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The effect of chloral hydrate on the in-vitro T_3 binding to adult rat cerebral nuclei

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Abstract

Chloral hydrate is a widely used hypnotic drug for children and animals but the possible interactions of its sedative action and thyroid hormones has not been investigated. In this study the effect of chloral hydrate on the in-vitro binding of triiodothyronine (T_3) to cerebral nuclei of adult rats and on the thyroid hormones' synaptosomal and plasma availability were examined. Our results show that during deep anaesthesia caused by a single intraperitoneal administration of chloral hydrate (100 mg kg^{-1}), the maximal number of nuclear thyroid hormone receptors (Bmax) and the equilibrium dissociation constant (Kd) were decreased. These changes returned to normal values when rats woke up (2½ h after chloral hydrate administration). Plasma or synaptosomal levels of thyroid hormones were unaffected during chloral hydrate treatment. Our study demonstrates that the nuclear T_3 binding in adult rat brain is affected by the sedative action of chloral hydrate.

Introduction

Chloral hydrate, first introduced for clinical use in 1869, displays hypnotic, sedative and anticonvulsant properties (Pershad et al 1999). In man and rodents, chloral hydrate is reduced within minutes to the lipophilic 2,2,2-trichloroethanol, its major active metabolite (Breimer 1977; Beland et al 1998). Other metabolites, which are formed in smaller amounts and accumulate for 1 or 2 days after chloral hydrate administration, include trichloroacetic acid and trichloroethanoglucuronide (Garrett & Lambert 1973). The trichloroethanol metabolite appears to be more important clinically than the parent compound; however, it is possible that chloral hydrate effects also contribute (Gruner et al 1973). Recent studies indicate that chloral hydrate, and to a lesser extent trichloroethanol, affects sperm function and fertilization in man and rodents (Forkert et al 2003; Xu et al 2004).

Chloral hydrate is still widely used for inducing light sedation or sleep during several neurodiagnostic procedures, such as electroencephalogram, neuroimaging and lumbar puncture (Greenberg et al 1993). In young children chloral hydrate administration may induce paradoxical excitation (Cote et al 2000) whereas in older children and adults it produces adequate sedation (Pershad et al 1999). When used for inducing sleep, chloral hydrate decreases sleep latency and number of awakenings without suppressing rapid eye movement sleep (Hartmann 1996). In man, a single dose of approximately 50 mg kg^{-1} should produce sleep within 1 h that lasts approximately 1 h (Anonymous 1998).

Recent studies indicate that the depressant effect of chloral hydrate in the brain may be due to interactions with several neurotransmitter-operated ion channels; chloral hydrate enhances $GABA_A$ receptor-mediated chloride currents (Weight et al 1992; Lovinger & Zhou 1993) and inhibits excitatory amino-acid receptor-activated ion currents in neuronal cultures (Peoples & Weight 1998; Scheibler et al 1999). Additionally, chloral hydrate and trichloroethanol inhibit AMPA-induced calcium influx in cultured cortical neurons (Fischer et al 2000).

Thyroid hormones are important in maintaining cognitive and affective homoeostasis (Bauer & Whybrow 1988) and are involved in the occurrence of anxiety and

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depression (Endo 1983; Hall 1983; Whybrow 1986; Kragie 1993). Triiodothyronine (T_3), the active form of thyroid hormone at the cellular level, exerts its physiological role mainly through binding to specific nuclear receptors, which are encoded by two different protooncogenes, *c-erbA*α and *c-erbA*β (Lazar 1993). Each gene has several alternative mRNA splicing products, including the predominant isoforms of thyroid hormone receptors – TRα1, TRα2, TRβ1 and TRβ2 (Thompson et al 1987; Koenig et al 1988; Mitsuhashi et al 1988; Hodin et al 1989). TRα1, TRβ1 and TRβ2 isoforms are known to bind T_3 with high affinity and also bind to thyroid hormone response elements (TREs) on chromatin, thus regulating transcriptional processes in several target tissues, including adult rat brain (Yen 2001).

The regulation of the T_3-dependent gene expression, dependent directly upon the cytoplasmic thyroid hormone availability and the density of the T_3 nuclear receptors, is very important in maintaining cognitive homoeostasis, especially during childhood. Despite the accumulated knowledge regarding the mechanism of action of thyroid hormones in brain at the cellular level, little is known of the possible interaction between thyroid hormones and hypnotic or sedative drugs (Borner et al 1995).

