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Effects of adult dysthyroidism on the morphology of hippocampal granular cells in rats

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Thyroid hormones are essential for normal brain development and very important in the normal functioning of the brain. Thyroid hormones action in the adult brain has not been widely studied. The effects of adult hyperthyroidism are not as well understood as adult hypothyroidism, mainly in hippocampal granular cells. The purpose of the present study is to assess the consequences of adult hormone dysthyroidism (excess/deficiency of TH) on the morphology of dentate granule cells in the hippocampus by performing a quantitative study of dendritic arborizations and dendritic spines using Golgi impregnated material. Hypo-and hyperthyroidism were induced in rats by adding 0.02% methimazole and 1% L-thyroxine, respectively, to drinking water from 40 days of age. At 89 days, the animals' brains were removed and stained by a modified Golgi method and blood samples were collected in order to measure T4 serum levels. Neurons were selected and drawn using a camera lucida. Our results show that both methimazole and thyroxine treatment affect granule cell morphology. Treatments provoke alterations in the same direction, namely, reduction of certain dendritic-branching parameters that are more evident in the methimazole than in the thyroxine group. We also observe a decrease in spine density in both the methimazole and thyroxine groups.

Key words: adult dysthyroidism, hippocampal granular cells

INTRODUCTION

Thyroid hormones are essential for normal brain development, and are important for the normal functioning of the brain. A lack or deficiency of thyroid hormones during critical periods of brain development results in a permanent deficit in brain functioning, including severe cognitive and neurological impairment. Thyroid hormones action in the mature brain is not as well understood (Anderson 2001, Diez et al. 2008). Thyroid hormones deficiency in the mature brain does undergo structural changes identical to but less exuberant than those observed during development (Madeira et al. 1991a). In fact, hormonal imbalances are involved in many pathologies, such as neurodegenerative and psychiatric disorders. Hypothyroidism in adulthood has been clearly linked to cognitive dysfunction, disturbed attention and depressed moods (Dugbartey 1998, Jackson 1998). Likewise, adult hypothyroid rats show deficits in learning tasks (Fundaro 1989, Alzoubi et al. 2009) and impaired long-term potentiation (LTP) (Alzoubi et al. 2005, Taskin et al. 2011); and also exhibit increased immobility in the Porsolt forced swim test, mimicking animal models of depression (Kulikov et al. 1997). All of this suggests the possibility of compromised thyroid status in adults leading to morphological changes in those regions of the brain that are strongly involved in learning, memory and mood, such as the hippocampus (i.e. adult-onset hypothyroidism significantly decreases hippocampal neurogenesis (Desouza et al. 2005).

In spite of this, we still know little about the mechanisms responsible for these alterations and disorders. The hippocampus is a highly sensitive neural structure to the actions of thyroid hormones due to its high content of thyroid receptors. The persistence of these

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receptors into adulthood would indicate a role of thyroid hormones in the mature CNS (Lee et al. 2003). Regions involved in hippocampal formation are the dentate gyrus, CA3 and CA1. *Via* mossy fibbers, the cells of dentate gyrus project upon the dendrites of CA3 pyramidal cells. At the same time, these cells contribute a major input system (the Schaffer collaterals) to CA1.

A reduction in the number of neurons in the hippocampus has been reported in adult hypothyroid rats (Alva-Sanchez et al. 2004, Alva-Sanchez et al. 2009a). Hypothyroidism reduces the density of pyramidal cells in the CA3 region (Alva-Sanchez et al. 2004) and leads to a decrease in the total number of pyramidal cells in the CA1 region (Madeira et al. 1992). Likewise, the dentate gyrus of adult rodents is also vulnerable to thyroid hormones deficiency. Madeira and coworkers (1991a) observed reduction in the volume of the granular layer and the numerical density of its neurons. DeSouza and colleagues (2005) provide the first evidence of thyroid hormones playing a role in hippocampal neurogenesis in the adult mammalian brain. They observed that adult-onset hypothyroidism decreases the survival and neuronal differentiation of dentate granule cell progenitors. Montero-Pedrazuela and others (2006) showed that short-term adult-onset hypothyroidism significantly impairs dendrite arborization of immature neurons in the sub-granular zone of the dentate gyrus.

