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## Accepted Manuscript



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**Peroxisome Proliferator-activated Receptor- $\gamma$  (PPAR $\gamma$ ) modulates  
hypothalamic *Trh* regulation *in vivo***

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**ABSTRACT**

Thyroid hormone receptor (TR) and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) co-regulate numerous peripheral metabolic responses. To examine potential crosstalk between PPAR $\gamma$  and TR $\beta$  in the hypothalamus, thyrotropin-releasing hormone (*Trh*) regulation in the newborn mouse hypothalamus was followed. QPCR showed PPAR $\gamma$  to be expressed in the hypothalamus at this developmental stage. Intra-cerebral injection of PPAR $\gamma$  agonists modified transcription from a *Trh-luc* construct introduced into the hypothalamus and increased serum thyroxine levels. Furthermore, shRNA-based *in vivo* PPAR $\gamma$  knockdown amplified T<sub>3</sub>-independent transcription and PPAR $\gamma$  overexpression dose-dependently abrogated T<sub>3</sub>-dependent *Trh* repression. Over-expression of retinoid X receptor- $\alpha$  (RXR $\alpha$ ), the common heterodimeric partner of PPAR $\gamma$  and TR $\beta$ , rescued PPAR $\gamma$  abrogation of T<sub>3</sub>-dependent repression. Thus, competition for RXR could represent one mechanism underlying this hypothalamic crosstalk between PPAR $\gamma$  and TR $\beta$ . These demonstrations of PPAR $\gamma$  effects on hypothalamic *Trh* transcription *in vivo* consolidate the role of the TRH neuron as a central integrator of energy homeostasis.

**Key words:** peroxisome proliferator-activated receptor type  $\gamma$  (PPAR $\gamma$ ), thyroid hormone receptors (TRs), mouse hypothalamus, thyrotropin-releasing hormone (TRH), transcriptional regulation, *in vivo* gene transfer (iGT).

## INTRODUCTION

Maintenance of energy homeostasis relies on tight regulation linking peripheral metabolism to central controls in the brain through complex cellular and molecular mechanisms (Havel, 2001). Hypothalamic control of energy balance integrates body metabolic signals, allowing adaptation to environmental challenges (Gao and Horvath, 2008). In the hypothalamus the thyrotropin-releasing hormone (TRH) neurons are crucial actors in these energy regulating, homeostatic processes (Herwig et al., 2008; Hollenberg, 2008; Lechan and Fekete, 2006). The cellular integration of various metabolic signals relies on stringent control of transcriptional responses, mainly through the coordinated action of transcription factors and associated co-regulators (Lonard and O'Malley, 2006). Several members of the nuclear receptor (NR) superfamily play critical roles in central and peripheral energy balance control. Their actions as metabolic gene-expression regulators has become a major focus in the development of preventive and therapeutic strategies (Francis et al., 2003).

In an era marked by the increasing prevalence of obesity, diabetes and cardiovascular diseases (Lehrke and Lazar, 2005), the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), a member of the NR superfamily, has emerged as a major transcriptional regulator of metabolism. PPAR $\gamma$  is highly expressed in adipose tissue where it regulates adipocyte differentiation and plays key roles in adipose tissue function (Sharma and Staels, 2007). PPAR $\gamma$  is also involved in glucose and lipid metabolism and energy balance in many other peripheral tissues (Cho et al., 2008).

All the PPAR subtypes ( $\alpha$ ,  $\gamma$ , and  $\delta$ ) are expressed in the central nervous system (CNS) (Wada et al., 2006). However, of the three, it is PPAR $\gamma$  signaling pathway that is best characterized in the brain. Indeed, PPAR $\gamma$  and its cognate agonists appears to be an attractive therapeutic target for various disorders of the central nervous system (Heneka and Landreth, 2007). PPAR $\gamma$  agonists have shown promising results in animal models of Alzheimer's disease, stroke, multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis and pituitary adenoma (Sundararajan et al., 2006). Clinical trials of PPAR $\gamma$  agonists are currently underway in several CNS diseases (Jiang et al., 2008; Schintu et al., 2009). More importantly, it was confirmed that the PPAR $\gamma$  isoform is expressed in key neuronal subsets regulating energy homeostasis (Moreno et al., 2004), particularly in the PVN neurons of the hypothalamus (Sarruf et al., 2009). However, there is as yet no indication of a role of PPAR $\gamma$  signaling in central metabolic regulations.

The thyroid hormone (TH) signaling pathway is also a major regulator of metabolism. Early "loss- and gain-of-function" experiments demonstrated the importance of TH in human metabolism and disease (Hollenberg and Forrest, 2008). The active form of TH is tri-iodothyronine (T<sub>3</sub>), mainly produced in peripheral tissues by deiodination of the precursor thyroxine (T<sub>4</sub>). T<sub>3</sub> regulates both energy expenditure in the peripheral tissues (Kim, 2008) and energy balance in the brain (Herwig, 2008). Regulation of gene expression by T<sub>3</sub> is mediated *via* thyroid hormone receptors (TRs) (Wu et al., 2001). Three major thyroid hormone receptor isoforms (TR $\alpha$ 1, TR $\beta$ 1, and TR $\beta$ 2) bind T<sub>3</sub>. TR $\alpha$  distribution is fairly ubiquitous, whereas that of TR $\beta$  is more restricted and tissue-specific (Bernal, 2007). Specifically, the TR $\beta$  isoforms play important roles in the central control of the hypothalamic-pituitary-thyroid (HPT) axis, by regulating hypothalamic *Trh* gene expression and production in a classic negative feedback fashion (Guissouma et al., 1998). Both TR $\beta$  isoforms bind to the *Trh* promoter at an unusual TH responsive element (TRE) as a heterodimer with the 9-cis retinoic acid X receptor, RXR (Shibusawa et al., 2003).