The aim of this study was to investigate whether administration of chloral hydrate affects thyroid hormone nuclear binding in adult rat cerebral hemispheres, and thus the response of this tissue to thyroid hormones. In particular, the kinetic characteristics of nuclear T_3 binding were examined in the cerebral hemispheres of adult rat brain after a single hypnotic dose of chloral hydrate. The plasma and synaptosomal levels of thyroid hormones were also examined to delineate whether the hypnotic effect of chloral hydrate can influence the thyroid hormone availability both in blood circulation and in cerebral tissue since it is known that T_3 is concentrated in the nerve ending fractions of adult rat brain (Dratman et al 1976) deriving, presumably, from local de-iodination of T_4 (Dratman et al 1976; Dratman & Crutchfield 1978).

Materials and Methods

Animals and treatment

Adult Wistar rats, 40 days old, of both sexes, bred in our Laboratory, were housed 4 per cage and had free access to laboratory food and water. Rats were treated according to international statutes on animal handling (89/609/EEC). They were exposed to a regular 12-h light–dark cycle at 22 ± 1°C for at least 1–2 weeks before chloral hydrate treatment and until sacrifice. Rats received an intraperitoneal injection of chloral hydrate (100 mg kg^{-1}); an equal volume of 0.9% NaCl was administrated to the control groups. After the injection, all the chloral-hydrate-treated rats were kept in individual cages and were continually observed until they passed into deep sleep. Rats that did not develop hypnosis were studied as a separate experimental group. Some groups of chloral-hydrate-treated rats were sacrificed 1.5 h after chloral hydrate administration (deep-anaesthesia stage), whereas other groups of chloral-hydrate-treated rats were sacrificed 2.5 h after chloral hydrate administration (waking-up stage). After sacrifice, brains were rapidly removed in a sterile cooled glass plate and the cerebral hemispheres were isolated and weighed. For binding studies and determination of synaptosomal thyroid hormone levels, fresh cerebral hemisphere tissue was used.

To elucidate whether chloral hydrate-induced hypnosis affects the free fraction of thyroid hormone levels in blood circulation, serum T_3 (triiodothyronine) and T_4 (thyroxine) concentrations were determined in all chloral-hydrate-treated and control rats by radioimmunoassay (T_3-RIA and T_4-RIA kits; Hellenic Center of Natural Research, Demokritos).

In-vitro nuclear T_3 binding assay

The effects of chloral hydrate on the in-vitro specific nuclear binding of T_3 were evaluated by performing equilibrium competitive binding assays of cerebral nuclei prepared from control and chloral-hydrate-treated rats (1.5 h after administration). Nuclei isolation was performed according to the method of Eberhardt et al (1978). Briefly, the cerebral hemispheres were homogenized (20% w/v) in ice-cold 0.32 M sucrose by ten up and down strokes in a glass homogenizer with a loose-fitting motor-driven Teflon pestle (1000 rev min^{-1}). The homogenates were then centrifuged at 1000 *g* for 10 min (4°C). The crude nuclear pellets were re-suspended in 0.32 M sucrose according to the initial dilution and re-centrifuged as above. To obtain pure nuclei, the pellets were suspended in 1.7 M sucrose and centrifuged at 53 000 *g* for 90 min (4°C) in a Beckman SW 27 ultracentrifuge rotor. The final nuclear pellets were suspended in potassium phosphate buffer (25 mM KH_2PO_4, 1 mM $MgCl_2$, 20 mM β-mercaptoethanol, pH 7.5) containing 0.5% Triton X-100 and were centrifuged at 10 000 *g* for 10 min (4°C). The pellets were then re-suspended in potassium phosphate buffer without Triton X-100 and were washed at 10 000 *g* for 10 min (4°C). The final pure nuclear preparations were sampled and the DNA content in each one was estimated by the method of Burton (1956) and the protein levels by the method of Bradford (1976).

