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Thyroid hormones are essential to preserve non-proliferative cells of adult neurogenesis of the dentate gyrus

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ABSTRACT

Thyroid hormones (THs) regulate adult hippocampal neurogenesis, a process that involves both cell populations that dynamically switch between pools of proliferative and quiescent cells, and cells that definitely leave the cell cycle to maturate into granular neurons. This investigation was carried out to determine the role of THs on the mitotic activity of specific proliferative cell populations and the preservation of non-proliferative cells participating in the neurogenic process of the dentate gyrus (DG) of the hippocampus. Hypothyroidism was induced in male adult Wistar rats with methimazole for 28 days. We quantified the total number of proliferative cells (BrdU+), proliferative type 1 (BrdU+/GFAP+), and 2b and 3 (BrdU+/DCX+) cells. Early non-proliferative cells (BrdU-/DCX+ cells lacking dendritic process), postmitotic neuroblasts (Tuj 1+ cells lacking dendritic process), and immature granular neurons (IGN; DCX+ or Tuj 1+ and the presence of dendritic processes into granular or molecular layer) were also included. The evidence showed that the proliferation of Type 1, 2b and 3 cells is not modified by hypothyroidism. In contrast, hypothyroidism reduced the number of early non-proliferative cells and also leads to a decrement in the number of IGN. Our results show that proliferative cells of the DG are not sensitive to thyroid perturbations. However, THs are essential to preserve cell populations that leave the cell cycle in the DG of the hippocampus.

1. Introduction

Adult neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus (DG) (Altman, 1963; Gould et al., 1992). In this process, new granular neurons are formed once the mitotic and postmitotic phases are completed. During the mitotic phase, Type 1 (radial and horizontal), 2a and 2b cells divide consecutively until they reach the stage of Type 3 cell (also with mitotic activity) (Kronenberg et al., 2003; Seri et al., 2001; Steiner et al., 2006). Interestingly, the mitotic phase involves also quiescent cells, which are non-proliferative cells that are able to re-enter the cell cycle (Cheung and Rando, 2013), since Type 1 and intermediate progenitor cells (IPCs; Type 2 and 3 cells) can eventually leave the cell cycle and continue shuttling between the proliferative and non-proliferative pools (Lugert et al., 2010; Plumpe et al., 2006). Meanwhile, the postmitotic phase is carried out exclusively by non-proliferative cells that irreversibly remain in the G_0 state of the cell cycle. These cells are called postmitotic cells and include neuroblasts and immature granular neurons (IGN). During this phase, postmitotic cells migrate to the granular layer (GL), mature and integrate into preexisting circuits (Zhao et al., 2006).

This particular process that takes place in the adult brain is finely modulated by several signals; among these, THs play a relevant role predominantly in the postmitotic phase. In vivo studies have shown that THs are essential for the dendritic maturation and the survival of the IGN (Ambrogini et al., 2005; Desouza et al., 2005; Montero-Pedrazuela et al., 2006). These effects are very similar to those exerted by brain derived neurotrophic factor (BDNF), a neurotrophin synthesized and released locally in the hippocampus (Balkowiec and Katz, 2002; Katoh-Semba et al., 1997; Scharfman et al., 2005; Wang et al., 2015). Although the molecular mechanism associated to the effects of THs on the postmitotic phase is yet to be known, the fact that the BDNF promoter is responsive to THs (Sui et al., 2010) suggests a genomic mechanism by which THs induce the expression of BDNF in the DG of the hippocampus.

The role of THs on the mitotic phase remains under discussion. A recent study showed that THs preserve the population of Type 2b and 3 cells in the SGZ of the DG (Kapoor et al., 2012). However these results were not conclusive about the action of THs on the mitotic activity of each cell population due to the absence of cell proliferation assays. Moreover, studies carried out by a single labeling with 5-bromo-2-deoxiuridine (BrdU) have provided contradictory results. Ambrogini et al. (2005) and Desouza et al. (2005) reported that thyroid deficiency

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does not modify the number of proliferative cells in the SGZ. In contrast, Montero-Pedrazuela et al. (2006) found a significant reduction of these cells in thyroidectomized rats.

The background supports that THs are active modulators of the neurogenic process. Based on the fact that adult neurogenesis is a process that involves the dynamic transition between pools of proliferative and non-proliferative cells, its regulation might be exerted by different strategies depending on the cell stage of the different cell populations. Therefore, this investigation was conducted to determine the role THs play in the mitotic activity of specific proliferative cell populations and the preservation of non-proliferative cells participating in the neurogenic process. Additionally, a shallow evaluation to the involvement of BDNF in the actions of THs was carried out. Our results indicate that the proliferation of Type 1 cells and some IPCs is not TH-dependent. Conversely, THs are essential to preserve non-proliferative cells probably by regulating the expression of BDNF.

2. Materials and methods

2.1. Animals and antithyroid treatment

Ninety day-old male Wistar rats (n = 37) were individually housed in a temperature (21 ± 2 °C) and light (12:12 h) regulated room. Food and water were available ad libitum. All experimental procedures described in this study are in accordance with the guidelines of Mexican laws and codes expressed in The Seventh Title of the Regulation of General Health Law regarding Health Research. All efforts were made to minimize the number of animals used and their suffering.

