Disruption of neuromedin B receptor gene results in dysregulation of the pituitary–thyroid axis

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Abstract

The level of thyrotropin (TSH) secretion is determined by the balance of TSH-releasing hormone (TRH) and thyroid hormones. However, neuromedin B (NB), a bombesin-like peptide, highly concentrated in the pituitary, has been postulated to be a tonic inhibitor of TSH secretion. We studied the pituitary–thyroid axis in adult male mice lacking NB receptor (NBR-KO) and their wild-type (WT) littermates. At basal state, NBR-KO mice presented serum TSH slightly higher than WT (18%, $P < 0.05$), normal intra-pituitary TSH content, and no significant changes in α and β TSH mRNA levels. Serum thyroxine was normal but serum triiodothyronine (T3) was reduced by 24% ($P < 0.01$) in NBR-KO mice. Pituitaries of NBR-KO mice exhibited no alteration in prolactin mRNA expression but type I and II deiodinase mRNA levels were reduced by 53 and 42% respectively ($P < 0.05$), while TRH receptor mRNA levels were importantly increased (78%, $P < 0.05$). The TSH-releasing effect of TRH was significantly higher in NBR-KO than in WT mice (7.1- and 4.0-fold respectively), but, while WT mice presented a 27% increase in serum T3 ($P < 0.05$) after TRH, NBR-KO mice showed no change in serum T3 after TRH. NBR-KO mice did not respond to exogenous NB, while WT showed a 30% reduction in serum TSH. No compensatory changes in mRNA expression of NB or other bombesin-related peptides and receptors (gastrin-releasing peptide (GRP), GRP-receptor and bombesin receptor subtype-3) were found in the pituitary of NBR-KO mice. Therefore, the data suggest that NB receptor pathways are importantly involved in thyrotroph gene regulation and function, leading to a state where TSH release is facilitated especially in response to TRH, but probably with a less-bioactive TSH. Therefore, the study highlights the important role of NB as a physiological regulator of pituitary–thyroid axis function and gene expression.

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Introduction

The level of thyrotropin (TSH) secretion is determined mainly by the balance of the stimulatory effect of hypothalamic TSH-releasing hormone (TRH) and the strong inhibition exerted by thyroid hormones. However, other modulators of TSH secretion have been described, and among those, neuromedin B (NB). NB is a mammalian peptide structurally and functionally related to the amphibian peptide bombesin (Minamino *et al.* 1983). Bombesin-like peptides elicit several biological effects when injected into mammals, acting through three related types of receptors (Battey *et al.* 1991, Wada *et al.* 1991, Weber *et al.* 1998); one of these binds preferentially NB (Wada *et al.* 1991). NB receptor (NB-R) is present in the pituitary gland (Houben *et al.* 1993), although it has a wide distribution, predominantly in the central nervous system (Wada *et al.* 1991) and gastrointestinal cells (Von Schrenck *et al.* 1989). NB-R is a G-protein-coupled receptor (Wada *et al.* 1991) and NB binding results in activation

of multiple signaling pathways, depending on the cell studied (Wang *et al.* 1992, Lach *et al.* 1995, Moody *et al.* 1995). NB-R signal transduction pathways had not been studied at the pituitary level.

Bombesin-like peptides have been shown to decrease TSH release in rodents and humans (Pontiroli & Scarpignato 1986, Pazos-Moura *et al.* 2003); however, only for NB is there compelling evidence for a physiological role. NB reduced serum TSH when administered into normal and hypothyroid rats (Rettori *et al.* 1989, 1992) and the blockade of endogenous NB employing a highly specific antiserum against the peptide injected into the third cerebral ventricle induced a rise in serum TSH in both euthyroid and hyperthyroid rats (Rettori *et al.* 1992). NB acts directly at the pituitary, since isolated rat pituitaries released less TSH in the presence of the peptide (Rettori *et al.* 1989, Tajima *et al.* 1989). In addition, NB is abundantly expressed in human and rat pituitaries (Jones *et al.* 1992, Houben *et al.* 1993). In fact, in the rat, pituitary is the tissue that