Adult hyperthyroidism effects, however, are less fully understood. Similar to adult hypothyroid, L-thyroxine administration in adult rats produces an impairment of synaptic plasticity and induces spatial memory task deficits (Taskin et al. 2011). Gould and coauthors (1990) showed a significant decrease in the density of apical dendritic spines in the CA1 region, but no changes were observed in the spine density of the basal dendrites in these cells. In contrast, no changes in any of these morphological variables were detected in the pyramidal cells of CA3. These researchers indicated that the difference in responsiveness between CA1 and CA3 indicates inherent possession of a greater degree of structural plasticity in adult CA1 pyramidal neurons than in adult CA3 pyramidal neurons.

However, from both methimazole and thyroxin treatments, our group observed that adult dysthyroidism provokes alterations in the same direction in hippocampus pyramidal cells, i.e., reducing dendritic branching and increasing spine density. These alterations were more pronounced in thyroxin than in methimazole treatment (Sala-Roca et al. 2008).

Recalling that the granular cells of the dentate gyrus project upon the dendrites of CA3 pyramidal cells, and that there have been few studies of the effect of dysthyroidism on the hippocampus granule cells in adulthood, the purpose of the present study was to assess the consequences of adult hormone dysthyroidism (excess/deficiency of TH) on the morphology of dentate granule cells in the hippocampus by performing a quantitative study of dendritic arborizations and dendritic spines using Golgi impregnated material.

METHODS

Subjects

Subjects were 36 males (8 control, 14 hypothyroid and 14 hyperthyroid) Wistar rats bred in our laboratory. At 40 days, the rats were individually housed with ad libitum access to food and water. A light-dark circadian rhythm of 12 h (LP, between 08:00 AM and 08:00 PM) was established. Temperature was regulated between 22 ± 2ºC; humidity between 40–60%.

Chemical induction of hypo- and hyperthyroidism

Subjects were randomly allocated to the experimental groups (C = Control rats; M = Methimazole-treated rats; T = Thyroxine-treated rats). Treatment was administered *via* drinking water from 40 days until the end of the experiment, when the rats reached 89 days of age. The onset of puberty in male rats occurs at around 40 days (Korenbrot et al. 1977, Chappel and Ramaley 1985, Engelbregt et al. 2000, Pinilla et al. 2001). Important changes in thyroid function occur during puberty as an adaptation to corporal and sexual development (Farwell et al. 2005). Hypothyroidism and hyperthyroidism were induced *via* drinking water by adding methimazole (20 mg/100 ml) in the first case and L-Thyroxine (1 mg/100 ml) in the second. No difference was observed in water consumption among animals in the three groups.

This method (oral treatment) was used to induce dysthyroidism as it avoids the stress caused by daily injections or thyroidectomy, which could affect the

analyzed behavior patterns. This method has been proven to be effective in the induction of dysthyroidism (Berbel et al. 1994, Darbra et al. 1995, Sala-Roca et al. 2002a,b, 2008).

Procedure

T4 serum levels were measured in order to establish the degree of dysthyroidism induced by our treatment. Animals were sacrificed by decapitation at 89 days between 10:00 AM and 11:00 AM. Blood samples were collected and centrifuged, and serum was immediately frozen and stored at −40°C. T4 Serum was determined by radioimmunoassay (reference values 4.5–12.5 μg/dl). The kit (Coat-a-Count®; DPC) was equipped with standard T4 values ranging from 1 to 24 μg/dl. Antiserum was highly specific for T4. The procedure is capable of detecting as little as 0.25 μg/dl. Each sample required a duplicate sample to confirm reliability. The recovery percentage for evaluations fell within the standard and acceptable limits for these measures. Coefficient of variation between duplicate samples was always <5%.

