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Increased anxiety and fear memory in adult mice lacking type 2 deiodinase

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ABSTRACT

A euthyroid state in the brain is crucial for its adequate development and function. Impairments in thyroid hormones (THs; T3 or 3,5,3′-triiodothyronine and T4 or thyroxine) levels and availability in brain can lead to neurological alterations and to psychiatric disorders, particularly mood disorders. The thyroid gland synthetizes mainly T4, which is secreted to circulating blood, however, most actions of THs are mediated by T3, the transcriptionally active form. In the brain, intracellular concentrations of T3 are modulated by the activity of type 2 (D2) and type 3 (D3) deiodinases. In the present work, we evaluated learning and memory capabilities and anxiety-like behavior at adult stages in mice lacking D2 (D2KO) and we analyzed the impact of D2-deficiency on TH content and on the expression of T3-dependent genes in the amygdala and the hippocampus. We found that D2KO mice do not present impairments in spatial learning and memory, but they display emotional alterations with increased anxiety-like behavior as well as enhanced auditory-cued fear memory and spontaneous recovery of fear memory following extinction. D2KO mice also presented reduced T3 content in the hippocampus and decreased expression of the T3-dependent gene *Dio3* in the amygdala suggesting a hypothyroid status in this structure. We propose that the emotional dysfunctions found in D2KO mice can arise from the reduced T3 content in their brain, which consequently leads to alterations in gene expression with functional consequences. We found a downregulation in the gene encoding for the calcium-binding protein calretinin (*Calb2*) in the amygdala of D2KO mice that could affect the GABAergic transmission. The current findings in D2KO mice can provide insight into emotional disorders present in humans with DIO2 polymorphisms.

1. Introduction

Thyroid hormones (THs, T3 or 3,5,3′-triiodothyronine and T4 or thyroxine) play an essential role in the developing and adult central nervous system (CNS) by the modulation of gene expression patterns. Although the thyroid gland synthesizes mainly T4, most actions of THs are mediated by the binding of T3 to specific nuclear receptors (Bernal, 2007). Type 1 and 2 deiodinases (D1 and D2 respectively) catalyze the removal of the iodine atom in the 5′ position of T4 generating the transcriptionally active T3 while type 3 deiodinase (D3) catalyzes the deiodination of T4 and T3 into rT3 and T2 respectively, inactivating TH action (St Germain and Galton, 1997). In the CNS, T4 acts as an important source of intracellular T3 through D2 activity.

D2 is predominantly expressed in astrocytes throughout the brain (Guadaño-Ferraz et al., 1997) and at tanycytes lining the lower part of the third ventricle controlling local T3 production in brain and hypothalamic-pituitary axis (Guadaño-Ferraz et al., 1997; Tu et al., 1997). The current model for T3 availability in the late postnatal and adult brain proposes that T3 in the brain has a double origin: a fraction, which can be up to 50%, would be available directly from the blood circulation and the rest would be produced locally from T4 in astrocytes by D2 (Morte and Bernal, 2014).

The generation of a knockout mouse lacking D2 (D2KO) (Schneider et al., 2001) revealed an important role of D2 in the regulation of the hypothalamic-pituitary-thyroid axis, as D2KO mice exhibited high levels of T4 and TSH with normal levels of T3 in serum. These mice presented a 50% reduction of the brain T3 content, but did not show gross neurological abnormalities in contrast to hypothyroid mice,

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which indicates the presence of important compensatory mechanisms that minimize functional abnormalities in the absence of D2 (Galton et al., 2007). However, analyses of the motor phenotype of D2KO mice at 6-months of age revealed clear alterations in gait, coordination, and muscle strength (Bárez-López et al., 2014), suggesting that the homeostatic processes that compensate for the lack of D2 cannot fully do so at later ages.

For a long time it has been known that alterations in TH levels induce modifications in cognition, memory, and mood state. Some studies have characterized irreversible learning and memory deficits caused by TH deficiency during critical points in development (Rovet, 2014). In addition, in experimental animals it has been demonstrated that adult-onset hypothyroidism impairs brain and synaptic plasticity inducing depressive-like behaviors and alterations in learning and memory processing, including the potentiation of fear memories (Fernández-Lamo et al., 2009; Montero-Pedrazuela et al., 2011, 2006). Furthermore, mice expressing a dominant-negative TH receptor TRα1, which reduces the affinity to T3 10-fold, present extreme anxiety and reduced recognition memory (Venero et al., 2005). On the contrary, mice lacking D3, that present increased brain T3 content, display decreased anxiety and reduced depression-like behaviors (Stohn et al., 2016).

Interestingly, recent studies have characterized an anxiety-depression-like behavior in 3-month-old mice with specific inactivation of D2 at the astrocytes (astro-D2KO) (Bocco et al., 2016). This contrast with the phenotype observed in global-D2KO mice at similar ages, which only exhibited minimal differences in the level of anxiety and exploratory behavior, and did not present abnormalities in locomotion, olfaction and spatial learning and memory (Galton et al., 2007).