exhibits the higher concentration of NB, predominantly found in thyrotrophs (Steel *et al.* 1988). The relative abundance of the peptide mRNA in pituitary indicates that NB is locally produced, and when its action was blocked by incubating normal or hyperthyroid glands with a specific antiserum, TSH release increased (Rettori *et al.* 1992), suggesting that pituitary NB exerts a tonic inhibitory effect on TSH secretion, acting as an autocrine/paracrine regulator. Conditions with increased TSH release, such as hypothyroidism and acute cold exposure, are associated with decreased pituitary NB expression (Jones *et al.* 1992, Ortiga-Carvalho *et al.* 2003), while suppressed TSH secretion induced by hyperthyroidism, and also observed in experimental fasting and diabetes mellitus, was associated with increased pituitary NB (Jones *et al.* 1992, Ortiga-Carvalho *et al.* 1997). Moreover, TRH acute administration rapidly decreased the pituitary content of the peptide and its mRNA (Ortiga-Carvalho *et al.* 2003), while acute injection of thyroid hormones increased NB expression (Ortiga-Carvalho *et al.* 1996).

Taken together, our previous work proposed that NB is a constitutive inhibitor of TSH release, acting mainly as an autocrine/paracrine factor, and that the control of its pituitary expression by TSH secretagogues or TSH release inhibitors may serve to modulate the final action of these hormones. However, the focus of those was on acute NB effects on TSH release, and here we studied in an animal model the long-term consequences of the disruption of the NB-R gene on pituitary–thyroid axis function and pituitary gene expression. NB-R knockout (NBR-KO) mice have been previously described (Ohki-Hamazaki *et al.* 1999). They presented a reduced response to the central hypothermic effect of exogenously administered NB (Ohki-Hamazaki *et al.* 1999), and behavioral changes, such as decreased marble-burying behavior and difficulties in learning and memory related to stress situations (Yamada *et al.* 2002a, 2003) as well as alterations in maternal behavior induced by stress (Yamada *et al.* 2002b). Here we report for the first time that NBR-KO mice present alterations in pituitary gene expression, with functional consequences for the thyrotroph–thyroid axis.

Materials and methods

Experimental animals

All experiments were conducted in adult male mice at 3–4 months old, homozygous for the deletion in NB-R (NBR-KO) and their wild-type (WT) littermates. Heterozygous NB-R +/– mice generated as described by Ohki-Hamazaki *et al.* (1999), were interbred to generate litters containing homozygous NB-R –/– and NB-R +/+ progeny. Mice studied were from

F16 to F23. To confirm the genotype of the mice, genomic DNA was obtained from tail samples and analyzed by PCR using specific primers as described previously (Ohki-Hamazaki *et al.* 1999).

Animals were maintained under a controlled temperature ($22 \pm 1^\circ\text{C}$) and a 12 h alternating darkness and artificial light cycle (lights on at 0700 h) and fed laboratory chow and water freely. All animals were killed in the morning between 0930 and 1130 h. In all experiments body weight (BW) of NBR-KO mice was similar to WT (25–28 g).

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.

Basal state of pituitary gene expression and pituitary–thyroid axis function

In order to evaluate the consequences of the deletion of NB-R on the pituitary–thyroid axis at steady state, NBR-KO and WT mice were taken from cages and immediately killed by asphyxia with CO_2 followed by decapitation. Trunk blood samples were centrifuged and serum was stored at -20°C for triiodothyronine (T3), thyroxine (T4) and TSH determinations by specific RIAs. To determine TSH content, each pituitary was homogenized in 200 μl phosphosaline buffer, pH 7.6, and supernatants stored at -20°C until assayed at a final dilution of 1:2000. Another set of pituitaries was immediately frozen in liquid nitrogen until processed to evaluate mRNA levels of α - and β -subunits of TSH, TRH-receptor (TRH-R), prolactin, 5'-deiodinase type I (DI) and 5'-deiodinase type II (DII) by fluorescence real-time RT-PCR.

