Molecular and Cellular Endocrinology 381 (2013) 115–123

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Retinoic X receptor subtypes exert differential effects on the regulation of *Trh* transcription

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ARTICLE INFO

Article history:
Received 4 October 2012
Received in revised form 19 July 2013
Accepted 19 July 2013
Available online 26 July 2013

Keywords:
Paraventricular nucleus of hypothalamus (PVN)
Thyrotropin-releasing hormone (TRH)
Retinoid X receptor (RXR)
Thyroid hormone (T_3)
Negative regulation
Repression

ABSTRACT

How Retinoid X receptors (RXR) and thyroid hormone receptors (TR) interact on negative TREs and whether RXR subtype specificity is determinant in such regulations is unknown. In a set of functional studies, we analyzed RXR subtype effects in T_3-dependent repression of hypothalamic thyrotropin-releasing hormone (*Trh*). Two-hybrid screening of a hypothalamic paraventricular nucleus cDNA bank revealed specific, T_3-dependent interaction of TRs with RXRβ. *In vivo* chromatin immuno-precipitation showed recruitment of RXRs to the TRE-site 4 region of the *Trh* promoter in the absence of T_3. *In vivo* over-expression of RXRα in the mouse hypothalamus heightened T_3-independent *Trh* transcription, whereas RXRβ overexpression abrogated this activity. Loss of function of RXRα and β by shRNAs induced inverse regulations. Thus, RXRα and RXRβ display specific roles in modulating T_3-dependent regulation of *Trh*. These results provide insight into the actions of these different TR heterodimerization partners within the context of a negatively regulated gene.

1. Introduction

Thyroid hormones (THs), in particular the biologically active form tri-iodothyronine (T_3), exert pleiotropic effects during vertebrate development and on homeostasis in the adult. The hypothalamic–pituitary–thyroid (HPT) axis drives the production of THs. In turn, T_3 controls its own production *via* negative feedback loops acting at the levels of thyroid-stimulating hormone (TSH) production in the pituitary and thyrotropin-releasing hormone (TRH) production in the hypothalamic paraventricular nucleus (PVN) (Chiamolera and Wondisford, 2009). Central regulation of the thyroid axis through the TRH neurons in the PVN is required for maintenance of homeostatic TH levels. TRH neurons in the PVN are regulated by multiple pathways that determine TRH production set point (Hollenberg, 2008).

T_3 represses TRH production at the transcriptional level through the thyroid hormone receptors (TRs) (Lezoualc'h et al., 1992; Abel et al., 2001; Guissouma et al., 1998). TRs belong to the steroid/thyroid nuclear receptor super-family and are both ligand-dependent and -independent transcription factors. Vertebrate TR isoforms derive from two genes, *c-erbAα* (NR1A1) (for TRα1 and TRα2 isoforms) and *c-erbAβ* (NR1A2) (for TRβ1and β2 isoforms) (review in Flamant et al., 2007). Functional TRs, TRα1, TRβ1 and TRβ2 co-localize in the PVN (Clerget-Froidevaux et al., 2004; Lechan and Fekete, 2004; Lechan et al., 1994). TRs play isoform-specific roles in T_3-dependent repression of *Trh* gene, each TR isoform exerting specific transcriptional activities on the *Trh* promoter (Dupre et al., 2004; Decherf et al., 2010), differences which are mainly due to variations in their N-terminal sequence (Guissouma et al., 2002).

TRs activate or repress target gene transcription as monomers, homodimers or heterodimers with retinoid X receptors (RXRs) *via* binding to T_3-response elements (TREs) (for review, see Cheng et al., 2010 and Yen et al., 2006), TREs are qualified as positive TREs (pTRE) or negative TREs (nTRE). Three separate nTRE half-sites (site 4 from −55 to −60 base pairs (bp); site 5 from +14 to +19 bp; and site 6 from +37 to +42 bp) have been identified in the *Trh* proximal promoter, which act in combination permitting negative regulation (Hollenberg et al., 1995; Satoh et al., 1996). Among these nTREs, the *Trh* promoter site 4 appears to be the most important for TH regulation, as it transduces both T_3-independent transcriptional activation and T_3-dependent repression (Guissouma et al., 2002; Hollenberg et al., 1995; Satoh et al., 1996). Moreover, unlike the other sites, the *Trh* site 4 preferentially binds TR/RXR heterodimers (Hollenberg et al., 1995).

