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Rise of Intrasynaptosomal Ca^{2+} Level and Activation of Nitric Oxide Synthase in Adult Rat Cerebral Cortex Pretreated with 3-5-3′-L-Triiodothyronine

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Accumulation of free, ionized calcium (Ca^{2+}) and stimulation of nitric oxide synthase (NOS) activity in depolarization-induced synaptosomes prepared from adult rat cerebral cortex have been demonstrated after addition of various doses (0.1–1,000 nM) of 3,5,3′-L-triiodothyronine (T_3). The effects of T_3 doses on those parameters are found to occur in a dose-dependent manner. The T_3 (100 nM)-induced optimum rise in intrasynaptosomal Ca^{2+} level $[Ca^{2+}]_i$ seems to be an early event occurring within 5 s; whereas, the maximum stimulation of NOS activity is observed during 10 to 30 s of T_3 (100 nM) administration, indicating a delayed effect. T_3 has no such effects on those parameters in synaptosomes at nondepolarized condition. Although the rise in $[Ca^{2+}]_i$ and stimulation of NOS activity after application of T_3 seem to be sequential events, the present data indicate a definite role of T_3 in nongenomic signal generation and transfer in mature rat cerebral cortex.
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KEY WORDS: *Triiodothyronine; Calcium; Nitric oxide synthase; Synaptosomes; Rat; Cerebral cortex*

Thyroid hormone (TH) influences the development and growth of the maturing mammalian brain and is involved in the alterations in nuclear-mediated transcription mechanisms (Oppenheimer et al. 1994; Brent 1994). However, in adults these long-term genomic responses are largely replaced by rapid, nongenomic effects (Davis and Davis 1996). Specific membrane- binding sites of 3,5,3′-L-triiodothyronine (T_3) have been demonstrated in the synaptosomal fraction of adult rat brain (Mashio et al. 1982, 1983; Sarkar and Ray 1998). Direct membrane effects of T_3 lead to the inhibition of synaptosomal Na^+-K^+-ATPase activity (Sarkar and Ray 1998), involvement of a GTP-binding protein (Giguere et al. 1996) and the increased activity of adenylate cyclase (Waltz and Howlett 1987), which seem to be the possible mechanism of nongenomic response of TH in neurones. The rapid influx of Ca^{2+} in the depolarization-induced adult rat cerebrocortical synaptosomes is also supportive to the extranuclear action of TH (Mason et al. 1990). Influx of Ca^{2+} in the nerve terminal in response to membrane depolarization is a prerequisite for neurotransmission (Turner et al. 1993; Sudhof 1995)and the rise of intrasynaptosomal Ca^{2+} level($[Ca^{2+}]_i$) is found to activate the constitutive nitric oxide (NO) synthase; thereby, producing NO (Garthwaite and Boulton 1995). NO has been found to be involved in the release of glutamate from the adult rat brain synaptosomes (McNaught and Brown 1998), and TH has been reported to influence depolarization-evoked release of

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GABA in adult rat cerebrocortical synaptosomes (Hashimoto et al. 1991). All this evidence indicates a relationship between TH, Ca^{2+}, and NO in neurotransmission in adult mammalian brain. To elucidate the matter further, we demonstrate the rise of $[Ca^{2+}]_i$ and NO production under in vitro conditions in the synaptosomes of adult rat cerebral cortex pretreated with T_3.

MATERIALS AND METHODS

T_3, choline chloride, fura-2/ acetoxymethylester (AM), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), oxyhaemoglobin, NADPH, L-arginine, N^G-monomethyl-L-arginine (NMMA) were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade, and deionized doubled distilled water was used throughout the study. The experiments were performed on brain tissue prepared from adult (3 months old) male Charles Foster rats (180–200 g body weight).