Rats were anesthetized and transcardially perfused with 4.0% paraformaldehyde in 0.1 M phosphate buffer with 1.5% picric acid. Brains were postfixed in a solution with the same composition as the above-described perfusate for 24 h until processing for single-section Golgi impregnation using a modified version of the protocol previously outlined by Gabbott and Somogyi (1984). Coronal sections (150 μm) of the hippocampus were obtained using a slicing microtome in a bath of 3.0% potassium dichromate in distilled water and subsequently incubated in this solution for 24 h. Following this, sections were briefly washed in distilled water and mounted on uncoated glass slides. Coverslips were glued over the tissue section at each corner and the slide assemblies were incubated in a solution of 1.5% silver nitrate in distilled water in darkness for 24 h. Following this, the slide assemblies were dismantled, the tissue sections removed and then briefly rinsed in distilled water, dehydrated in 95% ethanol solution followed by absolute ethanol and mounted onto slides.

Neurons were sampled at different localizations. The exact localization was registered for each granular cell. The morphological criteria employed in the selection of neurons were integrity, homogenous impregnation, and relative isolation from the blood vessels and silver deposits of other impregnated cells located nearby. A minimum of one neuron and a maximum of five neurons (mean = 3.64; mode = 5) per subject in each hippocampal region were drawn individually. As a whole, 131 neurons (30 control, 50 hypothyroid and 51 hyperthyroid) were drawn individually at 625×, using a camera lucida (Nikon, mod Drawing tube L). Dendrites and dendritic spines were drawn at 1552.5× magnification.

The branching density of dendritic trees was evaluated by applying the method of concentric rings. Rings were calculated at 50 μm intervals (0–50, 50–100, 100–150). Each neuron was therefore examined for number of primary dendrites, for number of dendritic arborizations (branch points),

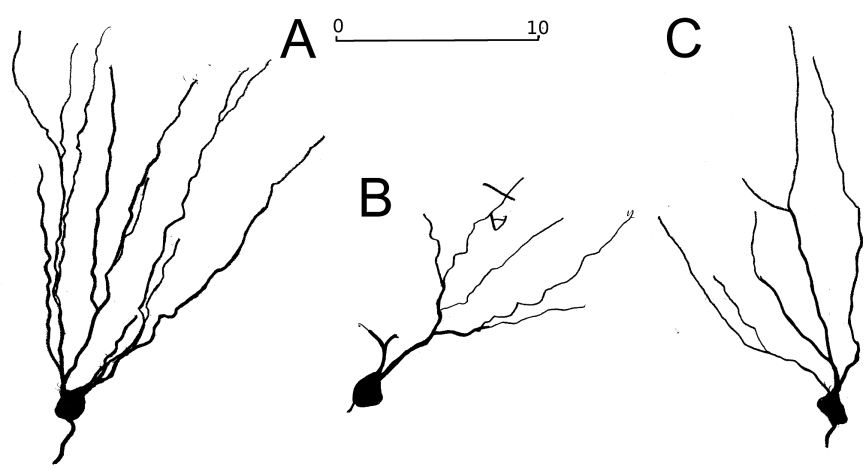

Fig. 1. Dendritic tree of granular cells. (A) Control; (B) Methimazole treatment; (C) Thyroxine treatment.

Table I

Effect of dysthyroidism on granule cells

Parameter		Thyroxine group (mean ± SEM)	Methymazole group (mean ± SEM)	Control group (mean ± SEM)
0–50 μm	Number of terminal segments	1.31 ± 0.22	1.66 ± 0.20	0.50 ± 0.20
	Dendritic intersections	3.90 ± 0.21	4.54 ± 0.30	4.93 ± 0.39
	Dendritic arboritzacions (branch points)	2.98 ± 0.22	3.90 ± 0.24	3.33 ± 0.31
50–100 μm	Number of terminal segments	2.12 ± 0.29	2.65 ± 0.32	2.67 ± 0.33
	Dendritic intersections	3.24 ± 0.35	3.06 ± 0.32	4.23 ± 0.55
	Dendritic arboritzacions (branch points)	1.40 ± 0.18	1.25 ± 0.18	1.90 ± 0.28
100–150 μm	Number of terminal segments	2.30 ± 0.23	2.48 ± 0.30	2.56 ± 0.41
	Dendritic intersections	2.50 ± 0.39	1.62 ± 0.34	3.00 ± 0.49
	Dendritic arboritzacions (branch points)	0.88 ± 0.18	0.76 ± 0.17	1.15 ± 0.28

