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Mild thyroid hormones deficiency modifies benzodiazepine and mu-opioid receptor binding in rats

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Abstract

The effects of a mild hypothyroidism condition on benzodiazepine (BDZ) and mu opioid receptor levels was investigated. Female Wistar rats were randomly divided into two groups: 1) hypothyroid rats (n=7), which received methimazole (60 mg/kg per day) in drinking water for four weeks, and 2) euthyroid rats (n=8), which drank only tap water. Animals were sacrificed and their brains were used for autoradiography experiments. When compared to the euthyroid group, the hypothyroid group presented reduced benzodiazepine receptor binding in medial amygdala (24%) and high mu-receptor levels in frontal (25%), sensorimotor (65%) and temporal (29%) cortices, basolateral amygdala (50%) and ventroposterior thalamic nucleus (49%). The present data suggest that alterations in BDZ and mu-receptor binding could be associated with the higher excitability observed in animals with triiodothyronine (T_3) deficiency.

Keywords: Hypothyroidism; Methimazole; T3 (triiodothyronine); Benzodiazepine receptors; Opioid receptors; Autoradiography

1. Introduction

Thyroid hormones have been shown to play a crucial role in the development of the central nervous system (CNS) (Porterfield and Hendrich, 1993). This fact is dramatically illustrated by the severe cognitive impairment that accompanies thyroid hormone deficiency during prenatal and perinatal age (Porterfield and Hendrich, 1993). Even so, hypothyroidism induced postnatally has been associated with lower structural protein expression, like tubulin (Aniello et al., 1991) and neurochemical alterations in the brain due to a different metabolic regulation (Calzá et al., 1997). Moreover, thyroid hormones exert a complex action on the mature brain, either through a direct gene expression regulation or through some more general biochemical and metabolic adjustments, including mRNA stability, protein degradation and energy balance (Aniello et al., 1991; Calzá et al., 1997).

It has long been established that thyroid hormones are associated with brain excitability. A positive correlation between serum T_4 levels and susceptibility to audiogenic seizures was described in DBA/2J mice (Seyfried et al., 1979). In fact, those mice present a subnormal brain deiodinase activity, which could produce a deficiency of 3,5,3′-triiodothyronine (T_3) in the brain, but not in the rest of the body (Sawitzke et al., 1988). In addition, cerebral T_3 requirements are highly dependent on the supply of T_4 and its local conversion to T_3 (Silva et al., 1982; Dratman et al., 1983). This group of evidence suggests that the higher seizure susceptibility could be more related to the T_3 brain deficiency, rather than a compensatory increase in T_4.

There are reports indicating that the development of amygdala kindling in rats is not modified by the adminis-

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tration of propylthiouracile (a drug known to induce hypothyroidism) over 10 days (Kim et al., 1996). On the other hand a treatment with methimazole (another antithyroid drug) (60 mg/kg) for four weeks, induces dual effects: it enhances the seizure threshold and prolongs the duration of convulsions induced by acute metrazole administration (Pacheco-Rosado et al., 1997). Recently, we have described that this methimazole in rats is associated with an increased susceptibility to metrazole and lidocaine-kindling (Pacheco-Rosado et al., 2001). These results suggest that the hypothyroidism can alter the epileptic process.

On the other hand, opioid peptides and Gabaergic systems are known to play an important role in the regulation of brain excitability (Hong et al., 1993). Evidence suggests that mu opioid receptors induce dual effects; i.e. the enhanced mu receptors facilitate the amygdala kindling process and increase the threshold for subsequent seizures during the postictal period (Rocha et al., 1993). In addition, up regulation of mu receptors facilitates the kainic acid-induced seizures and reduces the postictal mortality (Cano-Martinez et al., 2001).

Concerning opioid peptide changes induced by a thyroid state, it has been found that hypothyroid rats present decreased mu receptors in the median eminence (Calzá et al., 1992), whereas the chronic treatment with thyroxine enhances mu receptor binding in the striatum (Bhargava et al., 1989).