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http://dx.doi.org/10.1016/j.mce.2013.07.016

TRs preferentially bind DNA as heterodimers with retinoid X receptors (RXRs) (for review, see Yen, 2001). Three mammalian RXR subtypes exist each encoded by separate genes: RXRα, RXRβ and RXRγ (Mangelsdorf et al., 1992; Mark et al., 2006). RXRs are involved in several metabolic and regulatory pathways because of their heterodimerization roles and because they can modulate transcription through ligand activation. Therefore, RXR plays a dual role in NR signaling. On the one hand, subtypes can function as a homodimer and regulate transcription in response to RXR ligands, or rexinoids. Originally it was thought that an endogenous RXR ligand might be 9-cis retinoic acid (9cRA) (Ahuja et al., 2003; Heyman et al., 1992; Mak et al., 1994). However, to date, 9cRA has only been identified *in vivo* in pancreas (reviewed in Kane, 2012). In mouse brain, polyunsaturated fatty acid and docosahexaenoic acid have been secondarily identified as natural RXR ligands (Lengqvist et al., 2004). Usually, heterodimerization strongly increases TR binding to the TRE and potentiates ligand-dependent regulations, thus amplifying the transcriptional activity (Velasco et al., 2007; Yu et al., 1991). If the role of RXR/TR heterodimers in ligand-dependent gene activation has been quite comprehensively studied, and a model proposed whereby RXR increases stimulatory T_3 responses (Castillo et al., 2004; Denver and Williamson, 2009; Leng et al., 1993), understanding RXRs in T_3-dependent repression has proved more complex.

RXR/TR heterodimers play roles in both basal transactivation and T_3 suppression of negatively regulated genes. In particular, RXRs increase the dominant negative effect of some mutant TRs on specific nTREs (Takeda et al., 1997). However, the endogenous function of RXRs in hypothalamic *Trh* regulation has not been investigated, least of all in the more relevant *in vivo* context. RXR ligands are without effect on *Trh* expression or preproTRH levels (Sharma et al., 2006; Janssen et al., 2011), whilst *in vitro* results showed RXR subtypes to improve T_3 dependent *Trh* regulation, independently of their DNA-binding properties (Hollenberg et al.,1995).

To help resolve these contradictory results we examined the function of RXRs in hypothalamic *Trh* regulation in a more physiological set-up. Through a set of functional *in vivo* studies targeting the mouse hypothalamus, we examined the effects of each RXR subtype on the T_3-dependent negative transcriptional regulation of the *Trh* gene. First, double-hybrid screening of a hypothalamic paraventricular (PVN) cDNA bank revealed a specific, T_3-dependent interaction of TRβ with the RXRβ subtype. Second, *in vivo* chromatin immuno-precipitation (ChIP) on PVN extracts showed RXRs associated with the region around the nTRE site 4 in the *Trh* promoter. Specific roles of each RXR (RXRα, RXRβ and RXRγ) were then analyzed using a polyethylenimine (PEI) based *in vivo* gene transfer (iGT) approach. PEI is one of the most exploited non-viral gene transfer agents (Boussif et al., 1995) and can be used in the same manner as its viral counterparts (e.g. adeno-associated virus or lentivirus). Previous results using this technique established that it provides region- and neuron-specific reporter gene expression and regulation (Decherf et al., 2010; Guissouma et al., 2000; Hassani et al., 2007; Lemkine et al., 2002). iGT was used in mouse hypothalamus to overexpress RXRs and to introduce small hairpin RNA (shRNA) against RXR subtypes. Our results reveal distinct roles for RXRα and RXRβ in modulating *Trh* negative regulation. This is particularly interesting in the under-investigated context of gene repression mechanisms, removing all doubt about the implication of RXRs as TR partners even in negative regulations.