for dendritic terminal segments in each concentric ring and for number of dendritic intersections crossing each concentric ring. In addition, one measurement of spine density was obtained from secondary-dendrite segments proximal to the soma (Gould et al. 1990). All spines in the selected dendritic segment (one per neuron) were counted, and spine density values were expressed as the number of spines/l0 μm dendrite.

The experimental protocol was in agreement with the European Community Council Directive (EEC directive 86/609) for the care and use of laboratory animals and was therefore approved by the Ethical Committee on Animal and Human Experimentation at the Universitat Autònoma de Barcelona.

Statistical analysis

Data were analyzed using a commercial statistical package (SPSS/PC+). Normal distribution of variables was confirmed by a Kolmogorov-Smirnov test. Logaritmic transformation was applied to spine density values because normal distribution was not obtained. Two-tailed analyses of variance (ANOVA) were performed to compare experimental groups. DMS *post-hoc* test values were considered when significant findings appeared in the ANOVA test. Statistical significance was considered to be attached at $P<0.05$ level.

RESULTS

Thyroxin levels

Serum samples were collected in order to analyze the serum levels of T4. Treatments were effective in altering thyroid hormones levels ($F_{2,35}=61.966$, $P<0.001$). Methimazole-treated rats showed significantly reduced serum levels of T4 (methimazole-treated rats: 1.17 ± 0.32 μg/dl; control rats: 5.89 ± 0.40 μg/dl; $P<0.005$) whereas thyroxine-treated rats showed higher serum levels of T4 than the control group (thyroxine-treated rats: 20.66 ± 2.63 μg/dl; control rats: 5.89 ± 0.40 μg/dl; $P<0.001$).

Granular cells

On the basis of cell localization, no significant differences were found for any morphologic parameter. In addition, the results show equal involvement regardless of the analyzed layer.

Table I shows means and SEM of morphological variables. The analysis of these variables showed that dendritic arborizations are shorter and fewer in neurons of treated groups:

Differences were observed in the number of dendrite intersections for the 0–50 concentric ring ($F_{2,128}=2.994$, $P<0.05$). *Post-hoc* analysis showed that neurons of animals treated with thyroxine (T) had

fewer dendrite intersections than neurons of control (C) animals (P<0.021)

Differences were observed in the number of dendrite terminal segments in the 0–50 concentric ring ($F_{2,128}$=6.582, P<0.002). *Post-hoc* analysis showed that neurons in the methimazole (M) group (P<0.001) and neurons in the T group (P<0.012) had more terminal segments than neurons in the C group.

Differences were observed in the number of dendrite intersections for the 100–150 concentric ring ($F_{2,106}$=3.008, P<0.05). *Post-hoc* analysis showed that neurons in the M group had fewer dendritic intersections than neurons in the C group (P<0.021).

There are no significant differences in the number of primary dendrites ($F_{2,128}$=0.325, P<0.7) and or the number of dendritic arborizations and dendritic intersections for the 50–100 concentric ring. However, some differences appeared when only neurons of one treatment were compared with neurons in the C group as shown in Fig. 1 and Fig. 2.

Finally, an important effect on spine density was observed ($F_{2,113}$=7.72, P<0.001). *Post-hoc* analysis showed reduced spine density in neurons in both M (P<0.001) and T (P<0.003) groups.