The T_3 binding constants (maximal binding capacity: Bmax, and equilibrium dissociation constant: Kd) in cerebral nuclei from control and chloral-hydrate-treated rats were determined by performing equilibrium competitive binding assays as described previously by Bolaris et al (1995). Briefly, nuclear samples (40–60 μg of DNA) were incubated at 37°C for 15 min in 1 mL of potassium phosphate buffer (without Triton) containing various concentrations ($0.5–10 \times 10^{-10}$ M) of ^{125}I-T_3 (specific activity 1200 μCi μg^{-1}; New England Nuclear). At all concentrations of radioactive T_3 examined, identical incubations were carried out with 100 M excess of unlabelled T_3. Duplicate assays were performed at each concentration employed. The incubation was terminated by placing the samples on ice for 2 min. Samples were then centrifuged at 10 000 *g* for 10 min (4°C). The pellets were subsequently washed once in potassium

phosphate buffer containing 0.5% Triton X-100 and were re-centrifuged as above. The final pellets were then counted in a Beckman γ-counter (5500 model) and the specific binding was determined by subtracting the radioactivity of non-specific bound T_3 (i.e., the radioactivity that could not be competitively displaced by incubating the nuclei in 100-fold molar excess of unlabelled T_3) from the total bound radioactivity. The results were expressed as moles T_3 bound per μg DNA. The DNA content in each sample was determined by the method of Burton (1956) and the protein content by the method of Bradford (1976). Finally, the binding constants were estimated by Scatchard analysis (Scatchard 1949) of the binding data.

The in-vitro total ^{125}I-T_3 binding in cerebral hemispheres from control and chloral-hydrate-treated rats (1.5 and 2.5 h after administration) was estimated by incubating, in duplicate, the nuclei at 37°C for 15 min in 1 mL potassium phosphate buffer (25 mM KH_2PO_4, 1 mM $MgCl_2$, 20 mM β-mercaptoethanol, pH 7.5) containing saturating levels (5×10^{-10} M) of ^{125}I-T_3, while identical incubations were performed in the presence of 100 M excess of cold T_3. The experiments were repeated three times to determine the significance of difference in the data obtained from experimental and control groups.

In a separate experimental procedure we examined whether chloral hydrate exerts any direct in-vitro effect on T_3 binding. In this case, the kinetic characteristics of the in-vitro T_3 binding were determined by performing an equilibrium competitive binding assay, as described previously, in the presence of 10^{-4} M chloral hydrate.

Isolation of cerebral synaptosomes and determination of the synaptosomal thyroid hormone levels

The preparation of synaptosomes was performed according to the method of Sarkar & Ray (1992) with slight modifications. Briefly, the cerebral hemispheres (≅ 800 mg) were homogenized (10% w/v) in ice-cold 0.32 M sucrose (pH 7.4). The homogenate was centrifuged at 1000 *g* for 10 min, the supernatant was layered slowly on top of a sucrose gradient composed of 8 mL of 1.20 M and 8 mL of 0.32 M sucrose (4°C, pH 7.4) and was centrifuged at 34 000 *g* for 50 min. The crude synaptosomal fraction was banded between the 0.32 M and the 1.20 M sucrose layers and was carefully removed by suction at 4°C. The sucrose concentration was adjusted to 0.32 M by slowly adding ice-cold bi-distilled water (≈ 1:1.8). Then the samples were layered slowly on top of a sucrose gradient composed of 8 mL of 0.85 M and 8 mL of 0.32 M sucrose (4°C, pH 7.4) and centrifuged at 34 000 *g* for 30 min. The bottom pellet thus obtained was the synaptosomal fraction, which was further purified by washing once with 5 mL of 0.32 M sucrose at 4°C and re-pelleted at 20 000 *g* for 20 min to finally obtain pure synaptosomes. The synaptosomes were then ruptured hypo-osmotically in 1.5 mL of 5.5 mM imidazole-HCl buffer (4°C, pH 7.4).

The ruptured synaptosomal suspensions were used immediately for T_4 and T_3 radioimmunoassay utilizing specific kits (T_3-RIA and T_4-RIA; Hellenic Center of Natural Research, Demokritos, Greece). Before analysis, in each synaptosomal suspension sample (100 μL), 10 μL of 0.1 M NaOH was added to obtain an appropriate alkaline environment for the dissolution of thyroid hormones from the membrane fraction of synaptosomes. All samples were analysed in triplicate. Protein concentration was evaluated by the method of Bradford (1976), using bovine serum albumin as standard.

Statistics

Results were expressed as the mean ± s.e. For the estimation of the statistical significance of the differences between two groups, Student's *t*-test was used. For estimation of the variability of the data from more than two groups, one-way analysis of variance, with a Bonferroni correction for multiple comparisons, was employed. All statistical tests were performed using a SPSS program for MS Windows, Release 6.0.