In view of this, the main goal in the present study was to evaluate possible alterations in amygdala-dependent and/or hippocampal-dependent processes in the absence of D2. To this aim, we explored the possible alterations in anxiety-like behavior as well as in learning and memory processes in global-D2KO mice at later stages, specifically in 6-month-old mice, as these mice present a late onset motor phenotype (Bárez-López et al., 2014). We found that global-D2KO mice at 6 months of age did not show deficits in learning acquisition and spatial memory; however, they presented increased levels of anxiety in the elevated plus maze and enhanced fear memory in the auditory-cued fear conditioning test with an exacerbation of the spontaneous recovery of fear memory following extinction. Furthermore, we provide direct evidence in the hippocampus and indirect in the amygdala that points to a hypothyroid state in both structures. D2-deficiency leads to alterations in the expression of the gene encoding the calcium-binding protein calretinin (*Calb2*) in the amygdala, which could be underlying the emotional alterations observed in D2KO mice due to impaired GABAergic transmission. The data suggest that intracerebral T3 generated by D2 activity plays an important role in the modulation of mood and amygdala-dependent learning and memory processes at adult stages. In addition, D2KO mice might be a suitable model to understand *DIO2* polymorphisms related with emotional disorders.

2. Materials and methods

2.1. Animals

All experimental procedures were performed following the European Union Council guidelines (2010/63/EU) and Spanish regulations (RD 1201/2005 and RD 53/2013) for the use of laboratory animals, and were approved by the ethics committee of our Institution (Consejo Superior de Investigaciones Científicas, CSIC; approval numbers PN2007-116 and PN2010-55). All efforts were made to minimize suffering. D2KO mice (Schneider et al., 2001) and wild type (WT) counterparts were initially provided by Dr. VA Galton and a colony was established at our Research Institute. *Dio2* mutation was backcrossed for 3 further generations onto the C57BL/6J background. Mice were housed in plastic cages in an air-conditioned and light controlled room at 24 ± 2 °C and 60 ± 5% humidity with automatic light/dark cycles of 12/12 h.

For the behavioral tests, two cohorts of mice were used and tested at different facilities. Each cohort consisted of WT and D2KO mice coming from first litters of at least 2 different dams with no differences in litter size. The first cohort was tested at the Universidad Nacional de Educación a Distancia and the testing schedule was as follows: Barnes maze during days 1–4, fear conditioning test during days 8–10, extinction sessions during days 22–24, spontaneous recovery of the fear memory at day 31. The second cohort was tested at the Instituto de Investigaciones Biomédicas "Alberto Sols" where the elevated plus maze test was performed. Due to possible stress-related alterations derived from the behavioral tests and to differences in sample processing, biological samples for corticosterone assessment, *in situ* hybridization, D2 activity measurements, TH determinations and gene expression analyses were obtained from additional and different cohorts of mice for each test. All tests were performed in 6-month-old male animals and the number of animals used in each study is stated in the results section. Further details on animal handling and tissue processing are presented in the Supplementary Information.

2.2. Behavioral tests

2.2.1. Barnes maze

This task tests hippocampal-dependent spatial reference learning and memory by using visual cues (Barnes, 1979). The test was conducted on a Barnes maze apparatus that consisted of a 1.2 m diameter white Plexiglas circular platform elevated 1 m from the ground with 20 equidistant holes of 45 mm in diameter. The platform was virtually divided into 4 quadrant sections, each quadrant representing one quarter of the platform with 5 holes. The only hole the mice could escape through was a target hole located in Q1 that had an escape box (30 × 20 × 7 cm) underneath (Fig. 1A). Different extra-maze visual cues were positioned in each of the cardinal points. The animal's behavior was recorded using a video camera with an automated video tracking system and analysis software (Etho Vision 1.9, Noldus IT).

The experimental design is schematically represented in Fig. 1B. Mice were trained for 3 consecutive days so they could memorize the position of the escaping hole. Training sessions consisted of 4 trials per day with 2 initial consecutive trials followed by a 30 min rest and then another 2 consecutive trials. In these sessions, mice were left to freely explore the platform for 5 min or until they found the escape hole and the variables measured were: a) the number of primary errors defined as the number of holes the animals explored before exploring the escape hole, and; b) the number of secondary errors defined as number of holes explored after having located the escape hole.

The memory test session was performed the day after the last training session and on this occasion the target hole was blocked so mice could not escape. The animals were left to explore the platform for 2 min and the measured variables were: a) the number of primary errors, b) the number of secondary errors, c) the number of times the animals explored the escape hole, and d) the percentage of time mice spent on Q1 where the target hole was located.

2.2.2. Elevated plus maze

This test is widely used to assess anxiety behavior of rodents (Walf and Frye, 2007). The elevated plus maze apparatus consisted of two opposing open arms (30 × 5 cm) and two opposing enclosed arms (30 × 5 x 15) with an open roof connected by a central square (5 × 5 cm) elevated 38 cm from the ground. The animals were placed on the center square facing one of the opens arms and were allowed to freely explore the environment for 350 s. Only one trial per animal was performed. The apparatus was cleaned with 0.1% acetic acid between animals. The session was recorded with a video camera and the behavior scored was open and closed arm entries (defining an arm entry as all four paws inside the arm), the time spent in the open and closed