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Hypothalamic–pituitary thyroid axis alterations in female mice with deletion of the neuromedin B receptor gene

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ABSTRACT

Neuromedin B, a peptide highly expressed at the pituitary, has been shown to act as autocrine/paracrine inhibitor of thyrotropin (TSH) release. Here we studied the thyroid axis of adult female mice lacking neuromedin B receptor (NBR-KO), compared to wild type (WT) littermates. They exhibited slight increase in serum TSH (18%), with normal pituitary expression of mRNA coding for α-glycoprotein subunit (*Cga*), but reduced TSH β-subunit mRNA (*Tshb*, 41%), lower intra-pituitary TSH content (24%) and increased thyroid hormone transporter MCT-8 (*Slc16a2*, 44%) and thyroid hormone receptor β mRNA expression (*Thrb*, 39%). NBR-KO mice exhibited normal thyroxine (T4) and reduced triiodothyronine (T3) (30%), with no alterations in the intra-thyroidal content of T4 and T3 or thyroid morphological changes. Hypothalamic thyrotropin-releasing hormone (TRH) mRNA (*Trh*) was increased (68%), concomitant with a reduction in type 2 deiodinase mRNA (*Dio2*, 30%) and no changes in MCT-8 and thyroid hormone receptor mRNA expression. NBR-KO mice exhibited a 56% higher increase in serum TSH in response to an acute single intraperitoneal injection of TRH concomitant with a non-significant increase in pituitary TRH receptor (*Trhr*) mRNA at basal state. The phenotype of female NBR-KO mice at the hypothalamus–pituitary axis revealed alterations in pituitary and hypothalamic gene expression, associated with reduced serum T3, and higher TSH response to TRH, with apparently normal thyroid morphology and hormonal production. Thus, results confirm that neuromedin B pathways are importantly involved in secretory pathways of TSH and revealed its participation in the *in vivo* regulation of gene expression of TSH β-subunit and pituitary MCT8 and *Thrb* and hypothalamic TRH and type 2 deiodinase.

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1. Introduction

Neuromedin B (NB) is a bombesin-like peptide, present in several mammalian tissues, where it modulates a wide array of biological functions [1]. Neuromedin B receptor (NB-R), encoded by *Nmbr* gene, belongs to the mammalian bombesin family of receptors, and it is a G protein-coupled receptor that binds preferentially neuromedin B [2]. Neuromedin B and its encoding mRNA are present in high concentrations in human and rat pituitary gland [3,4], and in the latter, the peptide was detected only in thyrotropin (TSH) producing cells [5]. Functional *in vivo* and *in vitro* studies have shown that neuromedin B inhibits TSH secretion, acting as an autocrine/paracrine factor [6–9]. NB pituitary expression is under the control of the main signals that determine the physiological rate of TSH secretion: thyroid hormones and hypothalamic thyrotropin-releasing hormone (TRH). Thyroid hormones inhibit TSH secretion and increase pituitary NB, while TRH is a potent secretagogue for TSH, and decreases the pituitary production of NB [10–13]. Therefore, it had been postulated that NB modulates the action of these hormones on TSH secretion.

In accordance with that, we had recently reported that male neuromedin B receptor knockout (NBR-KO) mice exhibited slightly increased serum TSH at basal state and, higher TSH release after TRH acute administration, consistent with the concomitant finding of elevated pituitary expression of TRH receptors (*Trhr*) mRNA [14]. However, even though male NBR-KO mice had a facilitated TSH release, they exhibited normal serum thyroxine (T4) and slightly reduced serum triiodothyronine (T3), as well as a lower increase in serum T3 after TRH-induced TSH release. Those findings raised the question of whether the disruption of NB pathways may alter the biological activity of TSH or the thyroid response to TSH. In addition, the ability of male NBR-KO mice to increase serum TSH in response to drug-induced hypothyroidism was reduced

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[15]. Hypothalamic TRH is a major determinant of TSH synthesis and biological activity [16], and therefore in the present study we investigate the thyroid axis phenotype of NBR-KO mice at the hypothalamus and thyroid.

2. Material and methods

2.1. Experimental animals

All experiments were conducted in adult female mice, at 3–4 months old, homozygous for the deletion in neuromedin B receptor (*Nmbr*) and wild-type littermates. Heterozygous NBR +/− mice generated as described by Ohki-Hamazaki et al. (1999) [17], were interbred to generate litters containing homozygous NB-R −/− and NB-R +/+ progeny. To confirm the genotype of the mice, genomic DNA was obtained from tail samples and analyzed by polymerase chain reaction (PCR) using specific primers as described previously [17].

Animals were maintained under 12 h alternating darkness and artificial light cycles (light on at 7 am), controlled temperature (22 °C ± 1 °C) and free access to standard rodent chow and tap water. In all of the experiments, the mice were euthanized in the morning by CO_2 inhalation followed by decapitation. Body weight of NBR-KO mice was similar to WT.

All procedures were performed in accordance with the Fund for the Replacement of Animals in Medical Experiments Guide for the care and use of laboratory animals and were approved by our Institutional Committee on Animal Care and Use.