Results

Effect of chloral hydrate on the plasma and synaptosomal levels of thyroid hormones

Plasma thyroxine (T_4, 58 ± 4 ng mL^{-1}) and triiodothyronine (T_3, 0.36 ± 0.15 ng mL^{-1}) levels were determined before and 1.5 and 2.5 h after chloral hydrate administration (100 mg kg^{-1}) to rats. Results show that plasma thyroid hormone levels were not affected by the administration of chloral hydrate.

The synaptosomal levels of T_3 in the cerebral hemispheres of chloral-hydrate-treated rats remained euthyroid (0.42 ± 0.03 ng (mg protein)$^{-1}$) 1.5 and 2.5 h after the administration of chloral hydrate. In agreement with the literature (Sarkar & Ray 1994), the synaptosomal T_4 was undetectable in all control and chloral-hydrate-treated rats examined in this study.

Effect of chloral hydrate on the in-vitro ^{125}I-T_3 binding to adult rat cerebral nuclei

The effect of chloral hydrate administration on the in-vitro total specific ^{125}I-T_3 binding to cerebral nuclei from adult rats is presented in Table 1. These data show that the total specific ^{125}I-T_3 in-vitro binding to nuclei from adult rat cerebral hemispheres was decreased (23%) 1.5 h after chloral hydrate administration (deep-sleep stage), an effect easily reversed to normal values upon waking up of the chloral-hydrate-treated animals (2.5 h after chloral hydrate administration).

To determine whether the decrease in the in-vitro ^{125}I-T_3 specific nuclear binding during deep sleep of the chloral-hydrate-treated rats was due to alterations in the maximal number of binding sites or in the equilibrium dissociation constant, we performed equilibrium competitive binding assays of nuclei prepared from control and chloral-hydrate-treated rats 1.5 h after chloral hydrate

Table 1 The effect of choral hydrate on the in-vitro total specific binding of ^{125}I-T_3 to nuclei isolated from adult rat cerebral hemispheres

	Specific ^{125}I-T_3 binding (10^{-17} mol (μg DNA)$^{-1}$)
Control	37.16 ± 3.7 (n = 5)
Chloral-hydrate-treated (1.5 h after administration) deep-anaesthesia stage	28.5 ± 2.9* (n = 4) (↓ 23%)
Chloral-hydrate-treated (1.5 h after administration) no anesthesia	33.4 ± 3 (n = 4)
Chloral-hydrate-treated (2.5 h after administration) waking-up stage	32.1 ± 1.4 (n = 3)

Choral hydrate (100 mg kg^{-1}) was injected intraperitoneally to adult rats. Control rats received equal volume of 0.9% NaCl. Data represent means ± s.e of n nuclear preparations (5 rats per preparation), which were analysed in duplicate. *$P < 0.05$ (analysis of variance, with a Bonferroni correction for multiple comparisons) vs control.

administration. Scatchard analysis of the binding data (Figure 1) showed that the increase in T_3 binding to cerebral nuclei can be attributed to a decrease (34%) of the maximal binding capacity (Bmax); however, a decrease (50%) in the dissociation constant (Kd) was also observed (Table 2).

The effect of chloral hydrate administration on the kinetic characteristics of T_3 binding to nuclei from cerebral hemispheres was not due to a direct pharmacological effect of chloral hydrate on the in-vitro ^{125}I-T_3 binding, since the presence of chloral hydrate (10^{-4} M) in the incubation medium of nuclei T_3 binding procedure did not cause any significant differences in the kinetic characteristics of the nuclear T_3 binding (Figure 2). The dose of chloral hydrate was chosen between a range of doses tested (10^{-3} to 10^{-5} M); however, the total in-vitro nuclear T_3 binding in cerebral hemispheres was not affected by the presence of chloral hydrate at any dose tested. Furthermore, in this study we observed that the in-vitro nuclear ^{125}I-T_3 binding to cerebral hemispheres from chloral-hydrate-treated rats that did not get into deep sleep was unaffected (Table 1).