Regarding the Gabaergic system, there are some studies indicating that the administration of T3 in adult rats does not alter benzodiazepine (BDZ) receptor levels (Sandrini et al., 1991).

The relevance of studying mild hypothyroidism is due to its relation to subclinical hypothyroidism associated with important metabolic alterations found in patients (Barnes and Galton, 1976; Cooper, 2001). Subclinical hypothyroidism has been defined biochemically as elevation of thyroid-stimulating hormone (TSH) levels and normal thyroid hormone concentrations (Eirís-Puñal et al., 1999). Patients with subclinical hypothyroidism present higher scores on scales of anxiety or depression (Monzani et al., 1993). In addition, longterm antiepileptic treatment with carbamazepine, valproate and phenopharbital in children with epilepsy may cause subclinical hypothyroidism (Eirís-Puñal et al., 1999; Cooper, 2001).

According to previous evidence, it is possible to suggest that mild hypothyroidism may be associated with an imbalance between the excitatory and inhibitory systems. The present study was performed to determine possible changes in BDZ and mu-opioid peptide receptor levels in mild hypothyroid adult rats.

2. Methods

2.1. Animal treatment

Female Wistar rats at two months of age (200–250 g), were used. The animals were kept under constant temperature (21 ± 1 °C) and a 12 h-light: 12 h-dark cycle (lights on at 8:00), with food (purina chow) and water ad libitum. All animal experiments were carried out 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. The rats were randomly divided into two groups as follows: 1) the mild hypothyroid group ($n = 7$), which received methimazole (60 mg/kg/day) (Sigma Chemical Co.) in drinking water daily for four weeks, dose was adjusted according to water intake and body weight every three days throughout the experiment as described before (Pacheco-Rosado et al., 1997; 2) The euthyroid group (n=8), which drank only tap water. Animals were killed by decapitation following 28 days of methimazole treatment in drinking water. The brains of control and experimental animals were quickly removed, frozen in pulverized dry ice and stored at −70 °C.

2.2. Serum triiodothyronine (T3) and thyroxine (T4) hormones

To observe the modifications of thyroid hormone serum levels, large enough to modify 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 (kit ICN Pharmaceuticals, USA).

2.3. Vaginal smears

The Papanicolaou technique using the vaginal smears was carried out daily during the last week of treatment for all animals to confirm the status of the estrous cycle (Fentanes and Guevara-Clavel, 1980). In order to avoid the variations of mu-opiate receptors in female animals during estrous cycle, all rats were sacrificed at estrous at 12:00 p.m. According to Maggi et al. (1993), the number of mu receptors is lower at that point in time.

2.4. Autoradiography experiments

Autoradiography experiments were performed in accordance with previously described procedures (Rocha et al., 1993; Rocha et al., 1996). Briefly, 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. Parallel sections from each animal

were obtained and processed for mu and BDZ receptor binding.

Autoradiography incubations were carried out in Tris-HCl buffer 50 mM (pH 7.4) for mu receptors and Tris-HCl buffer 170 mM (pH 7.4) for BDZ receptors. Initially, the brain sections were prewashed for 30 min at 25 °C. For mu receptors, the sections were subsequently incubated during 60 min at 25 °C in a solution of 2 nM of [^{3}H]DAMGO (a mu agonist), in the absence or presence of 2 mM of naloxone (a mu antagonist). For BDZ receptors, the brain sections were incubated during 45 min at 4 °C in a solution of 2 nM of [^{3}H]Flunitrazepam (a BDZ agonist), in the absence or presence of 1 mM of clorodiazepoxide (a BDZ agonist). Binding obtained in the presence of naloxone or clorodiazepoxide 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 film (Amersham Ultrafilm) for 10 (mu receptors) or three weeks (BDZ receptors) at room temperature. The films were developed using standard Kodak D11 and fixer at room temperature. Optical densities were determined using a video-computer enhancement program (JAVA Jandel Video Analysis Software). Parallel sections were stained with Nissl Technic to identify anatomically distinct brain regions and to allow comparison with the brain atlas of Paxinos and Watson (1996). For each structure, 10 optical density readings were taken from at least five sections and averaged. The optical density values of the standards were used to determine tissue radioactivity values for the accompanying tissue sections and to convert them to fmol/mg protein. Optical density readings went measured blind.