Preparation of Synaptosomes

Synaptosomes were prepared from the cerebral cortex according to the method of Whittaker (1969) with slight modifications. Briefly, cell debris and nuclei were removed from the 10% cortical homogenate in 0.32 M sucrose by centrifugation at 2000×*g* for 5 min at 4°C. The supernatant was layered on 1.2 M sucrose and centrifuged at 50,000×*g* for 50 min using ultracentrifuge (Beckman-L7). The fraction collected between 0.32 M and 1.2 M sucrose layer was diluted to 1:1.5 with ice-cold, double distilled water, further layered on 0.8 M sucrose and recentrifuged at 50,000×*g* for 30 min. The pellet obtained was washed and repelleted at 20,000×*g* for 5 min. This fraction, representing intact synaptosomes, was finally suspended in buffered salt solution (BSS) containing 20 mM Tris-HCl, 136 mM NaCl, 5.6 mM KCl, 1.3 mM $MgCl_2$, and 11 mM D-glucose at pH 7.4.

Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was measured using fura-2 fluorescence according to Grynkiewicz et al. (1985). Briefly, synaptosomes (1 mg/ml) suspended in BSS (pH 7.4) were loaded with 10 μM of fura-2/AM in DMSO and incubated at 37°C under constant shaking in the dark. After 45 min, the synaptosomes (0.5 mg/ml) were washed, resuspended in BSS replacing NaCl by equimolar choline-chloride, and transferred to a thermostated, magnetically stirred quartz cuvette of Hitachi spectrofluorimeter (Model 3010). 1.0 mM $CaCl_2$ was added to the synaptosomal suspension (0.1 mg/ml), and fluorescence (F) was measured at 37°C with excitation at 340 nm (slit 5 nm) and emission at 495 nm (slit 5 nm). Maximum fluorescence (F_{max}) was measured in the presence of 0.1% Triton-X100, which reflected the efficiency of fura-2 loading. Subsequent addition of 20 μM $MnCl_2$ caused quenching of Ca^{2+}-fura-2 fluorescence (F_{min}). The $[Ca^{2+}]_i$ was then obtained using the formula: $[Ca^{2+}]_i = 224\ (F\text{-}F_{min})/(F_{max}\text{-}F)$, where 224 was the association constant of Ca^{2+} with fura-2 at physiological pH. Autofluorescence was measured in synaptosomes sham-loaded with DMSO and subtracted from the values obtained in experimental samples.

Assay of Nitric Oxide Synthase (NOS)

The NOS activity was measured by exploiting the reaction of NO with oxyhemoglobin to form methemoglobin as described by several authors (Knowles et al. 1989, 1990; Hevel and Marletta 1994; Lowe et al. 1996) with slight modifications. Briefly, the synaptosomal pellete was lysed by osmotic shock with double distilled water for unmasking the latent enzyme. The preparation (0.1 mg/ml) was then incubated at 37°C for 5 min in an assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 10 μM oxyhaemoglobin and 1 mM NADPH. The reaction was started by addition of 1 mM L-arginine in the thermostated cuvette of Shimadzu Spectrophotometer (Model UV-2401 (PC)S) and A_{401} was monitored over 5 min against a blank incubation with 1 mM L-NMMA and no L-arginine. The initial rate of change in absorption was used to calculate the rate of NO formation, using the molar absorption coefficient of methemoglobin (112000).

Determination of Protein

The protein concentrations were estimated by the method of Vera (1988) using bovine serum albumin as standard.

Statistical Analysis

Data are expressed as mean ± SEM of the number of experiments indicated. Statistical significance was analyzed by two-tailed Student's *t*-test.

RESULTS

Effect of Pretreatment with T_3 on $[Ca^{2+}]_i$

The synaptosomes suspended in BSS containing choline-chloride instead of NaCl, and Ca^{2+} ($CaCl_2$-1mM) were depolarized with addition of K^+ (55 mM). Such depolarized synaptosomes showed a 2.8-fold (637.87 ± 28.48 nM vs. 228.73 ± 6.68 nM, $p < .001$, Figure 1C) higher level of $[Ca^{2+}]_i$ in comparison to that found in BSS-synaptosomal suspension. Addition of T_3 (100 nM) at the depolarization-induced synaptosomes further increased the $[Ca^{2+}]_i$ to a maximum of 1.5-fold (979.62 ± 7.96 nM, $p < .001$) in comparison to the depolarized value, within 5 s after application of the hormone and sustained up to 7 min. T_3 (100 nM) had no effect on both