DISCUSSION

In the present experiment, granular dendritic tree alterations in adult rats have been observed in dysthyroid status, i.e., dysthyroid status leads to dendritic retraction: reduction in dendritic intersections and reduction in the number of branch points in some concentric rings. Likewise, we find early termination of dendrites in neurons for both treatments. That is, although the number of primary dendrites is not altered in treated groups, we observe a higher number of terminal segments in the 0–50 concentric ring, mainly in the methimazole group.

Another relevant effect observed in this study is the important reduction in spine density in both methimazole- and thyroxine-treated groups.

Treatments therefore appear to block dendritic extension on granular cells. We have observed the same effect on pyramidal cells (Sala-Roca et al. 2008). This reduction in dendritic branching in neuronal cells is consistent with results obtained by many other researchers. In addition to the pioneering results obtained by the Ruiz-Marcos group (1980) for cortical neurons and by the Madeira group (1992) in granule cells, Montero-Pedrazuela and coauthors (2006) observed impairment in the dendritic ramification (fewer and shorter) of immature neurons in adult-on-

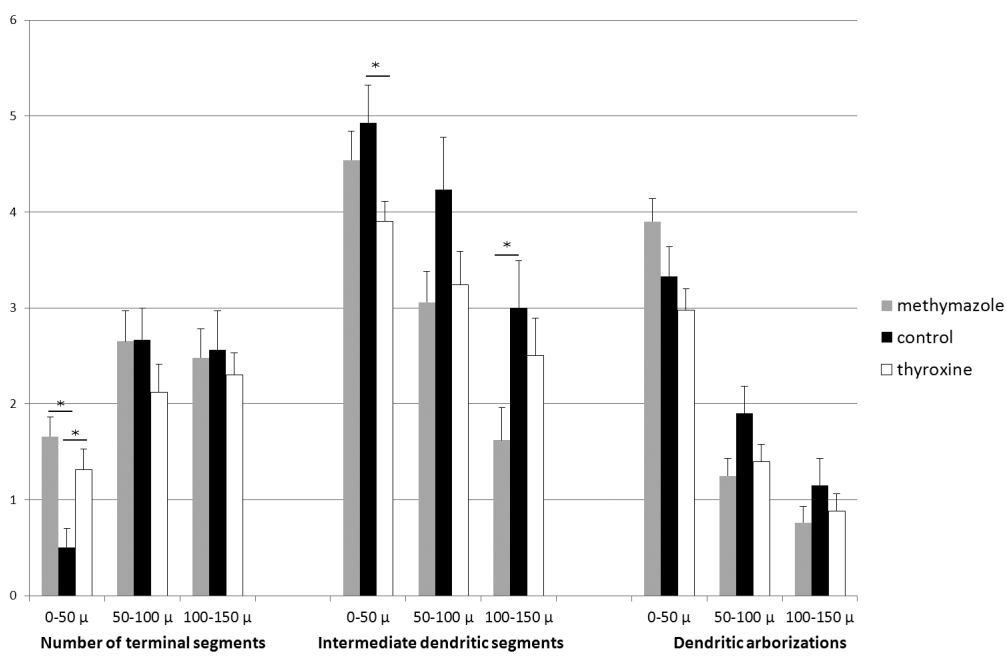

Fig. 2. Number of terminal segments, dendritic intersections, and dendritic arborizations at different concentric rings. *Significant differences between groups.