2. Materials and methods

2.1. Animals

Swiss wild-type mice were purchased from Janvier (Le Genest St Isle, France). All aspects of animal care and experimentation were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Animal Protection and Health, Veterinary Services Direction, Paris, France.

Hypothyroidism: 8-week-old male mice were given iodine-deficient food containing 0.15% 6-*n*-propyl-2-thiouracil (PTU) (Harlan, Gannat, France) and drinking water added with 0.5 g/L PTU (Sigma–Aldrich, St. Quentin Fallavier, France) for 13 days until sacrifice. To induce fetal and neonatal hypothyroidism, dams were given the iodine-deficient and PTU-diet from day 14 of gestation through lactation. *Hyperthyroidism*: adult mice were treated with 1.2 μg/ml of T_4 in drinking water 13 days before sacrifice.

2.2. Plasmids

The TRH-luciferase (TRH-luc) plasmid, containing the *Firefly luciferase* coding sequence cloned downstream the 5'flanking sequence of the *Trh* promoter, was a gift from W. Balkan (University of Miami School of Medicine, Miami, FL). RXRα, RXRβ, RXRγ expression plasmids (pSG5 backbone) were a gift from Patrick Kastner (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, CU de Strasbourg, France). TRβ1 plasmid was described previously (Dupre et al., 2004).

2.2.1. Design and cloning of the shRNA

The two sets of shRNA against RXR subtypes were designed as previously described (Hassani et al., 2007). Briefly, the double stranded shRNA coding sequence itself contains sense and antisense siRNA sequence against the target gene, separated by a loop sequence. The shRNA sequence is subcloned between XhoI and NotI sites in the pCMV-H1 backbone, downstream the hybrid promoter CMV-H1 following the Quick Ligation kit protocol. Plasmids coding for shRNA against RXRα and RXRβ were obtained by designing custom double stranded shRNA coding sequence with already published siRNA against each target gene (Diallo et al., 2007), whereas the two shRNA against RXRγ were designed with siRNA sequences available on MWG Biotech siRNA database. To knock-down endogenous RXRα, RXRβ and RXRγ, two shRNA coding plasmids were designed against each receptor, providing two shRNA sets against RXR subtypes. Each shRNA coding sequence was purchased from Eurogentec (Angers, France). Specific knock-down of endogenous RXRγ by the shRXRγ was assessed by qPCR on newborn mice hypothalami (data not shown). The control plasmids CMV-f.luc and CMV-r.luc, with the cytomegalovirus (CMV) promoter cloned upstream either the *Firefly* or the *Renilla luciferase* coding sequence, were purchased from Promega (Charbonnières-les-Bains, France).

All plasmid DNA preparations were prepared using commercial columns (Qiagen, Chatsworth, CA) and resuspended in 10 mM Tris–HCl, 1 mM EDTA, pH 8, sequenced (MWG Biotech, Roissy CDG, France) except for TRH-Luc which was routinely prepared by Plasmid Factory (Bielefed, Germany), and stored as aliquots at −20 °C.

2.3. Two-hybrid experiments

The two-hybrid experiments were done using the MATCHMAKER Library Construction and Screening Kit (Clontech). Procedures have been described previously (Froidevaux et al., 2006).

2.4. In vivo transfection and luciferase assays

DNA/PEI (polyethylenimine) complexes and *in vivo* gene transfer (*i*GT) were adapted from (Guissouma et al., 1998). Briefly, pups were anaesthetized by hypothermia on ice and transfected on postnatal day one. A glass micropipette was lowered 2.5 mm through the skull, 0.5 mm posterior to bregma, 0.1 mm lateral from