TRH stimulation of TSH release

NBR-KO and WT mice received a single s.c. injection of saline (control group) or TRH (Sigma) at doses of 0.05 or 0.5 $\mu\text{g}/\text{kg}$ BW and were killed 30 min after injection. Mice were killed by asphyxia with CO_2 followed by decapitation and trunk blood was collected and serum stored at -20°C for TSH and T3 determinations.

NB effect on TSH release

NBR-KO and WT mice received a single s.c. injection of 50 μg (44.16 nmol) NB (Sigma) per animal, 30 min before killing, or the same volume of saline (control group). Mice were killed by asphyxia with CO_2 followed by decapitation and trunk blood was collected and serum stored at -20°C for TSH determinations.

Expression of bombesin-related peptides and receptors in pituitary gland

In order to investigate whether the lack of NB-R could induce compensatory changes in pituitary expression of bombesin-like peptides and their related receptors, pituitaries from NBR-KO and WT mice were immediately frozen in liquid nitrogen until processed to evaluate mRNA levels of NB, gastrin-releasing peptide (GRP), GRP-receptor (GRP-R) and bombesin receptor subtype-3 (BRS-3) by fluorescence real-time RT-PCR.

Hormone measurements

Serum TSH was measured in 100 µl serum samples in duplicate determinations by a specific mouse TSH RIA using a mouse TSH/LH reference preparation (AFP51718 MP), a mouse TSH antiserum (AFP98991), and rat TSH antigen for radioiodination (NIDDK-rTSH-I-9, AFP-11542B). 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, Torrance, CA, USA). Samples or standards were incubated in phosphosaline buffer, pH 7.6, containing 1% (w/v) BSA, for 21 h at room temperature with the antiserum at a final dilution of 1:150 000 and ¹²⁵I-TSH (10 000 c.p.m./tube). At the end of the incubation period, a second antibody at a final dilution of 1:100 was added (goat anti-guinea pig IgG; Antibodies Incorporated, Davis, CA, USA) and after 3 h at room temperature the tubes were centrifuged (4 °C) at 1600 *g* for 30 min and radioactivity was measured in the precipitate. TSH was labeled with ¹²⁵I in our laboratory by the chloramine-T method (Hunter & Greenwood 1962). Minimum assay detection was 30 ng/ml. The intra-assay variations were <7%. All samples from each experiment were measured within the same assay.

Serum total T3 and T4 concentrations were measured by a coated tube RIA from ICN Pharmaceuticals (Costa Mesa, CA, USA) using 100 and 25 µl of serum respectively. The sensitivity of the assay is 0.76 µg/dl for T4 and 6.7 ng/dl for T3. The intra-assay variation was 4.7% for T4 and 2.6% for T3.

Fluorescence real-time RT-PCR

Analysis of TSH α- and β-subunits, TRH-R, prolactin, DI and DII mRNAs

Pituitary total RNA was purified using TRIZOL (Invitrogen, Carlsbad, CA, USA) and single-stranded cDNA was synthesized from 2 µg pituitary total RNA using Superscript II (Invitrogen). Real-time RT-PCR analyses were performed in fluorescence temperature cyclers (MyiQ, single color Real-time PCR Detection System; Bio-Rad), according to recommendations of the