set-hypothyroidism. Although it is well-established that thyroid hormones influence the formation of dendritic trees (Gould et al. 1990), and that the hippocampus exhibits morphological plasticity well into adulthood (Desouza et al. 2005), the mechanisms of neural damage in dysthyroidism are still unknown. The decrease in the dendritic shaft could perhaps be explained by altered neurogenesis in adult hypothyroid status. *In vivo* and *in vitro* results obtained by the DeSouza group indicate that thyroid hormones play a role in regulating adult hippocampal neurogenesis (Desouza et al. 2005), but an understanding of the involvement of thyroid hormones in adult neurogenesis is still incomplete (Ambrogini et al. 2005). Although our understanding of adult neurogenesis is still incomplete, thyroid hormone receptors might play a relevant role (Kapoor et al. 2010, 2011).Thyroid hormones deprivation negatively influences the survival of newborn cells, in particular immature neurons, and newborn cells show a delay in their neuronal differentiation from a morphological and biochemical point of view (Ambrogini et al. 2005). Moreover, both proliferative impairment and morphological alterations in granule cells are related to each other and are influenced by such proteins as BDNF (produced by a thyroid hormone-regulated gene) and Reelin. Various observations indicate that changes in *Reln* gene expression observed in hypothyroid animals might, in fact, be exerted through changes in *Bdnf* gene expression (Konig and Moura Neto 2002). BDNF plays an essential role in promoting neuronal survival throughout adulthood (Kirschenbaum and Goldman 1995) and appears to work in feedback loops, along with a number of other neurogenic factors, to promote the differentiation and survival of new neurons. BDNF can bind to a specific receptor trkB or a single pan-neurotrophin receptor, called p75. Binding to one or other receptor will produce opposite effects. Lu and colleagues (2005), predict that future studies will show that this yin–yang model will be applied to other aspects of neurotrophin function, such as neurogenesis, growth cone turning, dendritic and axonal growth, and synapse formation. In this regard, it has been found that proBDNF collapses neurites outgrowth and filopodial growth cones by activating RhoA through the p75NTR signalling pathway (Sun et al. 2012). Recent results found that in adult hypothyroidism an increase in BDNF in all regions of the hippocampus was accompanied by a decrease in TrkB, whereas p75 levels remain normal (Cortes et al. 2012). The dendritic retraction observed in our study could be produced by this mechanism.

On the other hand, Alva-Sanchez and coworkers (2009b) observed that adult-onset hypothyroidism causes dramatic changes in the morphology of the entire CA3 pyramidal-cell population, as shown by the increase in the number of atrophic pyramidal cells in this region, and also indicate that their results suggest that this neuronal damage requires activation of NMDA channels. In this regard, Losi and others (2008) were the first to show that thyroid hormones modulate nongenomically NMDA receptor activity. Moreover, Caria and colleagues (2009) also observed nongenomic neural actions of thyroid hormones in hippocampal cells of adult rats, in this case intimately related to those of norepinephrine. They proposed that thyroid hormones, similar to neurosteroids, may serve as modulators of adrenergic transmission or as alternate endogenous adrenergic neurotransmitters derived from thyroxine. The regulation in the adult brain of glutamatergic (Losi et al. 2008, Alva-Sanchez et al. 2009b), gabaergic (Wiens and Trudeau 2006, Puia and Losi 2011) and monoaminergic (Caria et al. 2009, Tousson et al. 2012) neurotransmission by thyroid hormones adds new insight into the pleiotropic effects of these hormones (Losi et al. 2008).

If thyroid hormones are needed at adult stages not only for the proper acquisition of new granular neurons but also for the maturation and maintenance of the dendritic tree, our result (decrease in dendritic branching) is therefore in accordance with the observations outlined above. In the methimazole group, new granule cells would thus fail to develop beyond the immature stage. DeSouza and coworkers (2005) suggest that the effects of thyroid hormones may be optimally permissive at euthyroid levels and that methimazole (but not thyroxin) treatment decreases neurogenesis in dentate gyrus (Desouza et al. 2005).Thus, the observed retraction in the dendritic shaft of granule cells could be expected to be more important in methimazole than in thyroxin treatment. Sandrini and others (1991) found that hypothyroidism in adult male and female rats had the same effect as hyperthyroidism. The brain would require an optimum level of thyroidal hormones beyond that (both for the deficit and excess of these hormones) dendritic development and neuronal survival would be affected.