Previous *in vitro* studies have underlined interactions between PPAR signaling and other pathways also involved in metabolic regulation, particularly TR-dependent pathways (Hyyti and Portman, 2006). Crosstalk between TR and PPAR signals could involve competition for their common heterodimeric-partner, the 9-cis-retinoic acid X receptor (RXR) (Juge-Aubry et al., 1995). Indeed, both PPAR $\gamma$  and TR heterodimerize with RXR on their respective responsive elements (RE) in target gene promoters. TR and PPAR can also compete for DNA-binding on their respective hormone-responsive elements as TR has been shown to bind PPAR $\gamma$  responsive elements (PPRE) (Araki et al., 2005; Miyamoto et al., 1997; Ying et al., 2003). However, despite increasing data showing interactions between PPAR and TR in peripheral tissues, their potential interactions in the hypothalamus remain to be investigated. This absence of data is all the more surprising given that this brain region is the central integrator of whole body energy homeostasis (Hollenberg, 2008).

One previous report has shown a peripheral effect of PPAR activation by a seven days treatment of rosiglitazone on hypothalamic-pituitary-thyroid axis concentrated on the PPAR $\gamma$  isoform, demonstrating a significant effect on thyroid hormone release (Festuccia et al, 2008). We thus investigated whether the hypothalamic PPAR $\gamma$  signaling pathway affected hypothalamic TH-dependent gene regulations. To this end, we focused on TH-dependent and independent regulations of *Trh* transcription, as *Trh* plays a key role in central metabolic homeostasis. The hypothesis tested was that hypothalamic PPAR $\gamma$  could modify TR $\beta$ -dependent regulation of the *Trh* promoter. To this end, a well documented non-viral reporter gene approach was applied to the newborn mouse model (Boussif et al., 1995; Guissouma et al., 1998). This polyethylenimine based method allows for both overexpression and gene silencing approaches to be carried out in an *in vivo* context, in defined brain areas. The regulations documented in this *in vivo*, tissue specific, context therefore reflect integrated, physiological conditions (Dupre et al., 2004; Guissouma et al., 1998).

The results reported here show first, that pharmacological activation of central, endogenous PPAR $\gamma$  signaling leads to increased T<sub>3</sub>-independent *Trh* transcription and increased circulating T<sub>4</sub> levels. In contrast, inhibition of PPAR $\gamma$  activity did not affect *Trh* transcriptional activity in the absence of T<sub>3</sub>, but interfered with T<sub>3</sub>-dependent *Trh* repression. Modifying local PPAR $\gamma$  protein levels by overexpression or knock-down also modulated transcription from the *Trh* promoter. Use of small hairpin RNA (shRNA) against PPAR $\gamma$  increased T<sub>3</sub>-independent *Trh* transcription, whilst PPAR $\gamma$  overexpression abrogated T<sub>3</sub>-dependent *Trh* repression. The effect of PPAR $\gamma$  overexpression was reversed by co-expression of either TR $\beta$ 1 or RXR $\alpha$  suggesting that PPAR $\gamma$  titrates the TR and PPAR $\gamma$  common heterodimeric partner RXR $\alpha$ .

Taken together the data demonstrate potential crosstalk between PPAR $\gamma$  and TR $\beta$ 1 signaling pathways at the level of the TRH neuron. Hypothalamic PPAR $\gamma$  signaling might thus play a critical but as yet unrecognized role in central control of metabolism through *Trh* transcription.

## MATERIAL AND METHODS

### Animals

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. Swiss wild-type mice were purchased from Janvier (Le Genest St Isle, France), bred in our animal facility and maintained on a 12h light, 12h dark cycle, with food and water *ad libitum*.

**Hypothyroidism:** Our experiments are generally realized on hypothyroid mice to reduce the variability between animals, being sure that they are all hypothyroid (that means, with homogenous, low level of T3). To induce fetal and neonatal hypothyroidism, pregnant female OF1 mice were given iodine-deficient food containing 0.15 % 6-*n*-propyl-2-thiouracil (PTU) (Harlan, Gannat, France) and drinking water with 0.5 g/l PTU (Sigma-Aldrich, St Quentin Fallavier, France) from day 14 of gestation through lactation. Hypothyroid 1-day-old pups were injected subcutaneously with 2.5 µg of T<sub>3</sub>/g of body weight (b.w) (in 0.9% saline). Controls received vehicle (saline 0.9%) injections.