BSS-synaptosomal suspension (nondepolarized, 229.66 ± 5.11 nM vs. 230.17 ± 5.09 nM, Figure 1A) and Ca^{2+}-added BSS-synaptosomal suspension (nondepolarized, 409.54 ± 21.69 nM vs. 430.89 ± 23.85 nM, Figure 1B). A dose-dependent stimulatory effect of T_3 on $[Ca^{2+}]_i$ was evident between 0.1 and 1.0 nM ($p < .05$), 1.0 and 10 nM ($p < .01$), 10 and 100 nM ($p < .01$) doses (727.24 ± 25.27 nM vs. 817.26 ± 25.23 nM vs. 926.84 ± 7.75 nM vs. 979.62 ± 7.96 nM, Figure 2). With higher doses of T_3 at 1,000 nM, an attenuation of $[Ca^{2+}]_i$ was found in comparison to the value obtained at 100 nM concentration (839.92 ± 16.23 nM vs. 979.62 ± 7.96 nM, $p < .001$).

Effect of Pretreatment with T_3 on NOS Activity

The NOS activity in depolarization-induced (by addition of 55 mM KCl in suspension containing BSS with choline-chloride substituted for NaCl) synaptosomes had been found to be 1.3-fold higher than that found in synaptosomes suspended in BSS only (35.99 ± 2.25 pmol NO/min/mg protein vs. 28.01 ± 2.16 pmol NO/min/mg protein, $p < .05$). The effect of T_3 on NOS activity is shown in Figure 3. The enzyme activity increased significantly as early as 10 s (59.65 ± 2.77 pmol NO/min/mg protein, $p < .001$) after T_3 (100 nM) treatment to the depolarized synaptosomes and sustained up to 30 s (65.91 ± 2.67 pmol NO/min/mg protein, $p < .001$) of T_3 administration in comparison to the value obtained in the depolarized condition only. There was a 1.7-fold rise of the enzyme activity in T_3 (100 nM)-treated synaptosomes, as compared to that in depolarized synaptosomes. Thereafter, the enzyme activity decreased and remained higher than the depolarized value, even up to 5 min (43.95 ± 2.49 pmol NO/min/mg protein, $p < .05$). It was further noted that T_3-induced changes in NOS activity were only observed in depolarized synaptosomes. T_3 (100 nM) was ineffective in both BSS-synaptosomal suspension (nondepolarized, 23.41 ± 2.84 pmol NO/min/mg protein, n = 7 vs. 28.01 ± 2.16 pmol NO/min/mg protein) and Ca^{2+}-added BSS-synaptosomal suspension (nondepolarized, 27.45 ± 1.83 pmol NO/min/mg protein, n = 6 vs. 28.92 ± 2.32 pmol NO/min/mg protein).The dose-dependent stimulatory effects of T_3 on NOS activity were evident between 0.1 nM and 1.0 nM ($p < .05$), 1.0 nM and 10 nM ($p < .05$) doses (45.34 ± 2.39 pmol NO/min/mg protein vs. 53.99 ± 2.94 pmol NO/min/mg protein vs. 63.75 ± 2.52 pmol NO/min/mg protein, Figure 4), and optimal activation was obtained at 10 nM of T_3 dose. No statistically significant difference in NOS activity was found between 10 nM and 100 nM (65.91 ± 2.67 pmol NO/min/mg protein) T_3 doses. NOS activity at 1,000 nM of T_3 (54.60 ± 2.66 pmol NO/min/mg protein, $p < .02$) dose showed attenuative effect of T_3 in comparison to the 100 nM T_3 dose.

