmacol. 136, 965–974.

Burmeister, L.A., Ganguli, M., Dodge, H.H., Toczek, T., DeKosky, S.T., Nebes, R.D., 2001. Hypothyroidism and cognition: preliminary evidence for a specific defect in memory. Thyroid 11, 1177–1185.

Caria, M.A., Dratman, M.B., Kow, L.M., Mameli, O., Pavlides, C., 2009. Thyroid hormone action: non genomic modulation of neuronal excitability in the hippocampus. J. Neuroendocrinol. 21, 98–107.

Chapell, R., Martin, J., Machu, T.K., Leidenheimer, N.J., 1998. Direct channel-gating and modulatory effects of triiodothyronine on recombinant GABA(A) receptor. Eur. J. Pharmacol. 349, 115–121.

Courtin, F., Zrouri, H., Lamirand, A., Li, W.W., Mercier, G., Schumacher, M., Goascogne, C.L., Pierre, M., 2005. Thyroid hormone deiodinases in the central and peripheral nervous system. Thyroid 15, 931–942.

Curia, G., Papouin, T., Seguela, P., Avoli, M., 2009. Downregulation of tonic GABAergic inhibition in a mouse model of Fragile X syndrome. Cereb. Cortex 19, 1515–1520.

Davis, P.J., Davis, F.B., Cody, V., 2005. Membrane receptors mediating thyroid hormone action. Trends Endocrinol. Metab. 16, 429–435.

Drasbek, K.R., Jensen, K., 2006. THIP, a hypnotic and antinociceptive drug, enhances extrasynaptic GABAA receptor mediated conductance in mouse neocortex. Cereb. Cortex 16, 1134–1141.

Dratman, M.B., Crutchfield, F.L., Axerold, J., Colburn, R.W., Thoa, N., 1976. Localization of triiodothyronine in nerve ending fractions of rat brain. Proc. Natl. Acad. Sci. U.S.A. 73, 941–944.

Dratman, M.B., Crutchfield, F.L., 1978. Synaptosomal [^{125}I]triiodothyronine after intravenous [^{125}I]thyroxine. Am. J. Physiol. 235, E638–E647.

Eravci, M., Pinna, G., Meinhold, H., Baumgartner, A., 2000. Effect of pharmacological and nonparmacological treatments on thyroid hormone metabolism and concentrations in rat brain. Endocrinolgy 141, 1027–1040.

Farrant, M., Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat. Rev. Neurosci. 6, 215–229.

Farwell, A.P., Dubord-Tomasetti, S.A., Pietrzykowski, A.Z., Stachelerk, S.J., Leonard, J.L., 2005. Regulation of cerebellar neuronal migration and neurite outgrowth by Thyroxine and 3,3,5- triiodothyronine. Brain Res. Dev. Brain Res. 154, 121–135.

Glykys, J., Mody, I., 2006. Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA(A) receptor alpha 5 subunit −deficient mice. J. Neurophysiol. 95, 2796–2807.

Gompf, H.S., Greenberg, J.H., Aston-Jones, G., Ianculescu, A.G., Scanlan, T.S., Dratman, M.B., 2010. 3-Monoiodothyronamine: the rationale for its action as an endogenous adrenergic-blocking neuromodulator. Brain Res. 1351, 130–140.

Gulseren, S., Gulseren, L., Hekimsoy, Z., Cetinay, P., Ozen, C., Tokatlioglu, B., 2006. Depression, anxiety, health-related quality of life, and disability in patients with overt and subclinical thyroid dysfunction. Arch. Med. Res. 37, 133–139.

Haggerty Jr., J.J., Garbutt, J.C., Evans, D.L., Golden, R.N., Pedersen, C., Simon, J.S., Nemeroff, C.B., 1990. Subclinical hypothyroidism: a review of neuropsychiatric aspects. Int. J. Psychiatry Med. 20, 193–208.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391, 85–100.

Hosie, A.M., Wilkins, M.E., da Silva, H.M.A., Smart, T., 2006. Endogenous neurosteroids regulate GABA(A) receptors through two discrete transmembrane sites. Nature 444, 486–489.

Hosie, A.M., Wilkins, M.E., Smart, T., 2007. Neurosteroid binding stes on GABA(A) receptors. Pharmacol. Ther. 116, 7–19.

Hosie, A.M., Clarke, L., da Silva, H., Smart, T., 2009. Conserved site for neurosteroid modulation of GABA(A) receptors. Neuropharmacology 56, 149–154.

Kastellakis, A., Valcana, T., 1989. Characterization of thyroid hormone transport in synaptosomes from rat brain. Mol. Cell. Endocrinol. 67, 231–241.

Losi, G., Garzon, G., Puia, G., 2008. Nongenomic regulation of glutamatergic neurotransmission in hippocampus by thyroid hormones. Neuroscience 151, 155–163.

Luo, J.H., Fu, Z., Losi, G., Kim, B.G., Prybylowski, K., Vissel, B., Vicini, S., 2002. Functional expression of distinct NMDA channel subunits tagged with green fluorescent protein in hippocampal neurons in culture. Neuropharmacology 42, 306–318.

Majewska, M.D., Schwartz, R.D., 1987. Pregnenolone sulfate: an endogenous antagonist of the γ-aminobutyric acid receptor complex in the brain? Brain Res. 404, 355–404.

Majewska, M.D., Mienville, J.M., Vicini, S., 1988. Neurosteroid pregnenolone sulfate antagonizes electrophysiological responses to GABA in neurons. Neurosci. Lett. 90, 279–284.

