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Full Length Research Paper

Triiodothyronine deficiency affects serotonergic- and muscarinic-receptor binding in the rat brain

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The study examined serotonergic- (5-HT) and muscarinic-receptor binding in the hypothyroid versus the euthyroid rat brain. Wistar rats were randomly divided into two groups; 1) hypothyroid, treated with methimazole (60 mg/kg per day) in their drinking water for four weeks, and 2) euthyroid, which were given only tap water. The animals were sacrificed and their brains were used for autoradiographic experiments. When compared to the euthyroid group, the hypothyroid rats had significantly enhanced ^{3}H-5-hydroxytriptamine-receptor binding in the cingulate (53%), parietal (19%), and temporal (32%) cortices, the caudate putamen (29%), anterior amygdala nucleus (39%), fields CA1-3 (54%), periaqueductal gray (45%), substantia nigra pars reticulata (71%), and compacta (19%). Autoradiograhic experiments revealed that in the hypothyroid group the ^{3}H-quinuclinidyl benzilate receptor binding (a muscarinic agonist) was reduced in the frontal (42%), parietal (46%), temporal (42%), and entorhinal (41%) cortices, the caudate putamen (45%), the anterior (40%), medial (33%), and basolateral (46%) amygdaloid nuclei, dentate gyrus (53%), fields CA1-3 (43%), periaqueductal gray (45%), substantia nigra pars reticulata (35%), and compacta (35%). The present data suggest that alterations in both 5-HT- and muscarinic-receptor binding could be associated with the changes in behavioral alterations and in the enhanced excitability seen in hypothyroid rats.

Keywords: Methimazole. Autoradiography.

INTRODUCTION

The thyroid hormone 3,5,3'-triiodothyronine (T3) has a fundamental role in the development, differentiation, physiology, and aging of the central nervous system (CNS) (Loosen 1992; Oppenheimer et al. 1994;Patel et al. 1980; Porterfield and Hendrich 1993). The effect of hypothyroid status on central neurotransmission is an important area of investigation because there is evidence indicating that neurotransmission in the CNS is modified by the status of the thyroid (Andersson and Eneroth 1987; Calzà et al., 1997). Several lines of data support the view that thyroid hormones influence the neurochemical organization of the brain in adults, including transmitter-identified pathways, neuropeptides, and growth factors (Calzà et al., 1997). There is evidence of the biological basis of the action of thyroid hormones on mature neurons. (Courtin et al., 1986) described that L-thyroxine (T4), L-3,5,3'-triiodothyronine (T3), and the enzyme deiodinase type II, which converts inactive T4 to the active form T3, are present in the adult brain. Receptors for T3 are located in the nuclei of glial cells and neurons in different areas of the brain (Bradley et al., 1992). The hippocampus, the cerebellar

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cortex, the olfactory bulb, the striatum, and some hypothalamic nuclei of the mature brain express high T3-receptor levels (Bradley et al., 1989;Bradley et al., 1992). The cellular action of thyroid hormones is mediated by nuclear receptors modulating the production of ligand-responsive transcription factors (Evans, 1988) able to enhance or repress the expression of target genes at transcriptional or posttranscriptional levels (Samuels et al., 1989; Dussault and Ruel, 1987). Some of these genes encode for proteins of general function, such as the Na+-K+-ATPase pump (Ismail-Beigi, 1992; Lin and Akera, 1978), laminin (Farwell and Dubord-Tomasetti, 1999), tubulin, or genes encoding for the growth hormone gene and the thyroid-stimulating hormone gene in the anterior pituitary gland (Glass and Holloway, 1990).

Much evidence suggests a neuromodulatory link between thyroid hormones and 5-HT alterations in regions like the limbic system (Tejani-Butt et al., 1993). Mason et al. (Mason et al., 1987) described that the administration of T3 or T4 for 7 days increased the number of cortical beta-adrenergic and 5-HT2 receptors, whereas a thyroidectomy produced a significant decrease in 5-HT2 receptors in the striatum. Other evidence indicates that severe hypothyroidism increases the density of 5-HT1 and 5-HT2 receptors in the rat brain at 31-32 days old. These receptor alterations are not associated with the degree of hypothyroidism or the rate of neonatal malnutrition (Vaccari et al., 1983).