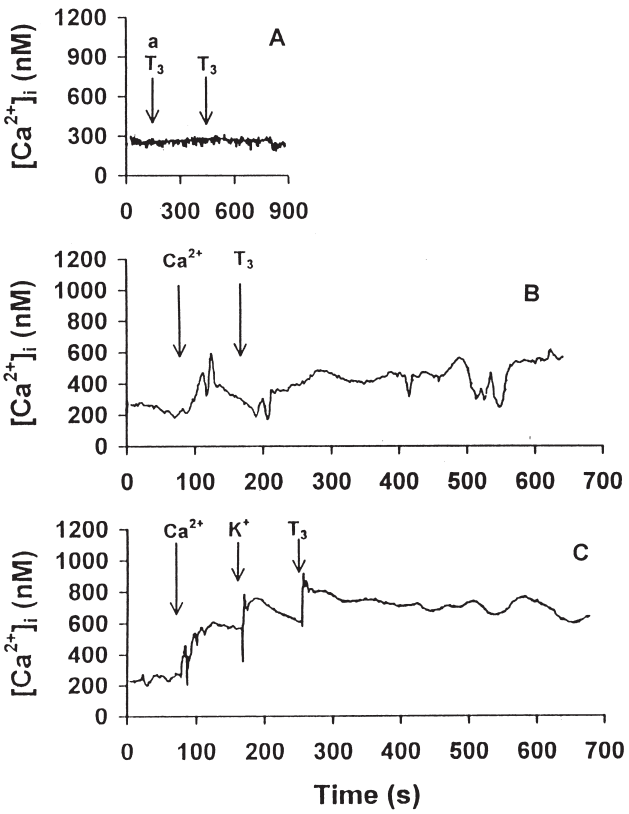

Figure 1. Time course of T_3 action on the $[Ca^{2+}]_i$ in rat cerebrocortical synaptosomes under nondepolarized **(A)** BSS-suspension; **(B)** Ca^{2+}-added BSS-suspension) and depolarized **(C)** conditions. Arrows indicate the time of addition of $CaCl_2$ (1mM), KCl (55 mM), T_3-a (1 nM) and T_3 (100 nM). The results are shown here as a representative case out of four (A,B) and six (C) experiments.

DISCUSSION

T_3 has many effects on adult brain function. Severe T_3 deficiency in adult human develops memory impairment and emotional labilities; whereas, hyperthyroidism produces tense dysphoria and marked motor retardation (Prange 1996). T_3 acts in a complex way at different cellular levels and time. Actions of T_3 at diverse cellular loci, such as plasmamembrane, cytoplasmic proteins, mitochondria, and nucleus are well-known (Hafner 1987; Luvisetto 1997). During postnatal period, the action of T_3 is mostly genomic in the cerebral cortex ,which gradually fades away at adulthood and is possibly replaced by a short-term rapid nongenomic effect (Eayrs 1961; Iniguez et al. 1992; Davis and Davis 1996). In our previous study, we demonstrated a correlation between the degree of saturation of the specific T_3-binding sites at the cerebrocortical synaptosomal membrane and gradual inhibition of the membrane Na^+-K^+-ATPase activity in adult male rat (Sarkar and Ray 1998). In the present study, the enucleated synaptosomal fraction prepared from adult rat cerebral cortical slices has been used as a model to investi-

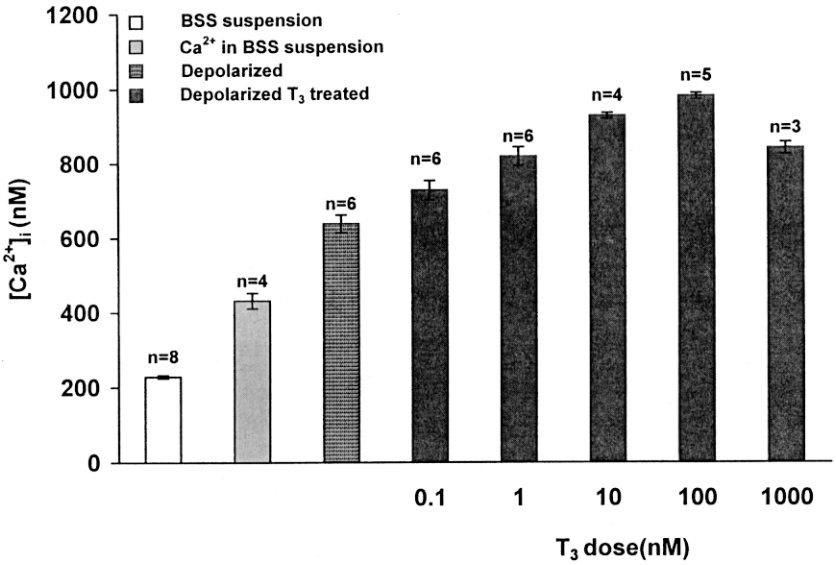

Figure 2. Effect of different concentrations of T_3 on $[Ca^{2+}]_i$ in rat cerebrocortical synaptosomes. Synaptosomes were depolarized by KCl (55 mM). Values are maximum changes in $[Ca^{2+}]_i$ within 5 s after T_3 addition on depolarized synaptosomes. Data represent mean ± SEM of 3 to 8 determinations.