### Plasmids

The *TRH* reporter gene construct consisting of 631 bp of the 5' *Trh* gene regulatory region cloned upstream the *Firefly* luciferase coding sequence (TRH-luc) was a gift from W. Balkan (University of Miami School of Medicine, FL) (Balkan et al., 1998). The *Glut4* reporter gene construct (Glut4-luc) was a gift from Hilde I. Nebb (University of Oslo, Norway) (Dalen et al., 2003). The pSG5/stop-mPPAR $\gamma$ 1 (pSG5-PPAR $\gamma$ ) expression vector construct was generously provided by Dr Liliane Mickalik (University of Lausanne, Switzerland). The pSG5-RXR $\alpha$  plasmid was a generous gift from Patrick Kastner (Kastner et al., 1994) (IGBMC, Illkirch, France). The pSG5-TR $\beta$ 1 has been described previously (Dupre et al., 2004).

### Design and cloning of the small hairpin RNAs

The double-stranded small hairpin RNA (shRNA) coding sequence itself contains sense and antisense siRNA sequence against the target gene separated by a loop sequence and is inserted in the pCMV-H1 plasmid backbone (Hassani et al., 2007). Plasmids coding for shRNA against PPAR $\gamma$  (shPPAR $\gamma$ ) were designed from previously published siRNA against PPAR $\gamma$  analyzed by immunoblot for their efficiency and validated for their capacity to completely abolish the rosiglitazone induced differentiation of 3T3-L1 pre-adipocytes into adipocytes (Liao et al., 2007). The shRNA coding sequence was purchased from Eurogentec (Angers, France). The following shRNA sequence against PPAR $\gamma$  mRNA (shPPAR $\gamma$ )

5' *TCGAGTGAATTATCTGATTGAGGCTTATCAAGAGTAAGCTTCAATCGGATGGTTC*TTT  
*TTTGC*3' was subcloned between XhoI and NotI sites in the pCMV-H1 backbone, downstream the hybrid promoter CMV-H1 following the Quick Ligation kit protocol (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). All plasmid DNA preparations were prepared using commercial columns (Qiagen, Chatsworth, CA, USA), suspended in 10 mM Tris-HCl, 1 mM EDTA pH 8, sequenced (Eurofins MWG Operon, Germany) and stored as aliquots at -20°C.

### Q-PCR

Hypothalami of pups were dissected under a binocular microscope (limits for hypothalamic dissection: posterior to the optic chiasma, anterior to the mammillary bodies, along both lateral sulcus and 2 mm in depth) immediately froze in liquid nitrogen, and stored at -80°C until processing. Total RNA was extracted following the protocol provided with RNable reagent (Eurobio, Les Ulis, France) and stored in Tris 10 mM /EDTA 0,1 mM (PH 7,4) at -80°C. To quantify mRNAs in hypothalami, 1µg of total RNA were reverse-transcribed using High capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France). Control reactions without reverse-transcriptase were done in parallel. Taqman probes for the detection of, TRH, PPAR $\gamma$ , RXR $\alpha$ , Glut4, Dio2 and Dio3 (Taqman Gene expression Assays) were purchased from Applied Biosystems (Courtaboeuf, France). Direct detection of the PCR product was monitored by measuring the increase in fluorescence generated by the TaqMan probe. Samples containing 2 µl of cDNA (diluted 20 fold),

1 µl of specific probe and 10 µl of 2x TaqMan<sup>R</sup> universal PCR Master Mix (Applied Biosystems) were prepared in a final volume of 20 µl. The gene-specific PCR products were measured continuously using ABI PRISM 7300 Sequence Detection System (Applied Biosystems) during 40 cycles. The threshold cycle (CT) of each target product was determined and  $\Delta$ CT between target and endogenous control was calculated. The difference in  $\Delta$ CT values of two groups ( $\Delta\Delta$ CT) was used to calculate the fold increase ( $F=2^{-\Delta\Delta CT}$ ) and to determine the target gene expression.

### Reagents

PPAR $\gamma$  agonists (pioglitazone and rosiglitazone) and antagonist GW9662 (2-chloro-5-nitrobenzanilide) were purchased from Alexis Biochemicals (Enzo Life Sciences Inc., Farmingdale, NY, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corporation, St. Louis, MO, USA) as a 5 mM stock solution at -80°C.

### ELISA assay

Euthyroid newborn mice were injected bilaterally in the hypothalamus with 2 µl of a 5% glucose solution containing Rosiglitazone at a final concentration of 1 µM, or DMSO in control animals. Blood samples were collected and pooled 20h and 36h post-treatment. Total T<sub>4</sub> blood concentrations were quantified using ELISA kits (AbCys, Paris, France) following the manufacturer's instructions.

### *In vivo* gene transfer

Transfection of plasmid/polyethylenimine (PEI) complexes was previously described (Guissouma et al., 1998). Briefly, pups were anaesthetized by hypothermia on ice and transfected on postnatal day one with 2x2 µl of a 125 ng/µl solution of TRH-luc complexed with PEI (500 ng/pup). To assess PPAR $\gamma$  overexpression effect, we added either pSG5-PPAR $\gamma$  (5-25-50-100 ng/pup), pSG5-TR $\beta$ 1 (100 ng/pup), pSG5-RXR $\alpha$  (100 ng/pup) or empty pSG5 expression vector (Promega, Madison, WI, USA) in the mixture. Luciferase activity was assayed 20h after transfection. In shRNA experiments, we added small hairpin expression vector in the complexes at 2x2 µl of a 100 ng/µl concentration (400 ng/pup) and luciferase activity was measured 48h later to allow for shRNA expression. To assess the pharmacological activation or inhibition of endogenous PPAR $\gamma$ , pioglitazone, rosiglitazone or GW9662 diluted stock solutions were added to the TRH-luc /PEI complexes solution to reach final concentrations (0.1; 0.5; 1 µM). In control animals, diluted DMSO was added to the plasmid/PEI complex solution.