In addition, hypothyroidism is associated with increased acetylcholinesterase activity in the cerebral cortex of both young and aged rats, and the enhanced density of M_1-Acetylcholine (Ach) receptors only in the former (Salvati et al., 1994). It is possible that the neurotransmitters involved in CNS excitability could also be altered by hypothyroidism. Our study was done to determine if 5-HT- and-or muscarinic-receptor levels are modified in the brain of the hypothyroid rat.

METHODS

Animal treatment

Female Wistar rats two-months old (200 – 250 g) were used. Animals were kept under constant temperature (21 ± 1 °C) and a 12-h light: 12-h dark cycle (lights on at 0800) with food (purina chow) and water *ad libitum*. All animal experiments were made in accordance with the National Institutes of Health guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). Water intake, colonic temperature, and body weight were recorded every three days. Rats were randomly divided into two groups; 1) the hypothyroid group (n = 11), which received methimazole (60 mg/kg/day) (Sigma Chemical Co.) in drinking water daily for four weeks, with the dose adjusted according to water intake and body weight every three days throughout the experiment as described before (Pacheco-Rosado et al., 1997; Pacheco-Rosado et al., 2001) and 2) the euthyroid group (n = 8), which drank only tap water. Animals were killed by decapitation after 28 days of the methimazole treatment. The brains of control and experimental animals were quickly removed, frozen in pulverized dry ice, and stored at -70 °C.

Serum T_3 and T_4 hormones

To observe the modifications of serum concentrations of the thyroid hormones large enough to modify the colonic temperature of the rats, blood samples from the rat-tail vein were taken at the end of the treatment. Serum was separated and stored at –4 °C until the day of analysis. T_3 and T_4 concentrations were determined by enzyme immunoassay (ICN Pharmaceuticals kit, U.S.A.).

Autoradiographic experiments

Frozen coronal sections of 20 µm were cut in a cryostat, thaw-mounted on gelatin-coated slides, and stored at -70 °C until the day of incubation. On the day of incubation, brain sections were washed for 30 min at 25 °C in TRIS·HCl buffer, pH 7.4, for 5-HT receptors and phosphate buffer, pH 7.4, for muscarinic receptors.

For 5-HT receptors the sections were subsequently incubated for 60 min at 25 °C in a solution of 2 nM of ^{3}H-5-hydroxytryptamine (a 5-HT agonist; sp. act. = 123 Ci/mmol, Amersham Pharmacia Biotech) in the absence or presence of 1 µM of 5-HT (a 5-HT agonist). For muscarinic receptors, the sections were subsequently incubated for 120 min at 25 °C in a solution of 2 nM of ^{3}H-quinuclidinyl benzilate (QNB) (a muscarinic agonist; sp. act. = 86.5 Ci/mmol; Amersham Pharmacia Biotech) (in accordance with procedures describe by Wamsley et al., (Wamsley et al., 1981) in the absence or presence of 1 µM of atropine sulphate (a muscarinic antagonist).

Binding obtained in the presence of 5-HT or atropine sulphate was considered nonspecific. Incubation was completed with two consecutive washes (1-min each) and a distilled water rinse (2 s) at 4 °C. The sections were then quickly dried under a gentle stream of cold air.

The slides were arrayed in X-ray cassettes with tritium standards (Amersham) and exposed to tritium-sensitive Amersham Ultrafilm for 60 days for 5-HT at room temperature or three weeks for muscarinic receptors at 4 °C. The films were developed using standard Kodak D11 and fixed at room temperature. Optical densities

Figure 1. Distribution of 5-HT with ^{3}H-5-hydroxytryptamine in coronal sections at the level of amygdala and dorsal hippocampus of euthyroid (A) and hypothyroid (B) rats. Areas with high 5-HT receptor binding appear black and gray, whereas white areas delineate structures with low receptor binding. Compared with the section of an euthyroid animal, the section of a hypothyroid rat shows high receptor binding.

were determined using a video-computer enhancement program (JAVA, Jandel Video Analysis Software). Parallel sections were stained with the Nissl technique to identify anatomically distinct brain regions and to allow comparison with the brain atlas of (Paxinos and Watson, 1999).