manufacturer. Briefly, after initial denaturation at 50 °C for 2 min and 95 °C for 10 min, reactions were cycled 40 times using the following conditions: 95 °C for 15s, 60 °C for 30s and 72 °C for 30s. SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. The sequences of the forward and reverse primers were respectively: 5'-GTG TAT GGG CTG TTG CTT CTC C-3' and 5'-GCA CTC CGT ATG ATT CTC CAC TCT G-3' for TSH α-subunit; 5'-TCT CGC CGT CCT CCT CTC CGT GCT T-3' and 5'-AGT TGG TTC TGA CAG CCT CGT G-3' for TSH β-subunit; 5'-AGC CAG CTC TAC GCG GC-3' and 5'-CCC TTG TAG CAG ATC CTG CC-3' for DI; 5'-CAT TCT GCT CAA GCA CGT GGC-3' and 5'-GAC GTG CAC CAC ACT GGA ATT-3' for DII; 5'-GCA GTC ACC ATG ACC ATG AA-3' and 5'-AGA TTG GCA GAG GCT GAA CA-3' for prolactin; 5'-CTG GAT CTC AAC ATC AGC ACC TAC-3' and 5'-GGA AGA TAC TGT GCT GTT GAA GCA-3' for TRH-R; 5'-CGG CTA CCA CAT CCA AGG AA-3' and 5'-GCT GGA ATT ACC GCG GCT-3' for 18S; 5'-GCA AGG ATG GCA AGG ATT GA-3' and 5'-AGC AAT TCT GCC TGG ATA GC-3' for cyclophilin.

Analysis of bombesin-related peptides and receptor mRNAs

Pituitary total RNA was purified using Rneasy kit (Qiagen) and the single-stranded cDNA was synthesized from 1 µg total RNA, using a high-capacity cDNA archive kit (Applied Biosystems). Real-time RT-PCR analyses were performed in an Applied Biosystems 7700 Sequence Detection System, according to recommendations of the manufacturer. PCR conditions were: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. The sequences of the forward and reverse primers were respectively: 5'-AAT TGG CTG CAA ACT GAT CCC-3' and 5'-TGG CCG TAC AAT GGC TTT GT-3' for GRP-R; 5'-AAA GCA CCC TGA ACA TAC CGA-3' and 5'-CAA CCA GCA GAG TGC GAA CA-3' for BRS-3; 5'-ATG AAT CCC CGT CCC TGT ATG-3' and 5'-AGG AGG TCC AGC AAA TCC CTT-3' for GRP; 5'-CGG TCA CTT CAT GGG CAA G-3' and 5'-GAG CTT TCT TTC GCA GGA GGA-3' for NB. β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were as described previously (Aoki *et al.* 2002).

The threshold cycle of each gene was determined as the PCR cycle at which an increase in fluorescence was observed above the baseline signal in an amplification plot. The normalized expression level of target (ΔCt) was

calculated as the difference in threshold cycles for target and reference (18S, cyclophilin, β -actin or GAPDH). Relative mRNA levels were determined by comparing the PCR cycle threshold (Ct) between groups. The purity of the PCR products was checked by analyzing the melting curves. Each sample was measured in duplicate and each experiment was repeated at least three times.

Statistical analyses

Data are expressed as means $\pm$ S.E.M. A two-tailed unpaired *t*-test was employed for assessment of significance of all data except for serum TSH and T3 in the TRH experiment, which was analyzed by one-way ANOVA followed by the Student–Newman–Keuls multiple comparisons test (GraphPad Prism, GraphPad Software, Inc., San Diego, CA, USA). Serum TSH was analyzed only after logarithmic transformation. Differences were considered to be significant at $P \leq 0.05$.

Results

Basal state of pituitary gene expression and pituitary–thyroid axis function

As depicted in Fig. 1A and B, TSH β - and α -subunit mRNA levels of NBR-KO mice were not significantly different from WT, although the β -subunit mRNA showed a trend toward reduction. The intra-pituitary TSH pool of NBR-KO mice was normal, since mice of both genotypes had similar pituitary TSH content (Fig. 1C). Serum TSH of NBR-KO mice was slightly higher than that of WT (approximately 18%, Fig. 1D, $P < 0.05$). There was no difference between NBR-KO mice and WT littermates in serum T4 (Fig. 1E). However, serum T3 was reduced by 24% in NBR-KO mice (Fig. 1E, $P < 0.01$).