### Statistical analysis

Q-PCR results boxes represent 5 to 95 percentiles around the median with whiskers for minimum and maximum values. Statistical analysis for Q-PCR data compared the medians of fold increase, using a Student's t-test to compare control and treated group. Independent experiments ( $5 \leq n \leq 8$ ) were repeated twice providing similar results, and data were pooled. *In vivo* transfection results are presented as means  $\pm$  SEM per group. ANOVA was used to compare groups followed by multiple comparisons Bonferroni's post-test. Each experiment was carried out with  $n \geq 10$ , repeated at least three times providing the same results. Statistical analysis for Elisa data compared the means of T<sub>4</sub> values  $\pm$  SEM, using Student's t-test to compare control and treated group at each time ( $4 \leq n \leq 6$ ). In all cases, representative experiments are shown. Differences were considered significant at  $p < 0.05$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

## RESULTS

### Hypothalamic expression of PPAR $\gamma$ and Glut4 in the newborn mouse

The main objective of this study was to test the hypothesis that crosstalk between PPAR $\gamma$  and TH signaling could occur in the mouse hypothalamus and play a physiological role in regulation of genes involved in central control of metabolism, such as *Trh*.

Q-PCR showed that mRNA encoding PPAR $\gamma$  and a key PPAR $\gamma$  target gene, Glut4, were expressed in euthyroid (Fig. 1A) and hypothyroid (Fig. 1B) newborn mouse hypothalamus. Measurable levels of both PPAR $\gamma$  and Glut4 mRNAs were found. Their expression levels were independent of thyroid status.

### Pharmacological modulation of endogenous PPAR $\gamma$ activity at the central level modifies HPT function

As PPAR $\gamma$  affects peripheral TR $\beta$  signaling, we studied whether pharmacological activation of endogenous PPAR $\gamma$  receptors modulated expression of *Trh*, PPAR $\gamma$  and certain genes known to be implicated in *Trh* regulation in the mouse hypothalamus. The well-characterized PPAR $\gamma$  agonist rosiglitazone was administered (1  $\mu$ M) to euthyroid newborn mice by hypothalamic injection. QPCR was used to assess expression of *Trh*, PPAR $\gamma$ , *RXR $\alpha$* , *Dio2* and *Dio3* gene levels after 20h (Fig. 2A) or 36h (Fig. 2B) exposure to PPAR  $\gamma$  agonist. Rosiglitazone administration was without significant effect on the global level of expression of these genes in the hypothalamus at the time points used.

To test more directly whether PPAR $\gamma$  activity affected the highly neuron-specific transcription an *in vivo* PEI-based *Trh* reporter paradigm (Guissouma et al., 1998, 2000) was exploited. The PPAR $\gamma$  agonists rosiglitazone and pioglitazone, were administered to euthyroid newborn mice by intracerebral hypothalamic injection simultaneous with a TRH promoter-luciferase reporter gene (TRH-luc) construct. Injecting increasing amounts (0.1, 0.5 and 1  $\mu$ M) of either rosiglitazone or pioglitazone, induced dose-dependent activation of TRH-luc expression by 68 % ( $p < 0.01$ ) and 50 % ( $p < 0.05$ ) respectively at 1 $\mu$ M (Fig. 3A).

The effects of these agonists were then tested on hypothyroid mice, as the hypothyroid state reduces variability in endogenous thyroid hormone levels and therefore in the transcriptional responses between animals. Both pioglitazone and rosiglitazone (each at 1 $\mu$ M) increased T<sub>3</sub>-independent TRH-luc transcription by 35% ( $p \leq 0.05$ ) and 50% ( $p \leq 0.05$ ) respectively. Moreover at this concentration, both pioglitazone (Fig. 3B) and rosiglitazone (Fig. 3C) increased levels of both T<sub>3</sub>-independent TRH-luc activation and T<sub>3</sub>-dependent TRH-luc repression. Thus, endogenous PPAR $\gamma$  activation by its agonists in the hypothalamus raised the set-point of *Trh* transcription, maintaining T<sub>3</sub>-dependent negative feedback on *Trh* transcription but at a higher transcriptional level than in the absence of PPAR $\gamma$  ligands.

The upward resetting of *Trh* promoter set-point by PPAR $\gamma$  agonists should lead to downstream repercussions on circulating thyroid hormone levels, endpoint of the HPT axis regulatory system. To examine whether this was the case, newborn mice were given a single intra-hypothalamic 1  $\mu$ M injection of rosiglitazone or vehicle, and total plasma T<sub>4</sub> levels were measured 20h and 36h later by ELISA assay. No changes in circulating T<sub>4</sub> were seen at 20h, whereas at 36h post-injection, rosiglitazone treatment significantly increased plasma total T<sub>4</sub> by nearly two-fold (see Fig. 4, 4.3 ng/ml  $\pm$  0.7 vs 6.9 ng/ml  $\pm$  0.93,  $p \leq 0.05$ , control vs 36h group). Thus, the upward shift in the *Trh* transcriptional set-point induced by the PPAR $\gamma$  agonist produces a coherent, physiological stimulation of the HPT axis.