For each structure, 10 optical density readings were taken from at least five sections and averaged. Optical density values of the standards were used to determine tissue radioactivity values for accompanying tissue sections and to convert them to fmol/mg protein. Specific binding was estimated by subtraction of nonspecific binding for all experiments. Optical density readings were made by a technician blind to the experiments.

The levels of 5-HT and muscarinic receptors were analyzed in the following structures: frontal, cingulate, piriform, parietal, entorhinal, and temporal cortices; caudate-putamen; anterior, medial, central, and basolateral amygdaloid nuclei; dentate gyrus and CA_{1-3} fields of the hippocampus; periaqueductal gray, substantia nigra pars reticulata, and compacta.

Statistical analysis

All results are presented as the mean ± SEM. Statistical analysis for serum thyroid hormone concentrations and 5-HT and muscarinic receptors were done using Student's *t*-test. Colonic temperature changes were evaluated by repeated measure ANOVA. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Serum concentrations of thyroid hormones and the colonic temperature

Serum levels of T_4 in the hypothyroid group (3.87 ± 0.27 µg/dL; $t = 0.44$) were similar to euthyroid rat values (4.04 ± 0.27 µg/dL). In contrast, serum levels of T_3 were significantly lower in the hypothyroid group (45.2 ± 1.9 ng/dL) compared to the euthyroid group (62.5 ± 2.43 ng/dL; $t = 5.59$; $P<0.001$). The colonic temperature was significantly decreased in the hypothyroid rats (36.5 ± 0.30 ℃) ($F_{1,19} = 17.88$; $P<0.001$) after four weeks of treatment with methimazole compared to the euthyroid-group values (37.8 ± 0.44 ºC). The hypothyroid group showed the expected changes according to their drug treatment. Additional observations, correlated with changes in T_3 and colonic temperature, included hypoactivity in hypothyroid rats.

Effect of hypothyroidism on ^{3}H-5-HT receptor binding

Compared to the euthyroid group, the hypothyroid group had enhanced ^{3}H-5-HT receptor binding in cingulate (53%), temporal (32%), and parietal (19%) cortices; anterior amygdala (39%), CA1-3 fields (54%), caudate-putamen (29%), periaqueductal gray (45%), substantia nigra pars reticulata (71%), and compacta (19%) (Figure 1 and table 1). Although medial (22%), basolateral (5%), and central (19%) amygdaloid nuclei; frontal (19%), piriform (28%), and enthorinal (16%) cortices, and dentate gyrus (52%) showed high levels, they were not statistically significant.

Effects of hypothyroidism on ^{3}H-quinuclidinyl benzilate (^{3}H-QNB)

Autoradiography experiments revealed that in the hypothyroid group had reduced ^{3}H-QNB binding in frontal (42%), parietal (46%), temporal (42%), piriform (49%), and entorhinal (41%) cortices; caudate-putamen (45%); anterior (40%), medial (33%), and basolateral (46%) amygdaloid nuclei; dentate gyrus (53%); CA1-3

Table1. Effect of hypothyroidism on ^{3}H-5-hydroxytryptamine-binding levels (fmol/mg protein) in specific regions of the rat brain.

STRUCTURE	EUTHYROID GROUP	HYPOTHYROID GROUP
Frontal Cx	54.9 ± 4.1	65.5 ± 5.9
Cingulate Cx	46.7 ± 3.0	71.4 ± 3.9 (1)
Parietal Cx	45.4 ± 1.7	54.0 ± 1.9 (4)
Caudate Putamen	44.3 ± 1.9	57.1 ± 3.5 (2)
Anterior AMG N.	46.9 ± 3.0	65.2 ± 5.6 (3)
Medial AMG N.	63.8 ± 7.1	78.1 ± 6.7
Basolateral AMG N.	64.9 ± 12.6	68.4 ± 6.4
Central AMG N.	54.2 ± 3.0	64.6 ± 8.3
Piriform Cx.	71.9 ± 9.1	92.3 ± 8.6
Dentate Gyrus	130.8 ± 23.4	198.8 ± 23.9
Fields CA1-3	56.8 ± 6.2	87.4 ± 6.8 (3)
Temporal Cx	46.5 ± 4.4	61.2 ± 4.9 (4)
Entorhinal Cx	71.8 ± 8.4	83.5 ± 4.6
Substantia Nigra, reticular part	163.6 ± 32.7	279.2 ± 32.3 (4)
Substantia Nigra, compact part	43.0 ± 1.8	51.2 ± 2.7 (4)
Periaqueductal Gray	56.8 ± 4.3	82.6 ± 6.7 (2)