As shown in Fig. 2A, NBR-KO mice exhibited a 78% increase in the expression of TRH-R mRNA ($P < 0.05$). Prolactin mRNA was similar between genotypes (Fig. 2B). However, both DI and DII mRNA levels were decreased in NBR-KO mice, by 53 and 42% respectively (Fig. 2C and D, $P < 0.05$).

TRH stimulation of TSH release

NBR-KO mice showed a markedly enhanced TSH response to acute administration of TRH, as shown in Fig. 3A. The lower TRH dose (0.05 μ g/kg BW) induced a 4.3-fold increase in serum TSH of NBR-KO mice ($P < 0.001$ vs saline control group), while the increment was of 2.9-fold in WT ($P < 0.001$ vs. saline control group). The higher TRH dose (0.5 μ g/kg BW) resulted in a more pronounced difference between the response

Figure 1 Pituitary–thyroid axis in NBR-KO mice (NBR-KO) and wild-type littermates (WT) at basal state. (A, B) Pituitary TSH β - and α -subunit mRNA analyzed by fluorescence real-time RT-PCR. Data were normalized for each mRNA level relative to WT animals ($n=8$ –12 per group). (C) Pituitary TSH content ($n=7$ –10 per group). (D) Serum TSH concentration ($n=28$ –34 per group). (E) Serum T4 and T3 concentrations ($n=16$ –22 per group). * $P < 0.05$ vs WT group. Data are means $\pm$ S.E.M.

of NBR-KO and WT mice (7.1- and 4.0-fold increase in serum TSH respectively, $P < 0.001$ vs saline control group). At both TRH doses, serum TSH concentrations reached in NBR-KO mice were significantly higher when compared with WT groups ($P < 0.01$ at lower dose; $P < 0.001$ at higher dose).

As shown in Fig. 3B, WT mice exhibited a 27% increase in serum T3 after TRH administration ($P < 0.05$). However, despite higher serum TSH, NBR-KO mice showed no increment in serum T3 after TRH. Therefore, the ratio of serum TSH/T3 after TRH was 8.6 ± 0.53 in NBR-KO mice, while it was 4.5 ± 0.48 in WT mice (Fig. 3C, $P < 0.001$).

Effect of NB administration on serum TSH

WT mice, 30 min after receiving a single s.c. injection of NB, showed a 30% lower serum TSH as compared with the saline-injected WT group ($P < 0.001$). This percentage of decrease in serum TSH induced by NB is similar

Figure 2 Pituitary gene expression in NB-R knockout mice (NBR-KO) and wild-type littermates (WT) at the basal state. (A) TRH-R. (B) Prolactin (PRL). (C, D) Pituitary 5'-deiodinase type I (DI) and type II (DII). mRNA levels were analyzed by fluorescence real-time RT-PCR. Data were normalized for each mRNA level relative to WT animals. $n=8-12$ per group. Data are means $\pm$ S.E.M. * $P<0.05$ and ** $P<0.001$ vs WT group.

to that previously described in rats (Rettori *et al.* 1989). Conversely, NBR-KO mice showed no significant response to the peptide injection (Fig. 4).

These results showed that in mice lacking NB-R, serum TSH concentration was not significantly affected by NB injection.

Expression of bombesin-related peptides and receptors in pituitary gland

NBR-KO mice showed no alterations in pituitary mRNA levels of NB and GRP as well as GRP-R and BRS-3, as illustrated in Fig. 5. Δ Ct values of each gene were similar in mice lacking NB-R and WT mice (GRP-R: WT = 17.8 ± 0.45 , NBR-KO = 18.1 ± 0.24 ; BRS-3: WT = 16.6 ± 0.28 , NBR-KO = 17.7 ± 0.52 ; GRP: WT = 13.6 ± 0.38 , NBR-KO = 13.1 ± 0.35 ; NB: WT = 6.3 ± 0.12 , NBR-KO = 6.2 ± 0.12). Therefore, deletion of NB-R did not elicit compensatory changes in the expression levels of bombesin-related receptors and peptides, including NB, the receptor's preferential ligand.