Next, the effects of the potent PPAR $\gamma$  selective antagonist GW9662 were investigated. GW9662 (0.1, 0.5 or 1  $\mu$ M) had no effect on T<sub>3</sub>-independent TRH-luc transcription (data not shown). However, GW9662 (1  $\mu$ M) completely abolished T<sub>3</sub>-dependent TRH-luc repression (Fig. 5, far right column) increasing mean levels by 33% ( $p \leq 0.05$ ) compared to controls (compare white columns in Fig. 5).

### Hypothalamic PPAR $\gamma$ knockdown affects basal *Trh* promoter activity

Having demonstrated that pharmacological modulation of PPAR $\gamma$  activity modulates the set point of *Trh* transcription, the effects of modulating hypothalamic PPAR $\gamma$  expression level on *Trh*

regulation were examined. First, the effect of hypothalamic PPAR $\gamma$  expression knockdown on *Trh* promoter activity was assessed using a plasmid-based RNA interference (shPPAR $\gamma$ ) approach (see *Materials and Methods*).

The shPPAR $\gamma$  strategy was validated exploiting the previously published effect of PPAR $\gamma$  ligands on Glut4 regulation, *i.e.* rosiglitazone induction of Glut4 expression (Armoni et al., 2003). shPPAR $\gamma$  plasmids were transfected into hypothyroid mice hypothalami together with a Glut4-luc construct. Effects of shPPAR $\gamma$  on Glut4-luc expression were tested in the absence or presence of rosiglitazone. Rosiglitazone significantly increased Glut4-luc transcription by 53 % ( $p < 0.001$ ) in control animals (Fig. 6A, left pair of columns). This potent rosiglitazone induction of Glut4-luc was completely abolished in presence of shPPAR $\gamma$  (Fig. 6A, right pair of columns). However, shPPAR $\gamma$  plasmids had no effect on basal *Glut4* promoter activity. Our results thus confirmed the specific role of liganded-PPAR $\gamma$  in activating *Glut4* gene transcription (Fig. 6A), and demonstrated the efficiency of the shPPAR $\gamma$  plasmid used.

Having validated that shPPAR $\gamma$  abolished rosiglitazone-dependent induction of *Glut4* promoter activity, we examined the effect of shPPAR $\gamma$  on T<sub>3</sub>-independent *Trh* transcription. As shown in Figure 6B, expressing shPPAR $\gamma$  in newborn mice hypothalami induced a somewhat unexpected, but highly significant, increase (*circa* 50%,  $p < 0.001$ ) of T<sub>3</sub>-independent TRH-luc transcription, showing that PPAR $\gamma$  knockdown enhanced T<sub>3</sub>-independent *Trh* promoter activity.

### Hypothalamic PPAR $\gamma$ overexpression interferes with T<sub>3</sub>-dependent *Trh* regulation

We next evaluated the effect of PPAR $\gamma$  over-expression on T<sub>3</sub>-dependent regulation of hypothalamic *Trh* transcription. Hypothyroid newborn mice were transfected in the hypothalamic region with a PPAR $\gamma$  expression vector (pSG5-PPAR $\gamma$ ) or a control pSG5 vector, and the TRH-luc construct (see *Materials and Methods*). Following transfection, mice were treated or not with T<sub>3</sub> (2.5  $\mu$ g/g of bw).

As shown in Figure 7A (left pair of columns), T<sub>3</sub> treatment significantly reduced TRH-luc expression ( $p < 0.001$ ) by more than 55% in control mice. In contrast, PPAR $\gamma$  overexpression (100 ng) totally abolished T<sub>3</sub>-dependent TRH-luc repression without affecting TRH-luc transcription levels in the absence of T<sub>3</sub> (right pair of columns fig. 7A). The effect of PPAR $\gamma$  on T<sub>3</sub>-dependent TRH-luc repression was dose-dependent, increasing amounts of PPAR $\gamma$  (5, 25, 50 and 100 ng) progressively reversing the T<sub>3</sub>-dependent inhibition of TRH-luc (Fig. 7B).

### Co-transfection of RXR $\alpha$ or TR $\beta$ 1 reverses PPAR $\gamma$ effects on *Trh* promoter activity

The demonstration that overexpressed PPAR $\gamma$  significantly ( $p < 0.01$ ) inhibited T<sub>3</sub>-dependent TRH-luc repression led to the hypothesis that this abrogation could be due to a PPAR $\gamma$ -dependent titration of RXR $\alpha$  and/or TR $\beta$ 1-required partners to achieve T<sub>3</sub>-dependent TRH-luc repression. To test this hypothesis, TRH-luc was co-transfected with expression vectors coding for PPAR $\gamma$  and RXR $\alpha$  or TR $\beta$ 1 into newborn mice hypothalami. As shown in Figure 8, co-transfection of RXR $\alpha$  (100 ng) with PPAR $\gamma$  (50 ng) completely restored the T<sub>3</sub> feedback on TRH-luc ( $P < 0.001$ ) (Fig. 8, 4<sup>th</sup> column). A similar reversal of the effect of PPAR $\gamma$  was seen when TR $\beta$ 1 (100 ng) was overexpressed (Fig. 8, far right column), confirming the hypothesis that PPAR $\gamma$  abrogation of T<sub>3</sub>-dependent TRH-luc repression was due to an imbalance and a competition between transcription factors.