gate the nongenomic action of the thyroid hormone. T_3 has been demonstrated to raise the intrasynaptosomal level of Ca^{2+} during the depolarization condition (Figure 1C), and the effects have been found to be dose dependent (Figure 2). However, T_3 has no such effects on nondepolarized synaptosomal suspension (Figure 1A, Figure 1B). The maximal rise in $[Ca^{2+}]_i$ within 5 s of T_3 application in depolarized synaptosomes indicates an early event of Ca^{2+} accumulation that may be mediated through the altered activity of the voltage-gated Ca^{2+} channel (Nachshen and Blaustein 1980; Leslie et al. 1983; Nachshen 1985; Suszkiw et al. 1986; McMahon and Nicholls 1991; Sihra et al. 1992; Turner et al. 1993). A dose-dependent rise in T_3-induced $[Ca^{2+}]_i$ is evident between 0.1 nM and 1.0 nM, 1.0 nM and 10 nM, 10nM and 100 nM doses, having a maximum significant rise at 100 nM dose. Such a phenomenon at low doses (nM) throws some light for the hormonal action of T_3 at the physiologic range. However, an attenuation of the rise in $[Ca^{2+}]_i$ with higher doses of T_3 at 1,000 nM could be attributable to the pharmacologic impedance of Ca^{2+}-accumulation in the synaptosomes. The present data on the T_3-mediated rise in $[Ca^{2+}]_i$ are in conformity with the ^{45}Ca uptake study of Mason et al. (1990). Our earlier data showed maximal (80%) saturation of specific T_3 binding sites on synaptosomal membrane at 100 nM T_3 concentration

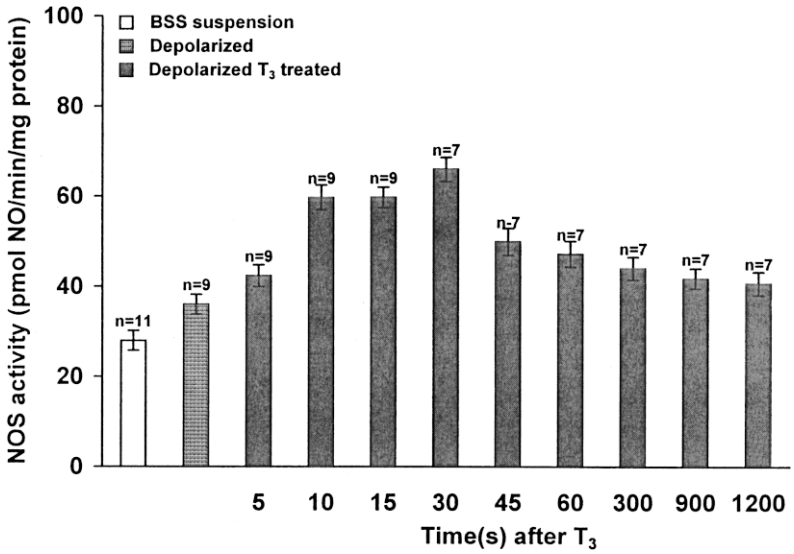

Figure 3. Time course of T_3 action on the NOS activity in rat cerebrocortical synaptosomes. Ca^{2+} (1 mM $CaCl_2$) was first added to the synaptosomes suspended in BSS containing choline-chloride instead of NaCl. Synaptosomes were then depolarized with K^+ (55 mM KCl), added externally and thereafter treated with T_3 (100 nM). Addition of Ca^{2+}, K^+ and T_3 maintained a regular interval of 1 min. The NOS activity of lysed synaptosomes was then measured as described in Materials and Methods. Data represent mean ± SEM of 7 to 11 determinations.