2.2. Basal state of hypothalamic–pituitary–thyroid axis function

To evaluate the consequences of the deletion of neuromedin B receptor on the hypothalamic–pituitary–thyroid axis at steady state, NBR-KO and wild type mice were taken from the cages and immediately sacrificed. Trunk blood samples were centrifuged and serum was stored at −20 °C for hormonal determinations by specific radioimmunoassays (RIA). To determine TSH content, each pituitary was homogenized in 200 μL of phosphosaline buffer, pH 7.6, and supernatants stored at −20 °C until assayed at a final dilution of 1:2000. Another set of pituitaries and hypothalamus (area corresponding approximately to −0.58 mm to −2.70 mm from bregma, and with 2 mm dorsal from the base of the brain, according to coordinates of the Paxinos and Franklin Mouse Atlas [18]) was immediately frozen in liquid nitrogen and storage at −80° until processed to evaluate mRNA levels by quantitative real-time RT-PCR. The enzymatic hydrolysis of thyroid glands was performed as described by Chomard and Austissier (1991) [19]. Briefly, after each thyroid gland was cut in small pieces, the hydrolysis was performed by 100 μL of 100 U/mL Pronase (protease XIV, Sigma, MO, USA) and 50 μL of 100 U/mL leucine aminopeptidase (type III-Cp, Sigma, MO, USA) in gas-free 0.1 M pH 8.6 tris-acetate buffer under anaerobic conditions and in the presence of 6-n-propyl-2-thiouracil (PTU, Sigma, MO, USA). Samples were storage at −20 °C for total T4 and T3 determination by specific RIA.

2.3. TRH stimulation of TSH release

NBR-KO and wild type mice received a single subcutaneous (s.c.) injection of saline (control group) or TRH (Sigma, MO, USA) at dose of 0.05 μg/kg body weight (BW) and sacrificed 30 min after injection. Serum was stored at −20 °C for TSH determination.

2.4. Hormone measurements

Serum TSH and pituitary TSH content were measured by specific mouse radioimmunoassay (RIA) as previously described in detail [14]. All reagents were obtained from Dr. A.F. Parlow at the National Hormone and Peptide Program (Harbor University of California at Los Angeles Medical Center, CA, USA). Minimum assay detection was 30 ng/mL. Serum total T4 and T3 and intra-pituitary T4 and T3 concentrations were measured by commercial coated tubes RIA from ICN Pharmaceuticals (CA, USA). The sensitivity of assay was 0.76 μg/dL for T4 and 6.7 ng/dL for T3. The intra-assay variation was less than 5%.

2.5. Real-time RT-PCR

Pituitary and hypothalamic total RNA were extracted using TRIZOL® (Invitrogen, CA, USA). Single-stranded cDNA was synthesized from 1 μg of total RNA using Reverse Transcription System (Promega, WI, USA). RT-PCR analyses were performed in fluorescent temperature cyclers (Mastercycler ep realplex real-time PCR system, Eppendorf, USA), using SYBR green PCR master mix (Applied Biosystems, CA, USA). Cycle parameters were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The sequences of the forward and reverse primers were, respectively: 5′-GGA TGC TGG CGT TTT GTG-3′ and 5′-TGT GAC TCC TGA CCT TCC A-3′ for thyrotropin-releasing hormone mRNA (*Trh*). The sequences of the primers used for *Tshb*, *Cga*, *Trhr*, *Dio2*, *Slc16a2*, *Thra*, *Thrb* and 36B4 were as described previously [14,20–22].

Relative mRNA levels were determined by comparing the PCR cycle threshold (Ct) between the groups, after correcting for control gene (36B4) using the ΔΔCt method [23]. The expression of 36B4 gene was stable under all experimental conditions. The efficiency of each reaction was calculated using a serial dilution and varied from 95% to 105%. Each sample was measured in duplicate and results are expressed relative to values of control group, which was set to 1. The purity of the PCR products was checked by analyzing the melting curves.

2.6. Thyroid gland histomorphology

Thyroids from WT and NBR-KO female mice were immersed in formalin's fixative solution, and embedded in paraffin. Sections were cut 5-μm thick and stained with hematoxylin–eosin. For histomorphometry, an image analysis system composed of a digital camera (Evolution, Media Cybernetics Inc., MD, USA) coupled to a light microscope (Eclipse 400, Nikon, NY, USA) was used. High quality images (2048 × 1536 pixels) were captured and analyzed using Pro Plus 4.5.1 software. Histomorphological parameters of the follicle cross-sections (follicle and colloid area) were quantified, according to Wade et al. (2002) [24], using 2 digital images of 04 slides/animal.

2.7. Statistical analyses

Data are expressed as mean ± SEM. A two-tailed unpaired *t*-test was employed for assessment of significance of all data except for serum TSH at TRH experiment, which was analyzed by one-way ANOVA followed by Student–Newman–Keuls multiple comparisons test (GraphPad Prism, GraphPad Software, Inc., CA, USA). Serum TSH was analyzed after logarithmic transformation. Differences were considered significant at $P < 0.05$.

3. Results

Neuromedin B receptor knockout (NBR-KO) female mice exhibited serum TSH slightly increased (18%, $P < 0.05$, Fig. 1A), together with a small, but significant decrease in intra-pituitary TSH content (24%, $P < 0.05$, Fig. 1B). Serum T4 of NBR-KO was similar to that of wild-type controls (Fig. 1D) while serum T3 was reduced (30%, $P < 0.001$, Fig. 1C) in NBR-KO mice.

As depicted in Fig. 2A, the rise in serum TSH in response to the administration of a single dose of TRH was of higher magnitude in NBR-KO female mice than that observed in WT mice (102% increase in relation to 46% in WT, $P < 0.01$ and $P < 0.05$, respectively). Concomitantly, the *Trhr* mRNA expression in the pituitary gland of NBR-KO mice

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