## DISCUSSION

### PPAR $\gamma$ agonists and antagonists modulate *Trh* transcription

The data presented here show that hypothalamic PPAR $\gamma$  and its cognate ligands interfere with T<sub>3</sub>-independent and T<sub>3</sub>-dependent *Trh* transcription, demonstrating that hypothalamic *Trh* regulation of metabolism can integrate information from PPAR $\gamma$  signaling pathways.

Although much data document how PPAR $\gamma$  signaling contributes to energy balance in the periphery by regulating glucose and lipid metabolism (Ferre, 2004; Sharma and Staels, 2007), little is known about the potential role of PPAR $\gamma$  in central metabolic regulations. The hypothalamus controls energy homeostasis at the central level. In particular, paraventricular TRH neurons exert a dominant role on the HPT axis regulation as key metabolic sensors (Lechan and Fekete, 2006) and notably as transducers of leptin and melanocortin signals that modulate the HPT axis (Garza et al., 2008). Here, the working hypothesis was that as PPAR $\gamma$  is expressed in the adult hypothalamus (Sarruf et al., 2009), it might contribute to central control of metabolic homeostasis by modulating TR-dependent *Trh* transcription.

This hypothesis was tested in newborn mice. Q-PCR confirmed that PPAR $\gamma$  is expressed in the newborn hypothalamus. Next, effects of two synthetic PPAR $\gamma$  agonists, rosiglitazone and pioglitazone, were assessed on *Trh* transcriptional activity. These thiazolidinediones (TZD) drugs are commonly used for the treatment of metabolic disease such as insulin resistance or cardiovascular dismetabolic syndrome (Murphy and Holder, 2000). Because of their poor blood-brain-barrier permeability (Pedersen and Flynn, 2004; Risner et al., 2006), they were administrated directly into the hypothalamus. Both agonists significantly increased T<sub>3</sub>-independent *Trh* transcription. Interestingly, a parallel activation of hypothalamic Glut4 transcription was seen within the same time-scale as for *Trh* transcription. Liganded-PPAR $\gamma$  induction of Glut4 transcription is well documented in peripheral tissues (Armoni et al., 2003) and so served as a positive control in these experiments on the effects of TZDs in the brain. PPAR $\gamma$  activates the Glut 4 promoter through direct binding of liganded PPAR $\gamma$  on the PPAR responsive element (PPRE) at -66/+163 bp from the transcription start site (Armoni et al., 2003). No such PPRE is present on the TRH-luc construct used for the reporter gene experiments, nor on the first 630 bp of the 5' region for the mouse *Trh* gene. Thus, the transcriptional activation observed is most probably due to an indirect effect of liganded PPAR $\gamma$ . Such a regulatory cascade could implicate liganded PPAR $\gamma$ -dependent gene pathways in other hypothalamic or extra-hypothalamic nuclei connected with TRH expressing neurons. One potential pathway could be the leptin pathway which provides information on energy stores to brain centers regulating appetite and energy expenditure, notably the HPT axis (Guo et al., 2004).

Here we show that PPAR $\gamma$  agonists increase not only T<sub>3</sub>-independent hypothalamic *Trh* transcription, but also importantly, T<sub>4</sub> production. Thus, TZDs or endogenous PPAR $\gamma$ -agonists activate hypophysiotropic *Trh*, which stimulates pituitary TSH, consequently stimulating TH production and release. In turn, TH affects peripheral metabolism at multiple levels including lowering lipids and atherogenic lipoproteins and increasing energy expenditure (Erion et al., 2007). These effects contrast with the peripheral effects of systemic TZDs treatment, which induces positive energy balance and weight gain. These differences are probably due to the fact that with systemic TZDs delivery only small amount of TZDs reach the brain (Festuccia et al., 2008; Risner et al., 2006).

### Overexpression or knock-down of PPAR $\gamma$ in the hypothalamus differentially affects T<sub>3</sub>-dependent and T<sub>3</sub>-independent *Trh* transcription

Using a previously published shRNA sequence to knockdown hypothalamic PPAR $\gamma$ , we observed a loss of Glut 4 regulation by PPAR $\gamma$  agonists and significant enhancement of T<sub>3</sub>-independent TRH transcription. Previous *in vitro* experiments have already described this phenomenon, referred as de-repression. Notably, in adipocytes, PPAR $\gamma$  deletion increases basal transcriptional activity of positively regulated PPAR $\gamma$  target genes (Guan et al., 2005; Bensinger and Tontonoz, 2008).