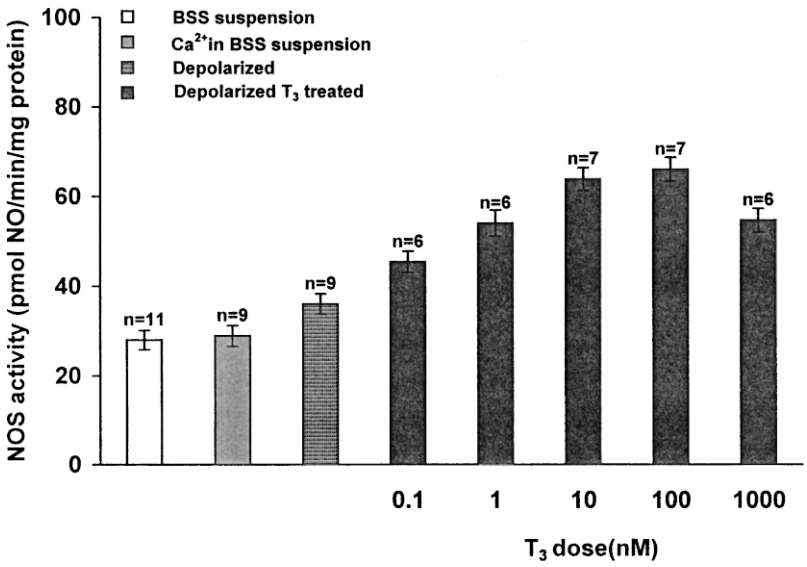

Figure 4. Effect of different concentrations of T_3 on the NOS activity in rat cerebrocortical synaptosomes. Depolarization-induced intact synaptosomes were pretreated with T_3 for 30 s and then the NOS activity of lysed synaptosomes was measured. Data represent mean ± SEM of 6 to 11 determinations.

(Sarkar and Ray 1998). From our present experiment, we may speculate that T_3-mediated rise in $[Ca^{2+}]_i$ could be a receptor-mediated process, having its maximal effect at 100 nM concentration of T_3. Considering the maximum rise in $[Ca^{2+}]_i$ at the present experiment and T_3 specific receptor saturation on the synaptosomal membrane as found earlier (Sarkar and Ray 1998), we have undertaken the time scanning of NOS activity with 100 nM T_3 dose. The occurrence of stimulated maximal NOS activity at 10 to 30 s (Figure 3) in depolarization-induced synaptosomes after 100 nM T_3 application seems to be a delayed effect of T_3. A dose-dependent rise in the T_3-mediated NOS activity (Figure 4) is also evident between 0.1 nM and 1.0 nM, 1.0 nM and 10 nM T_3 doses having maximum significant activation at 10 nM dose. It is evident from our experiment that the $[Ca^{2+}]_i$ raised after administration of 10 nM T_3 is sufficient to elicit the maximum NOS response. It has been reported that $[Ca^{2+}]_i$ is related to constitutive NOS activation vis-à-vis formation of NO (Garthwaite and Boulton 1995). In the present experiment, T_3 has been found to be ineffective to nondepolarized synaptosomes for NOS activation. Moreover, the optimal increase in the NOS activity at 10 and/or 100 nM concentration of T_3 preceded by a rapid maximal rise in intrasynaptosomal Ca^{2+} level at 100 nM concentration of T_3 seems to be a resultant effect of maximum saturation of T_3-binding sites on synaptosomal membrane at such T_3 level as evidenced in our previous experiment (Sarkar and Ray 1998). Furthermore, it seems that the rise in $[Ca^{2+}]_i$ being the initial T_3-mediated synaptosomal effect, initiates enhancement of NOS activity, resulting in modulation of the pre-existing enzyme (NOS) to a state of higher catalytic activity, and Ca^{2+} may serve as its endogenous activator for NO production (Bredt and Snyder 1994; Griffith and Stuehr 1995). Although, the present experiment shows a sequential and highly correlated ($r = 0.95$) relationship between T_3-mediated dose-dependent rise in $[Ca^{2+}]_i$ and enhancement of NOS activity in synaptosomes, it needs further investigation using Ca^{2+}-channel blocker as well as demonstrating the dose–response relationship between only $[Ca^{2+}]_i$ and NOS activity to dissociate the relationship between the two important events. Pending that, the present experiment deserves merit as the first report of its kind demonstrating simultaneous T_3-stimulated rise in $[Ca^{2+}]_i$ and elevation of NOS activity in adult rat cerebrocortical synaptosomes that could be relevant in T_3-mediated nongenomic signal generation and transfer in mature mammalian brain.

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