The data are means ± SE from 9 experiments. (1): $P = 0.006$; (2): $P = 0.004$; (3): $P = 0.014$; (4): $P = 0.045$; AMG = amygdala; N = Nucleus; Cx = cortex

Table 2. Effect of hypothyroidism on ^{3}H-QNB binding (fmol/mg protein) in specific regions of the rat brain.

STRUCTURE	EUTHYROID GROUP	HYPOTHYROID GROUP
Frontal Cx	588.5 ± 76.3	342.0 ± 44.0 (1)
Cingulate Cx	672.7 ± 120.0	371.03 ± 41.6
Parietal Cx	533.9 ± 59.1	286.7 ± 32.1 (2)
Caudate Putamen	591.3 ± 81.4	326.4 ± 33.8 (3)
Anterior AMG N.	224.9 ± 18.1	134.9 ± 17.47 (3)
Medial AMG N.	303.6 ± 31.4	204.1 ± 23.5 (4)
Basolateral AMG N.	659.6 ± 113.0	356.14 ± 40.3 (5)
Central AMG N.	442.8 ± 48.5	311.0 ± 29.3
Piriform CX.	673.4 ± 104.4	342.8 ± 24.4 (1)
Dentate Gyrus	900.1 ± 148.5	422.7 ± 51.7 (6)
Fields CA1-3	574.7 ± 70.1	327.2 ± 36.3 (7)
Temporal Cx.	532.8 ± 53.7	307.1 ± 38.4 (8)
Entorhinal Cx	527.88 ± 61.7	310.7 ± 46.1 (9)
Substantia Nigra, reticular part	132.7 ± 5.4	85.8 ± 11.1 (10)
Substantia Nigra, compact part	131.8 ± 11.3	86.0 ± 10.0 (1)
Periaqueductal Gray	270.3 ± 15.1	149.1 ± 27.1 (11)

The data are means ± SE from 9 experiments. (1): $P = 0.008$; (2): $P = 0.001$; (3): $P = 0.003$; (4): $P = 0.021$; (5): $P = 0.011$; (6): $P = 0.006$; (7): $P = 0.005$; (8): $P = 0.004$; (9): $P = 0.016$; (10): $P = 0.002$; (11): $P = 0.007$; AMG = amygdala; N = Nucleus; Cx = cortex

fields (43%); periaqueductal gray (45%), substantia nigra pars reticulata (35%), and compacta (35%). The Cingulate (45%) cortex and central (30%) amygdala nucleus showed reduced values, but they were not statistically different from that of the euthyroid group (Table 2).

DISCUSSION

Our study supports the notion that hypothyroidism causes significant changes in both 5-HT and muscarinic receptors in different areas of the rat brain. For the 5-HT receptors, our study is in agreement with the findings

that thyroid hormones influence 5-HT-receptor expression (Tejani-Butt et al., 1993; Sandrini et al., 1996; Mason et al., 1987; Kulikov et al., 1999). There is evidence that 5-HT deficiency and hypothyroidism cause similar effects, i.e. they modify the hipocampal brain-derived neurotrophic factor (BDNF) mRNA levels (Luesse et al. 1998; Zetterstrom et al., 1999). BDNF, a neurotrophic factor, influences the development, survival, maintenance, and plasticity of neurons in the immature and adult nervous system (Lewin and Barde, 1996; Thoenen, 1995). It has been recently shown that it causes rapid action potentials, thus influencing neuronal excitability (Kafitz et al., 1999). Moreover, 5-HT deficiency and hypothyroidism both decrease neurogenesis in the dentate gyrus and subventricular zone of adult rats (Madeira et al., 1991; Brezun and Daszuta, 1999), an effect that could be associated with functional deficits in the hypothyroidism state.