In contrast, overexpression of PPAR $\gamma$  in the hypothalamus of newborn mice hypothalamus did not modify T<sub>3</sub>-independent *Trh* activation, but abolished T<sub>3</sub>-dependent *Trh* repression. This effect of

PPAR $\gamma$  overexpression was dose-dependent. Previous *in vitro* studies have shown PPAR $\gamma$  to counteract T<sub>3</sub> signaling (Wang et al., 2005). Our data suggest that unliganded PPAR $\gamma$  acts as a dominant negative on hypothalamic TH signaling, preventing T<sub>3</sub>-induced *Trh* regulation. This inhibition could occur through at least two mechanisms. First, as PPAR $\gamma$  and TR $\beta$  recruit similar transcriptional machineries, there would be competition for common cofactors (co-activators and co-repressors) as described in the heart (Hyyti and Portman, 2006). The other possibility is competition for the common heterodimer partner RXR, as demonstrated in *in vitro* studies (Araki et al., 2005; Hunter et al., 1996; Juge-Aubry et al., 1995). This explanation is all the more probable given that RXR $\alpha$  enhances T<sub>3</sub>-dependent repression of *Trh* transcription (Hollenberg et al., 1995). The present data consolidate this hypothesis, as the inhibitory effect of PPAR $\gamma$  on T<sub>3</sub>-dependent *Trh* repression was abrogated by co-expression of either TR $\beta$ 1 or RXR $\alpha$  in a ligand-dependent manner. The formation of inactive PPAR $\gamma$ :RXR $\alpha$  heterodimers might be involved in the PPAR $\gamma$ -induced modification of *Trh* transcription regulation. In coherence with the roles of both TR $\beta$ 1 and RXR $\alpha$  in regulating *Trh* transcription (Dupre et al., 2004; Guissouma et al., 1998; Hollenberg et al., 1995), increasing TR $\beta$ 1 would titrate PPAR $\gamma$ :RXR heterodimer formation in favour of formation of active TR $\beta$ 1:RXR heterodimers that mediate T<sub>3</sub>-dependent *Trh* repression. The effects of PPAR $\gamma$  reported here could also be explained by direct interaction between hypothalamic PPAR $\gamma$  (expressed in PVN, Sarruf et al., 2009) and TR $\beta$ 1 (TR-PPAR heterodimers), as previously described in peripheral regulations on both TR and PPAR target genes (Araki et al., 2005; Bogazzi et al., 1994). The negative effect of overexpressed PPAR $\gamma$  on T<sub>3</sub>-dependent *Trh* repression could arise from sequestration of TR $\beta$ 1 in an inactive complex, through heterodimerisation with PPAR $\gamma$ , that would modulate T<sub>3</sub> action on *Trh* promoter. These possibilities are not mutually exclusive and in each case excess TR $\beta$ 1 would re-establish PPAR $\gamma$ -disrupted *Trh* regulation.

Together the data provide a basis for a model for PPAR $\gamma$  interference with *Trh* transcription. Liganded-PPAR $\gamma$  (holo-PPAR $\gamma$ ) would affect *Trh* transcription indirectly, via a third effector (hypothetically through leptin signaling, as PPAR $\gamma$  is expressed in the center of leptin signaling, the arcuate nucleus (Sarruf et al., 2009) whereas unliganded-PPAR $\gamma$  (apo-PPAR $\gamma$ ) would disrupt T<sub>3</sub>-induced *Trh* regulation more directly, competing with common partners as RXR $\alpha$ . It is likely that the two mechanisms are independent.

In conclusion, this study represents the first *in vivo* report of interplay between PPAR $\gamma$  and TR $\beta$  in the hypothalamus, depicting a functional role of PPAR $\gamma$  in regulating *Trh* transcription. Thus, PPAR $\gamma$ , besides being implicated in regulating peripheral metabolism, can also contribute to central control of metabolism. Furthering the understanding of the mechanisms by which TR and PPAR $\gamma$  crosstalk in TRH neurons will shed light on how this central group of neurons integrates a wide range of metabolic signals which in turn influence metabolism by setting TH levels. Such knowledge could open up therapeutic perspectives for pathologies implicating metabolic disorders.

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## FIGURE LEGENDS

### **Figure 1: PPAR $\gamma$ and Glut4 mRNA are expressed in the newborn mouse hypothalamus.**

Real-time PCR quantification of PPAR $\gamma$  and Glut4 mRNA on mRNA extracted from hypothalami of euthyroid (A) and hypothyroid (PTU) (B) newborn mice. Animals were treated for 8h with T<sub>3</sub> or by saline. The experiment was done twice, providing similar results. Boxes represent 5 to 95 percentiles around the median from pools of two independent experiments, with whiskers for minimum and maximum values, n between 5 and 8 samples per group. PPAR $\gamma$  and Glut4 mRNA levels are expressed as relative to TR $\alpha$ 2 mRNA levels.

### **Figure 2: Endogenous TRH expression and its regulatory genes are not modulated by the PPAR $\gamma$ agonist Rosiglitazone in the newborn mouse hypothalamus.**

Real-time PCR quantification of TRH, PPAR $\gamma$ , RXR $\alpha$ , Dio2 and Dio3 mRNA was done on mRNA extracted from hypothalami of euthyroid newborn mice. Animals were given intracerebral injections of rosiglitazone and sacrificed 20h (A) or 36h (B) later. Boxes represent 5 to 95 percentiles around the median from pools of two independent experiments, with whiskers for minimum and maximum values, n between 5 and 8 samples per group, derived from pools of two independent experiments. Gene mRNA levels are expressed as relative to TR $\alpha$ 2 mRNA levels.