Discussion

Previous studies have proposed a physiological role for NB as a negative modulator of TSH release, acting mainly as an autocrine/paracrine inhibitor, whose pituitary expression is under the control of TSH

Figure 3 Pituitary-thyroid axis response to TRH acute administration in NB-R knockout mice (NBR-KO) and wild-type littermates (WT). Mice received a single s.c. injection of TRH (0.05 or 0.5 μ g/kg BW) 30 min before killing. One group of each genotype remained as control (C) and received saline. Values are means $\pm$ S.E.M. of the increase in serum TSH (A), serum T3 (B) and TSH/T3 serum ratio (C) induced by TRH relative to control groups of the corresponding genotype. $n=8-10$ per group. * $P<0.001$ vs control group, ** $P<0.05$ vs control group.

secretagogues and TSH-releasing inhibitors. However, those studies focused on acute effects of the peptide or the antiserum, and therefore allowed drawing of conclusions related to the release of stored TSH, leading to the suggestion that NB acts to inhibit the TSH secretory mechanism. Here, studying mice lacking NB-R we were able to demonstrate the long-term role of NB in the regulation of pituitary-thyroid axis hormonal secretion and gene regulation.

At basal state, NBR-KO mice showed a slight higher serum TSH, without significant changes in TSH β -subunit mRNA levels or in intracellular TSH storage. More importantly, NBR-KO mice showed an exaggerated TSH response to acute administration of TRH. Together, the data suggest that NBR-KO mice have a higher rate of TSH release, which is consistent with the lack of NB action as an inhibitor of the TSH secretory mechanism. The facilitated TSH-releasing action of TRH in NBR-KO mice is also justified by the higher expression of TRH-R mRNA. This is in agreement with

Figure 4 Serum TSH decrease in response to a single injection of neuromedin B (NB) in NB-R knockout mice (NBR-KO) and wild-type littermates (WT). Mice received a single s.c. injection of NB (50 µg per animal) or saline 30 min before killing. Values represent the percentage decrease relative to saline-treated groups of the corresponding genotype. $n=8-9$ per group. * $P<0.001$ vs WT NB-treated group. Data are means $\pm$ S.E.M.

previous reports showing the ability of NB to reduce TRH-induced TSH release (Pazos-Moura *et al.* 1996) and may justify why TRH has a rapid and profound inhibitory effect on pituitary NB expression, as demonstrated previously in rats (Ortiga-Carvalho *et al.*

Figure 5 Pituitary mRNA expression of bombesin-related receptors: gastrin-releasing peptide receptor (GRP-R), bombesin receptor subtype-3 (BRS-3), and peptides: GRP and neuromedin B (NB) in NB-R knockout mice (KO) and wild-type littermates (WT) at basal state. The mRNA expression was evaluated by fluorescence real-time RT-PCR. The figure illustrates a representative agarose gel visualization obtained with reaction samples collected when their amplification curves were linear. MW=molecular weight (pUC19 DNA/MspI). The range of standard was 34–501 bp.

2003). Therefore, the data suggest that NB has an important antagonistic effect on TRH action on the release of stored TSH, and at least one possible mechanism is by regulating, directly or indirectly, TRH-R mRNA expression. However, there was not an overall increment in TRH action in thyrotrophs, since TSH α - and β -subunit mRNA levels were not increased as expected, since TRH is a well-known stimulator of expression of both mRNAs. Therefore, NBR-KO mice present other alterations in thyrotroph function.