Rats were randomly separated into two groups: control and hypothyroid (MMI) group. The control group received tap water while the MMI group was treated for 28 days with the antithyroid drug methimazole (60 mg/kg/day) dissolved in the drinking water. Water intake, colonic temperature, and body weight were recorded every 3 days throughout the exposure period. Water intake and body weight data were used to adjust the methimazole quantity throughout the treatment.

2.2. Thyroid status evaluation

Thyroid status was evaluated indirectly by monitoring colonic temperature and body weight (thyroid status dependent-variables). Additionally, and in order to confirm the induction of hypothyroidism by methimazole, triiodothyronine (T_3) and thyroxine (T_4) serum levels were measured at the end of the antithyroid treatment. Blood samples were taken from the rat tail vein in both experimental groups. Serum was separated by centrifugation (1095g × 15 min) and stored at −20 °C until analysis. Total T_3 and T_4 serum levels were determined by immunoassay system using a commercial kit (DGR, Germany). The lowest calibrator was 0.75 ng/mL for T_3 and 2 μg/dL for T_4 assays. The sensitivity of the assay was 0.2 ng/mL for T_3 and 0.2 μg/dL for T_4; such values were assigned to samples in which amounts were below the lowest calibrator.

2.3. BrdU labeling and tissue processing

At the end of antithyroid treatment, the subjects received six injections of BrdU (50 mg/kg), a thymidine analog that is incorporated into DNA during cell division (Cooper-Kuhn and Kuhn, 2002). BrdU was dissolved in saline solution (0.9%) and the injections were applied at 2 h intervals (ip). Twenty four hours after the last injection, the animals were anesthetized with phenobarbital (37 mg/kg), and perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS, pH 7.4). Brains were post fixed overnight in PFA and transferred subsequently to 10, 20 and 30% sucrose solution for 24 h each. Dorsal hippocampus (AP −2.6 to −4.4 mm, according to Paxinos and Watson (1986)) was serially sectioned on a cryostat obtaining slices of 20 μm width. Slices were mounted on gelatinized slides and stored at −60 °C until immunofluorescence (IF) process.

2.4. Immunofluorescence

In order to show the cell populations participating in the neurogenic process, brain slices were processed for IF. Sections were hydrated in PBS 1× (pH 7.4) and incubated in 1.3 N HCL at 37 °C for 45 min for DNA denaturalization. After acid treatment, slices were submerged in borate buffer (0.1 M, pH 8.5) for 15 min and then permeabilized using Triton X-100 (0.2% in PBS 1×). Sections were blocked (1 h) with a solution containing 0.1% Triton X-100, 2% goat serum (or horse serum) and 1% bovine serum albumin; they were then incubated with an antibody against BrdU (biotinylated mouse IgG, Invitrogen Kit) for 20 h at room temperature (RT). At the end of the incubation period, BrdU staining was revealed with fluorescein-streptavidin (1:50, Vector Laboratories) for 2 h at RT. Once BrdU staining was completed, the tissue was processed for double labeling for glial fibrillary acidic protein (GFAP) or doublecortin (DCX). Slices were incubated with anti GFAP (1:200, rabbit IgG, DakoCytomation) or anti DCX (1:50, goat IgG, Santa Cruz Biotechnology) antibodies for 24 h at RT. Finally, secondary anti rabbit IgG Texas red (1:100, Vector Laboratories) or Alexa Fluor 594 anti-goat IgG (1:100, Life technologies) antibody was applied for 1 h at RT.

Single labeling for neuronal class III β-tubulin (Tuj 1) was performed on sections hydrated, permeabilized and blocked as previously described. The anti Tuj 1 antibody (1:100, mouse IgG, Covance) was incubated overnight at RT and then a secondary antibody against mouse IgG conjugated to Texas Red (1:100, Vector Laboratories) was applied for 1 h at RT. In order to corroborate that Tuj 1 is only expressed by postmitotic cells, slices from a subset of control (n = 2) and hypothyroid (n = 3) rats were processed for BrdU/Tuj1 double labeling. Slices were hydrated, denaturalized with HCl and blocked. Next, they were consecutively incubated with the anti Tuj1 antibody for 24 h at RT and anti IgG conjugated to Texas Red for 1 h at RT. Finally, BrdU was evidenced using an antibody against BrdU and reveled with fluorescein streptavidin (see details above).

All slices were mounted using Vectashield supplemented with the nuclear marker DAPI (Vector Laboratories). Fluorescent signals were detected with a fluorescence microscope (Olympus BX51) and the images were processed with the Image Pro-Plus version 4.5 software (Media Cybernetics; Rockville, MD). Only general contrast adaptations were made and figures were not otherwise manipulated.

2.5. Cell counting

The present study was restricted to the dorsal area of the DG of the hippocampus; this region was completely sectioned in slices and cell counting was carried out in ten slices per rat (200 μm apart). Positive cells were counted in both brain hemispheres and the total number of cells per rat was estimated by adding the number of positive cells found in the ten slices. Data obtained from different animals, within the same experimental group, were averaged and shown in the graphs.

Positive cells were counted in three regions of the DG: GL, SGZ and the hilus. GL is the region constituted by the cell bodies of granular neurons; SGZ was defined as two or less than two cell bodies out of GL; and the hilus was considered the area located further than two cell bodies from the GL (Desouza et al., 2005). BrdU positive cells were counted in the GL/SGZ and the hilus; the rest of the study was restricted to the GL/SGZ.

2.6. Immunofluorescence and morphological criteria for cell identification

Proliferative cells were identified by the presence of BrdU in their cell nucleus; BrdU location was confirmed by colocalization with the