Discussion

Chloral hydrate is an old psychotropic agent and is continually used in paediatric units in a single dose for premedication before painful examinations or care procedures (Anonymous 1998; Pershad et al 1999) and recently it has been used in the anti-epileptic treatment of young children (Pranzatelli & Tate 2001; Krsek et al 2002). This study shows, for the first time, that during deep anaesthesia, 1.5 h after a single intraperitoneal dose of chloral hydrate (100 mg kg^{-1}), the in-vitro total nuclear T_3 binding was significantly decreased in adult rat cerebral hemispheres, an effect easily reversed upon awaking the rats 2.5 h after chloral hydrate administration (Table 1). The decrease in the in-vitro T_3 binding to cerebral nuclei has also been observed under treatment with diazepam, a commonly used tranquillizer (Constantinou et al 2005b); it has been shown that diazepam and chloral hydrate have similar sedative effects (Badalaty et al 1990), indicating that T_3-dependent gene expression displays a crucial role in the processing of sedation and consequently in the repression of neural activity. Moreover, recent studies have shown that increased neural activity, as is caused by the administration of pentylenetetrazole, induces a transient increase in the total in-vitro T_3 binding to cerebral nuclei (Bolaris et al 2005), suggesting that the in-vitro T_3 binding and the density of neural activity are proportionally related. The decrease in the nuclear T_3 binding was not a direct effect of chloral hydrate on the in-vitro nuclear T_3 binding, since addition of the drug in the incubation medium did not alter the kinetic characteristics of the nuclear T_3 binding (Figure 2). However, the

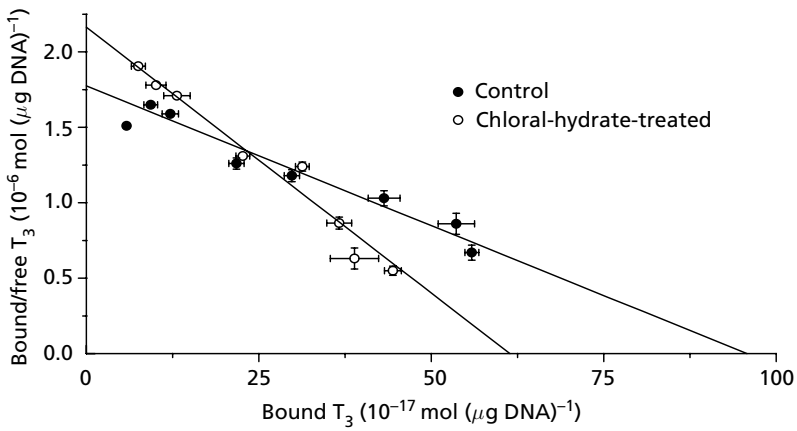

Figure 1 Scatchard analysis of the specific ^{125}I-T_3 binding to brain nuclei from control (●) and chloral hydrate-treated rats (○), which were sacrificed 1.5 h after chloral hydrate administration. Data are means ± s.e. ($P < 0.05$, Student's t-test) from six different nuclear preparations (5 rats per preparation) assayed in duplicate.

Table 2 The effect of chloral hydrate on the kinetic characteristics of the in-vitro specific binding of ^{125}I-T_3 to nuclei isolated from adult rat cerebral hemispheres

	Bmax (10^{-17} mol (μg DNA)$^{-1}$)	Kd (10^{-10} M)
Control	97.5 ± 0.5	5.5 ± 0.3
Chloral-hydrate-treated (100 mg kg^{-1}, 1.5 h)	64 ± 2.0* (↓ 34%)	2.7 ± 0.2* (↓ 50%)

Data represent means ± s.e. from six nuclear preparations (5 rats per preparation) assayed in duplicate. *$P < 0.05$ (Student's t-test) vs control.