Although TSH secretion seems to be facilitated in NBR-KO mice, it is likely that the hormone has decreased biological activity, since serum T3 was reduced, and more importantly, NBR-KO did not show the expected rise in serum T3 after TRH, even though their TSH release was 3.4-fold higher than that of WT. This *in vivo* indirect evidence should be confirmed by *in vitro* bioassay, although they are highly suggestive of a less-bioactive TSH. The serum T3 response to TRH was reduced in some patients with central hypothyroidism, whose basal TSH was slightly elevated and less bioactive (Faglia *et al.* 1983). Mice lacking TRH had slightly higher serum TSH, low serum T3 and T4, and showed diminished T3 response to endogenous TSH after TRH stimulation, which was considered as evidence of reduction in TSH bioactivity (Yamada *et al.* 1997). This is in agreement with the role of TRH in regulation of TSH biological activity (Persani 1998). Low serum T3, in the presence of normal T4 and normal or slightly reduced serum TSH, is present in initial stages of starvation or mild illnesses, the so-called non-thyroidal illness syndrome (De Groot 1999), which has a multifactorial cause, but exhibit in common the suppression of TRH gene expression (Lechan & Fekete 2004). Although there is no previous study on NB effects on TRH, NB-R had been localized in the paraventricular nucleus (Wada *et al.* 1992), and it is possible that NBR-KO mice have some degree of TRH deficiency, although mild, since serum T4 is normal. However, another possibility is that NB itself may be a direct pituitary regulator of TSH bioactivity, which remains to be tested in specific *in vitro* studies.

Another original finding of this study is the previously unsuspected involvement of NB in the regulation of DI and DII mRNA expression in the pituitary gland. The mechanism for reduction of both deiodinase mRNAs as well as the specific pituitary cells involved cannot be elucidated by the present study, and these alterations may represent direct or indirect effects of NB. T3 is a major regulator of deiodinase mRNAs, and up-regulates DI and down-regulates DII mRNA (Maia *et al.* 1995, Kim *et al.* 1998). Therefore, the lower serum T3 of NBR-KO mice could be associated with reduced pituitary DI mRNA, although it is uncertain since T4 was normal. However, the decrease in DII mRNA must have another cause, not related to T3. A previous report

has shown that TRH, dexamethasone and 8-Br-cAMP cause modest increases in DII mRNA levels in pituitary tumor cells (Kim *et al.* 1998). However, it remains to be studied if NBR-KO mice have any alterations in those factors or if NB is a direct regulator of mRNA expression of these enzymes. Independently of the underlying mechanisms, the reduction of pituitary DI and especially DII mRNA, leading to lower pituitary T3 generation, can potentially contribute to increased TSH release in response to TRH, since T3 is able to down-regulate TRH-R (Schomburg & Bauer 1995). The fact that pituitary deiodinase mRNAs are reduced raises the question of whether these enzymes are changed in other tissues and if this would contribute to reduced serum T3. However, thyroid axis abnormalities of NBR-KO mice strongly suggest that their primary defect is at the central level.

NBR-KO mice lost the ability to respond to NB by decreasing serum TSH, which indicates that the acute action of NB on TSH release is being mediated via NB-preferring receptors. Therefore, it is reasonable to assume that the phenotype observed in NBR-KO mice resulted from the lack of action of endogenous NB. This is further supported by the absence of a compensatory increase in expression of pituitary NB or other bombesin-like receptors, which have a much lower affinity for NB, and therefore, could not mediate NB actions at normal levels of the peptide.

In conclusion, this study reveals that NB-R pathways are involved, directly or indirectly, in regulation of pituitary genes, as the lack of NB-R resulted in increased TRH-R mRNA and decreased DI and DII mRNAs in association with increased TSH release, especially in response to TRH. Even so, serum T3 at the basal state and after TRH stimulation was decreased, suggesting a reduction in TSH biological activity. Therefore, the study highlights the important role of NB not only as an inhibitor of TSH release, but also in the regulation of pituitary gene expression, with functional consequences for thyrotroph–thyroid axis function.

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