Martin, J.V., Williams, D.B., Fitzgerald, R.M., Im, H.K., Vonvoigtlander, P.F., 1996. Thyroid hormonal modulation of the binding and activity of the GABA(A) receptor complex of brain. Neuroscience 73, 705–713.

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 GABA(A) receptor by metabolites of thyroid hormones: correlations with molecular modeling studies. Brain Res. 1004, 98–107.

Mason, G.A., Walker, C.H., Prange, A.J., 1987. Modulation of gamma-aminobutyric acid uptake of rat brain synaptosomes by thyroid hormones. Neuropsychopharmacology 1, 67–70.

Mason, G.A., Walker, C.H., Prange, A.J., 1993. L-Triiodothyronine: is this peripheral hormone a central neurotransmitter? Neuropsychopharmacology 8, 253–258.

Maguire, J.L., Stell, B.M., Rafizad, M., Mody, I., 2005. Ovarian cycle-linked changes in GABA(A) receptor mediating tonic inhibition alter seizure susceptibility and anxiety. Nat. Neurosci. 8, 797–804.

Morreale de Escobar, G., Calvo, R., Escobar del Rey, F., Obregan, M.J., 1994. Thyrod hormones in tissues from fetal and adult rats. Endocrinology 134, 241–245.

Mortensen, M., Ebert, B., Wafford, K., Smart, T.G., 2010. Distinct activities of GABA agonist at synaptic- and extrasynaptic-type GABA(A) receptors. J. Physiol. 588, 1251–1268.

Mtchedlishvili, Z., Kapur, J., 2003. A presynaptic action of the neurosteroid pregnenolone sulfate on GABAergic synaptic transmission. Mol. Pharmacol. 64, 857–864.

Murase, K., Ryu, P.D., Randic, M., 1989. Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. Neurosci. Lett. 103, 56–63.

Pinna, G., Broedel, O., Visser, T., Jeitner, A., Grau, H., Eravci, M., Meinhold, H., Baumgartner, A., 2002. Concentrations of seven iodothyronine metabolites in brain regions and the liver of the adult rat. Endocrinology 143, 1789–1800.

Puia, G., Santi, M.R., Vicini, S., Pritchett, D.B., Purdy, R.H., Paul, S.M., Seeburg, P.H., Costa, E., 1990. Neurosteroids act on recombinant human GABA(A) receptors. Neuron 4, 759–765.

Puia, G., Mienville, J.M., Matsumoto, K., Takahata, H., Watanabe, H., Costa, E., Guidotti, A., 2003. On the putative physiological role of allopregnanolone on GABA(A) receptor function. Neuropharmacology 44, 49–55.

Sarkar, P.K., 2008. L-Triodothyronine differentially and non genomically regulates synaptosomal protein phoshorylation in adult rat brain cerebral cortex: role of calcium and calmodulin. Life Sci. 82, 920–927.

Scimeni, A., Semyanov, A., Sperk, G., Kullmann, D.M., Walker, M.C., 2005. Multiple and plastic receptors mediate tonic GABA(A) receptor currents in the hippocampus. J. Neurosci. 25, 10016–10024.

Segal, J., 1990. In vivo effect of 3,5,3′-triiodothyronine on calcium uptake in several tissues in the rat: evidence for a physiological role for calcium as the first messanger for the prompt action of thyroid hormone at the level of the plasma membrane. Endocrinology 127, 17–24.

Semyanov, A., Walker, M.C., Kullmann, D.M., 2003. GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat. Neurosci. 6, 484–490.

Shen, W., Mennerick, S., Covey, D.F., Zorumski, C.F., 2000. Pregnenolone sulfate modulates inhibitory synaptic transmission by enhancing GABA(A) receptor desensitization. J. Neurosci. 20, 3571–3579.

Simon, N.M., Blacker, D., Korbly, N.B., Sharma, S.G., Worthington, J.J., Otto, M.W., Pollack, M.H., 2002. Hypothyroidism and hyperthyroidism in anxiety disorders revisited: new data and literature review. J Affect. Disord. 69, 209–217.

Vara, H., Martinez, B., Santos, A., Colino, A., 2002. Thyroid hormone regulates neurotransmitter release in neonatal rat hippocampus. Neuroscience 110, 19–28.

Westerholz, S., DeLima, A.D., Vogit, T., 2010. Regulation of early spontaneous network activity and GABAergic neurons development by thyroid hormones. Neuroscience 168, 573–589.

Wiens, S.C., Trudeau, V.L., 2006. Thyroid hormone and gamma-aminobutyric acid (GABA) interactions in neuroendocrine systems. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 144, 332–344.

Zamoner, A., Funchal, C., Heimfarth, L., Silva, F.H., Pessoa- Pureur, R., 2006. Short term effects of thyroid hormones on cythoscheletal proteins are mediated by GABAergic mechanisms in slices of cerebral cortex from young rats. Cell Mol. Neurobiol. 26, 209–223.

Zamoner, A., Frasson, Corbellini, P., Funchal, C., Menegaz, D., Mena Barretto Silva, F.R., Pessoa Pureur, R., 2005. Involvement of calcium–dependent mechanisms on the action of T3 in the in vitro phosphorylation of vimentin of immature rat testis. Life Sci. 77, 3321–3335.

Zhang, N., Wei, W., Mody, I., Houser, C.R., 2007. Altered localization of GABA(A) receptor subunits on dentate granule cell dendrites influences tonic and phasic inhibition in a mouse model of epilepsy. J. Neurosci. 27, 7520–7531.