BDZ and mu receptors were analyzed in the following structures: frontal, cingulate, piriform, sensoriomotor, entorhinal and temporal cortices; caudate nucleus; medial and basolateral amygdaloid nucleus; centromedial, ventroposterior, ventrolateral, lateral and medial thalamic nuclei; dentate gyrus and CA1-3 fields of Ammon's horn; periaqueductal gray; substantia nigra pars reticulata and pars compacta.

2.5. Statistical analysis

All results are presented as mean ± SEM. Statistical analysis of thyroid hormone levels, BDZ and mu receptor binding was performed using the Student's *t*-test. Colonic temperature changes were evaluated using repeated measure ANOVA. *p* values of less than 0.05 were considered statistically significant.

3. Results

Serum T_4 levels 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 T_3 levels were significantly lower in the hypothyroid group (45.2 ± 1.9 ng/dl) compared with the euthyroid group (62.5 ± 2.43 ng/dl; $t = 5.59$). Colonic temperature was significantly diminished in hypothyroid rats (36.5 ± 0.30 °C) ($F_{1,19} = 17.88$; $p < 0.001$) after four weeks of treatment with methimazole in contrast to euthyroid group values (37.8 ± 0.44 °C). So, 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.

Concerning autoradiography experiments, and in comparison with euthyroid rats, the hypothyroid group presented significantly enhanced ^{3}H-DAMGO binding in frontal (25%), sensorimotor (65%) and temporal (29%) cortices, basolateral amygdala (50%) and ventroposterior thalamic nucleus (49%). Although the Cingulate, piriform and enthorinal cortices, caudate putamen, medial amygdala nucleus, ventromedial and ventrolateral thalamic nuclei, dentate gyrus and CA_{1-3} fields showed high levels, they were not statistically significant (Fig. 1 and Table 1).

Autoradiography experiments revealed that in the hypothyroid group the ^{3}H-flunitrazepam receptor binding was reduced (24%) in medial amygdala. Parietal cortex, basolateral amygdala nucleus and CA_{1-3} fields presented reduced values, but they were not statistically different from the euthyroid group (Table 2).

4. Discussion

The present study indicates that mild hypothyroidism induces significant changes in both mu and BDZ receptor binding. These findings are in accordance with the notion that thyroid gland activity influences neurochemical organization of the brain (Calzá et al., 1997). Thus, it is possible that the changes observed in the present study could be associated with the altered cerebral excitability produced by the T_3 deficiency.

Concerning opioid peptide systems, there is evidence to demonstrate that hypothyroidism induces an up-regulation of enkephalin mRNA in both the granule cells of the dentate gyrus and the layers V/VI of the cingulate cortex and of dynorphin mRNA in the granule cells of the dentate gyrus (Giardino et al., 1995). With respect to opioid receptors, it has been shown that hypothyroid rats present decreased mu receptor levels in the median eminence (Calzá et al., 1992). In the present study, an important finding was the increase in mu receptors in several brain areas of mild hypothyroid rats. The dis-