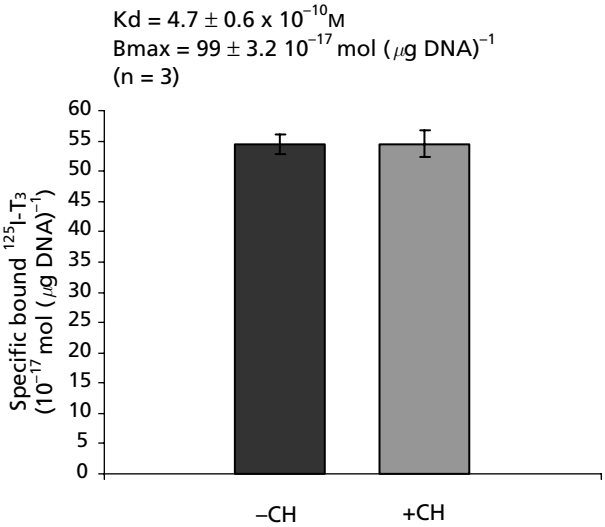

Figure 2 In-vitro effect of chloral hydrate (CH) on the specific T_3 binding to rat brain nuclei. Nuclei were incubated at 37°C for 15 min in 1 mL phosphate buffer containing 5×10^{-10} M ^{125}I-T_3 in the presence or absence of 10^{-4} M chloral hydrate in the incubation medium. The bars represent means ± s.e. ($P > 0.05$, Student's t-test) from five different preparations (3 rats per preparation) analysed in triplicate. In the inset are presented the kinetic characteristics of the in-vitro ^{125}I-T_3 binding to cerebral nuclei in the presence of 10^{-4} M chloral hydrate. No statistical difference was observed in the presence or in the absence of chloral hydrate ($P > 0.05$, Student's t-test).

possibility that trichloroethanol, or other chloral hydrate metabolites, exert any direct effect on the in-vitro nuclear T_3 binding cannot be excluded, since it is known that trichloroethanol and chloral hydrate reach their maximal levels in plasma almost concomitantly when chloral hydrate is administered in rodents, and also have similar half-times in blood circulation (Beland et al 1998). Further, the observation that experimental rats that did not develop hypnosis after the chloral hydrate treatment, or chloral-hydrate-treated rats that were at the awaking stage, did not show any changes in the in-vitro T_3 nuclear binding, similar to control rats, strongly suggests that the decrease in the T_3 binding is a result of the hypnotic action of chloral hydrate.

In addition, equilibrium competitive binding experiments revealed that the decrease in the in-vitro nuclear T_3 binding in cerebral hemispheres during chloral-hydrate-induced deep anaesthesia was accompanied by a proportional decrease in the T_3 Bmax and an inversely proportional increase of the T_3 binding affinity to its nuclear receptors (Table 1). No changes have been observed in the plasma thyroid hormone availability in any interval during chloral hydrate treatment examined in this study. The decrease in T_3 Bmax (Figure 1) indicates a down-regulation of the number of nuclear T_3 receptors in adult brain of chloral-hydrate-treated rats, whereas the increase in T_3 binding affinity could represent a compensatory mechanism through which adult rat brain tries to cope with the decreased number of nuclear T_3 receptors. Alterations in the number of T_3 receptors could consequently influence transcriptional and metabolic processes due to differential regulation of the T_3-dependent gene expression in several T_3-target tissues including adult rat brain (Lazar 1993). It could be very interesting to discover whether the regulation of the expression of the specific thyroid hormone receptor coding genes is affected directly by chloral hydrate or its metabolites and consequently to delineate if they belong to the long-known anaesthesia genes (i.e., genes, the products of which mediate or modulate anaesthesia (Nash 2002)). Recently, has been reported that the relative expression of thyroid hormone receptors is affected by other neuromodulatory drugs, such as lithium chloride, pentylenetetrazole and diazepam, in a time- and isoform-specific manner (Hahn et al 1999; Bolaris et al 2005; Constantinou et al 2005a, b)

Although the central depressant effect of chloral hydrate has been correlated with various neurotransmitter-operated ion channels, this study demonstrates that chloral hydrate also affects nuclear phenomena. The identification of the intracellular pathway(s) through which chloral hydrate – a commonly used hypnotic/sedative drug – affects nuclear phenomena, such as the maximal number of the nuclear T_3 receptors, would be very valuable in elucidating the communication between synaptic function and nucleus during sedation.

It has been shown that T_3 is concentrated in the nerve-ending fractions of adult rat brain (Dratman et al 1976), deriving presumably from local de-iodination of T_4 (Dratman et al 1976; Dratman & Crutchfield 1978); 70% of the intracellular T_3 levels in adult rat brain are derived from local de-iodination of T_4 to T_3 (Crantz & Larsen 1980; Crantz et al 1982). Our data show that chloral hydrate treatment did not affect the synaptosomal T_3 levels of the adult rat brain and consequently the intracellular levels of the active form of thyroid hormone.

Conclusion

This study demonstrates that nuclear T_3 binding to adult rat brain nuclei is affected by the sedative action of chloral hydrate (caused by a single intraperitoneal administration of chloral hydrate), whereas the intracellular availability

of the active form of thyroid hormone (T_3) and the thyroid hormone availability in blood circulation remain unaffected, indicating that the density of neural activity and the number of nuclear T_3 receptors are proportionally related.

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