The reduction in dendritic spine density observed in the present study is consistent with results

obtained by several other researchers but contrasts with what we found in pyramidal cells (Sala-Roca et al. 2008). The Ruiz-Marcos and Gould groups observed that adult-onset hypothyroidism decreased spine density in neocortical neurons (Ruiz-Marcos et al. 1980, Gould et al. 1990). Wang and coauthors (2000) found that dendrites in the inner neurons were characterized by varicosities and had only a few very thin dendrite spines. A late stage of neuronal maturation consists of synapse formation and the establishment of connectivity. Synaptogenesis is the result of a complex series of events that includes the acquisition of synaptic competence and the apposition of presynaptic and postsynaptic anatomical structures (Craig et al. 2006). Through the activation of its signaling pathway, reelin has an impact on the recruitment of postsynaptic proteins to the spines, thus favoring the development of anatomical synaptic structures, in addition to having an impact on the physiological activity of the synapse (Niu et al. 2008). Moreover, spinal structure is determined by its underlying actin cytoskeleton. Farwell and colleagues (2005) showed that both T4 and rT3, but not T3, directly regulate the F-actin content of elongating neurites in cerebellar neurons. Through altering proteins such as actin and reelin, hypothyroid treatment would therefore impair spine development in granule cells. Furthermore, Cortes and others (2012) have found in adult hypothyroidism an increase of BDNF in all regions of the hippocampus accompanied by a decrease in TrkB, whereas p75 levels remain normal. It has also been reported that the interaction of BDNF or ProBDNF with p75 has deleterious effects on glutamatergic neurons (Dechant and Barde 2002). In fact, BDNF binding with Trk will be necessary for LTP, whereas p75 will be for long term depression (LTD) (Lu et al. 2005); also the binding of BDNF with TrkB increases the density of dendritic spines, while binding to p75 decreases it (Zagrebelsky et al. 2005, Chapleau and Pozzo-Miller 2012). Over 95% of excitatory synapses on neurons occur on the dendritic spines, with each spine head typically receiving one synapse (Fiala et al. 2002). Spine growth accompanies LTP expression, whereas shrinkage or retraction of dendritic spines seems to be associated with LTD (Nagerl et al. 2004, Zhou et al. 2004, Becker et al. 2008). According to this data, it seems reasonable to assume that adult hypothyroidism could produce a decrease in spine density by this mechanism. Nevertheless, the reduction in dendritic spines of dentate gyrus granular cells is not in the same direction as the results obtained by our group in other areas of the hippocampus, in particular in pyramidal cells of CA1 and CA3 (Sala-Roca et al. 2008). However, both studies showed that the effects of the two treatments were in the same direction, indicating the need for optimal levels of thyroid hormones. It seems reasonable to assume that subtle changes in the levels of the substances involved in the mechanisms of production of the spines might cause these changes. The effects of thyroid changes on other molecules such as reelin might cause these topographical differences. In fact, several studies indicated that neonatal thyroxine treatment produces the hyperplasia of mossy fibers (Lauder and Mugnaini 1977, Lauder and Mugnaini 1980, Lipp et al. 1989, Schwegler et al. 1991). This effect may explain the increase in dendritic spines that we have found (especially in the CA3 field), as a compensatory mechanism of dendritic arborization reduction observed in this CA3 field (Sala-Roca et al. 2008).

It is likely that those morphological changes observed in dysthyroidism (i.e. altered neuronal citoarchitecture, neuronal growth and synapotogenesis) lead to altered hippocampus functions (Madeira et al. 1991b, Hosseini-Sharifabad and Hadinedoushan 2007, Koromilas et al. 2010). But future experiments are needed to address the contribution of adult morphological alterations to the cognitive and behavioral deficits associated with adult-onset hypothyroidism.

CONCLUSIONS

Our results indicate that (1) both methimazole and thyroxine treatment affect granule cell morphology; (2) treatments provoke alterations in the same direction, i.e., reduction of some dendritic-branching parameters and decrease in spine density; (3) both of these are more evident in the methimazole than in the thyroxine group. Altered neurogenesis and altered neurotransmitter systems may lead to morphological alterations in granule cells.

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