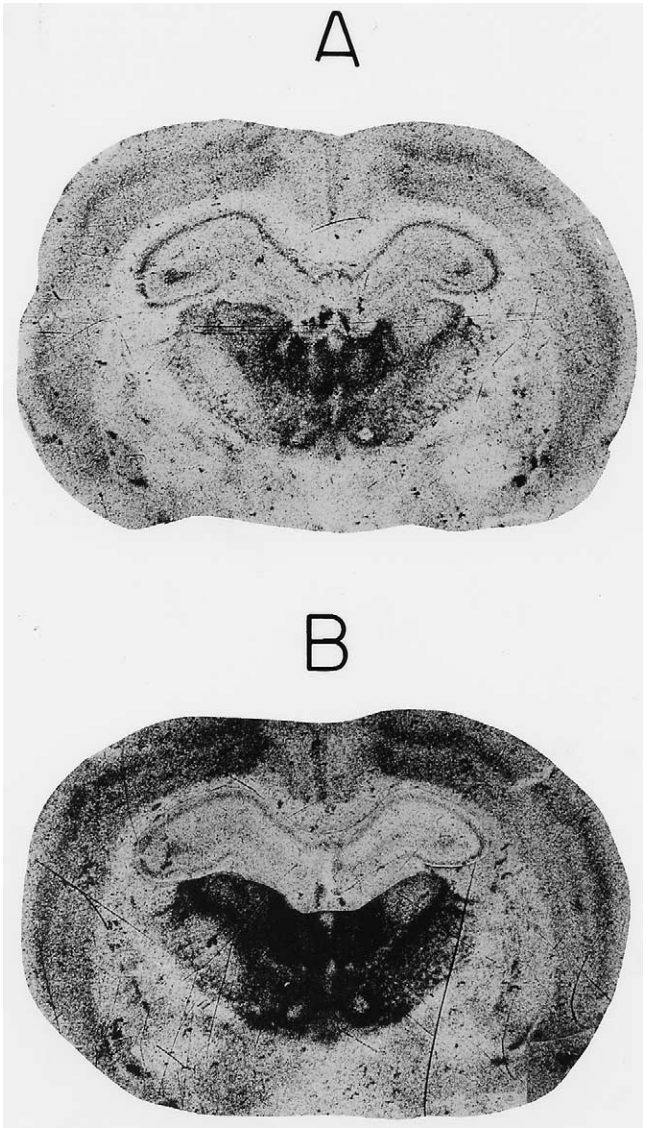

Fig. 1. Distribution of mu receptors radiolabeled with ^{3}H-DAMGo in coronal sections at the level of amygdala and dorsal hippocampus of euthyroid (A) and hypothyroid (B) rats. Areas with high mu receptor binding appear black and gray, whereas white areas delineate structures with low receptor binding.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

crepancy between these results and those of previous studies may be due to the different experimental conditions to induce hypothyroidism, since Calzá et al. (1992) used thyroidectomized rats while in the present study a mild hypothyroidism was induced.

With respect to the relationship between opioid receptors and brain excitability, we have demonstrated that the up-regulation of mu receptors, induced by the chronic administration of naloxone or morphine, produces two different effects: seizure activity facilitation and enhanced postictal seizure suppression (Rocha et al., 1993). This evidence may help to explain the controversy about reduced and enhanced seizure susceptibility associated with the deficiency of T_3 and T_4 (Pacheco-

Table 1
Effect of the hypothyroidism condition on ^{3}H-DAMGO binding (fmol/mg protein) in specific regions of the rat brain[a]

Structure	Euthyroid group	Hypothyroid group
Frontal Cx	105±6.8	132±13.7 (1)
Cingulate Cx	175±33.5	220±34.1
Piriform Cx	121±15.7	163±30.0
Sensorimotor Cx	101±16.0	167±25.0 (2)
Entorhinal Cx	118±17.8	156±30.3
Temporal Cx	139±12.2	179±25.2 (3)
Caudate Putamen	126±21.4	152±19.6
Medial AMG N	216±27.4	276±37.2
Basolateral AMG N	362±43.6	543±51.3 (4)
Ventromedial thalamic N	599±44.3	632±83.8
Ventroposterior thalamic N	131±27.5	195±24.2 (5)
Lateral thalamic N	192±27.1	173±29.8
Medial thalamic N	598±37.5	599±72.9
Ventrolateral thalamic N	310±19.4	381±70.3
Dentate gyrus	86±9.6	110±15.9
Fields CA_{1-3}	89±13.0	103±16.0
Substantia nigra pars reticulata	95±11.3	91±15.9
Substantia nigra pars compacta	138±19.0	107±21.5
Periaqueductal gray	137±1.3	105±18.2

[a] The data are means ± SE from 8 experiments (1) $p = 0.006$; (2) $p = 0.004$; (3) $p = 0.014$; (4) $p = 0.045$; (5) $p = 0.026$. AMG = amygdala; N = Nucleus; Cx = cortex.