The 5-HT also activates Na+-K+-ATPase preferentially in glial cells (Hernández and Condes-Lara 1992). This is important for glutamate uptake and excitatory neurotransmission (Amato et al., 1994). Thus, hypothyroidism-induced enhanced 5-HT receptors may be the consequence of reduced 5-HT extracellular-levels and this might lead to decreased Na^+-K^+pump activity and be associated with a higher glutamate level with altered brain excitability. Future experiments should be made to examine the effects of hypothyroidism on the subtypes of 5-HT receptors and the relation of these receptor changes and brain excitability.

For the muscarinic-receptor levels, we found a decrease in the hypothyroid group. The cholinergic neurons of the basal forebrain provide the widespread innervation of the cortical areas that are responsible for cognitive processes (Bierer et al., 1995). Adult hypothyroidism in humans is characterized by different neurological symptoms including affective abnormalities and cognitive deficits, which can be as severe as to be defined as “reversible dementia” (Loosen, 1992). Also (Tomei et al., 1988) have reported cortical atrophy in the brain of the hypothyroid human adult. These and other data suggest that the alterations of the cholinergic system and the resulting behavioral impairment are features shared by aging and hypothyroidism. (Calzà et al., 1997) suggested the possibility that common mechanisms, although triggered by different signals, may lead to cholinergic alterations in hypothyroidism and aging.

Controversial data exist about the effect produced by hypothyroidism on Ach activity and muscarinic-receptor levels in the rat brain. It has been shown that hypothyroidism causes an impairment of cholinergic neurotransmission in young rats leading to an upregulation of M1-Ach receptors (Salvati et al., 1994). Other results indicate that hypothyroidism in rats produced a decrease in acetylcholinesterase activity, whereas the T3 treatment of rats at age up to 30 days after birth causes an increased density of muscarinic receptors in the cortical-synaptic membrane fractions (Moskovkin et al., 1989; Hamburgh and Flexner, 1957). In this case, it is possible that a low acetylcholinesterase activity leads to a decreased Ach level that causes upregulation of receptor levels, which was shown by the T_3 treatment.

Our differences with the results found in the cited work above could be caused by the method used because these authors used membrane fractions.

Because the muscarinic receptor is located on different neurons and mediates different functional responses (Marchi and Raiteri, 1985), the decreased muscarinic-receptor levels in hypothyroid rats could be involved in memory disorders, hypoactivity, diminished alertness, and low reflexes in general.

In a previous study we demonstrated that hypothyroid rats had higher levels of mu-opioid receptors in some brain regions, the cortex, basolateral amygdala, and ventroposterior thalamic nucleus, whereas benzodiazepine (BDZ) receptor levels were decreased only in the medial amygdale (Ortiz-Butron et al., 2003). Because those receptors and the receptors evaluated in our study are altered by hypothyroidism, we propose that receptor density may be involved in changes of neuronal excitability.

One could assume that if the hypothyroidism were mild, the effects on brain receptors were a nonspecific effect of the agent (methimazole) rather than a specific effect of hypothyroidism. However, in previous work we found that the hypothyroidism, not the antithyroid drugs (methimazole or propylthiouracil), can cause anatomical changes in the hippocampus (Alva-Sánchez et al., 2002). We do not know if the alteration of the population of neuronal cells and-or the amount of neurotransmitter receptors is related to the increased susceptibility to seizure in hypothyroid rats (Pacheco-Rosado et al., 1997; Pacheco-Rosado et al., 2001), but it is well-known that aberrant-network reorganization in the hippocampus of the adult rat is associated with epileptogenesis (Parent, 2002). Considering the higher excitability found in rats with this mild condition, we postulated that this state is possibly one of the factors causing a convulsion. Further studies evaluating occult thyroid dysfunction in patients with epilepsy are necessary to investigate this possibility.

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