Table 2
Effect of the hypothyroidism condition on ^{3}H-Flunitrazepam binding (fmol/mg protein) in specific regions of the rat brain[a]

Structure	Euthyroid group	Hypothyroid group
Frontal Cx	321±45.3	330±56.6
Cingulate Cx	403±76.7	448±121.2
Parietal Cx	325±71.8	295±13.6
Piriform Cx	371±47.1	428±75.9
Entorhinal Cx	399±52.2	381±84.5
Temporal Cx	387±64.8	321±37.9
Caudate putamen	164±43.7	175±30.9
Anterior AMG N	236±35.1	212±17.9
Medial AMG N	380±20.0	287±28.9 (1)
Basolateral AMG N	413±52.4	349±33.3
Central AMG N	230±29.3	204±22.0
Dentate gyrus	350±50.3	336±24.6
Fields CA_{1-3}	328±53.5	281±29.0
Substantia nigra pars reticulata	283±15.4	273±84.8
Substantia nigra pars compacta	165±36.1	179±53.0
Periaqueductal gray	239±63.6	269±25.7

[a] The data are means ± SE from 6 experiments (1) $p = 0.021$ AMG = amygdala; N = nucleus; Cx = cortex.

Rosado et al., 1997). Presumably, the increased mu receptor binding associated with mild hypothyroidism plays both anti- and proconvulsant effects, depending on the state of excitability (Hong et al., 1993) i.e. the higher threshold to seizure activity and longer convulsion dur-

ation as well as faster acquisition of chemical kindling in rats with deficiency to T3 and T4 (Pacheco-Rosado et al., 1997; Pacheco-Rosado et al., 2001).

Regarding BDZ receptors, a group of evidence indicates that thyroidectomy increases BDZ binding sites (Medina and DeRobertis, 1985). In contrast, other studies suggest that BDZ receptors are not modified in the experimental condition of dysthyroidism (Sandrini et al., 1991). In the present study, we found that hypothyroidism reduces BDZ receptor binding in medial amygdala nucleus. These results may have physiological relevance since the amygdala is part of the limbic system playing an important role in several functions such as memory and brain excitability (Calzá et al., 1997). In fact, the amygdala is a brain area with lower threshold for seizure activity (Goddard et al., 1969). It is possible that reduced BDZ binding receptors in the amygdala could be associated with the memory alterations and higher seizure susceptibility produced by the mild hypothyroid state. The disagreement between the increased BDZ levels induced by thyrodectomy and decreased values found in the present study suggests a biphasic control of thyroid hormones on BDZ receptors. This effect could be explained by saying that the lack of triiodothyronine (thyroidectomy) may produce an increased amount of BDZ receptors whereas diminished thyroid hormone levels (the mild hypothyroid condition used in this paper) induces a reduction in the number of BDZ receptor binding sites. Future studies should be carried out to investigate this hypothesis.

It is well known that reduced BDZ receptor binding is associated with increased seizure susceptibility (Olsen et al., 1986). In fact, the decreased BDZ receptor binding, observed after single and repetitive metrazole administration, has been associated with a progressive increase in seizure susceptibility (Rocha et al., 1996). It is plausible that reduced BDZ receptor binding in amygdala as a consequence of mild hypothyroidism is related with higher neuronal excitability state and faster seizure acquisition (Pacheco-Rosado et al., 2001).

Although a general agreement exists that the majority of thyroid hormone effects at cellular level are mediated by nuclear receptors, it is not possible to exclude neurochemical alterations in the brain, due to a different metabolic regulation. Several thyroid hormone-regulated genes have been identified so far, including genes for proteins of general cellular functions like Na^+/K^+ ATPase (Lin and Akera, 1978). This notion is in accordance with the fact that mild T_3 deficiencies facilitate the development of the chemical kindling induced by metrazole and lidocaine, probably by reducing Na^+/K^+-ATPase activity in the brain (Pacheco-Rosado and Ángeles-López, 1997; Pacheco-Rosado et al., 2001). Future studies evaluating Na^+/K^+-ATPase in mild T_3 deficiency are necessary to investigate this possibility.

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