### **Figure 3: The PPAR $\gamma$ agonists Pioglitazone and Rosiglitazone activate T<sub>3</sub>-independent *Trh* promoter activity. A:**

Euthyroid newborn mice were transfected with TRH-luc reporter vector (500 ng per pup) and treated either with DMSO (vehicle) or with increasing concentrations of PPAR $\gamma$  ligands Pioglitazone and Rosiglitazone (0.1, 0.5 or 1  $\mu$ M). To ensure delivery to the hypothalamus, PPAR $\gamma$  ligands were added to the transfection mixture together with TRH-Luc. **B-C:** To further investigate the effect of activation of endogenous PPAR $\gamma$  on T<sub>3</sub>-dependent *Trh* regulation, hypothyroid newborn mice were transfected with TRH-luc reporter vector (500 ng). In each case, animals were treated either with DMSO (vehicle) or Pioglitazone (1  $\mu$ M) (B) or Rosiglitazone (1  $\mu$ M) (C) in the presence or absence of T<sub>3</sub> (2.5  $\mu$ g/g bw), as mentioned. Data are expressed in RLU as means  $\pm$  SEM; 10  $\leq$  n  $\leq$  33. The whole experiment was repeated three times. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001 vs control mice.

**Figure 4: Intracerebral injection of Rosiglitazone increases peripheral T<sub>4</sub> levels.** Euthyroid one day-old newborn mice were acutely treated by a single intra-hypothalamic injection of either Rosiglitazone (1  $\mu$ M) or DMSO (controls). Sixteen treated animals and sixteen controls were killed at 20h or 36h after treatment. Blood samples were collected and pooled (four individuals per pool) and T<sub>4</sub> assayed by Elisa. Data are expressed in ng/ml as means  $\pm$  SEM of pooled samples; n  $\geq$  4 for each point. \*, p<0.05 vs control mice.

### **Figure 5: The PPAR $\gamma$ antagonist, GW9662, inhibits T<sub>3</sub>-dependent TRH-luc repression.**

Hypothyroid newborn mice were transfected with TRH-luc reporter vector (500 ng) and animals treated either with DMSO as vehicle or with GW9662 (1  $\mu$ M). Test substances were added to the transfection mixture, in the presence or absence of T<sub>3</sub> (2.5  $\mu$ g/g bw). The data are expressed in RLU as means  $\pm$  SEM; n  $\geq$  10 per point. The whole experiment was repeated three times providing similar results. \*, p<0.05; vs control mice.

### **Figure 6: PPAR $\gamma$ mRNA knock-down upregulates T<sub>3</sub>-independent TRH-Luc transcription. A:**

intra-hypothalamic expression of shPPAR $\gamma$  abolishes specific rosiglitazone-dependent Glut 4 promoter activity. **B:** shPPAR $\gamma$  stimulates TRH-luc expression. Pups received intra-hypothalamic injection of a mix containing either DMSO (vehicle) or Rosiglitazone (1  $\mu$ M) together with Glut4-luc (500 ng/pup) (A) or TRH-luc (500 ng/pup) (B) and a shRNA expression vector (either pCMV H1-shPPAR $\gamma$  or the empty pCMVH1 (400 ng/pup), as specified). Luciferase activity was measured 48h later. Data are

expressed in RLU as means  $\pm$  SEM;  $n \geq 10$  per point. The whole experiment was repeated three times providing similar results. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  vs control mice.

**Figure 7: PPAR $\gamma$  overexpression abrogates T<sub>3</sub>-dependent repression of TRH-luc transcription in a dose-dependent manner.** Hypothyroid one-day-old mice were transfected in the hypothalamus with TRH-luc as reporter plasmid (500 ng/pup) and 100 ng (A) or increasing amounts (5-25-50-100 ng/pup) (B) of PPAR $\gamma$  expression vector (pSG5/stop-mPPAR $\gamma$ ). Empty expression vector, pSG5, was used to keep DNA concentration constant (ct vector). Animals were treated with T<sub>3</sub> (2.5  $\mu$ g/g of b.w.) or saline solution as vehicle (-T<sub>3</sub>). Luciferase activity was measured 20h post-transfection. Data are expressed in RLU as means  $\pm$  SEM;  $n \geq 10$  per point. The whole experiment was repeated three times with similar results. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  vs control mice.

**Figure 8: Overexpression of RXR $\alpha$  or TR $\beta$ 1 reverses PPAR $\gamma$  induced abrogation of T<sub>3</sub>-dependent TRH-luc regulation.** Hypothyroid one-day-old mice received intrahypothalamic injections of TRH-luc reporter vector (500 ng/pup) and PPAR $\gamma$  expression vector (pSG5/stop-mPPAR $\gamma$ , 50 ng/pup). RXR $\alpha$  or TR $\beta$ 1 overexpression was obtained when 100 ng of respectively RXR $\alpha$  or TR $\beta$ 1 expression vectors were cotransfected with PPAR $\gamma$  expression vector. Empty expression vector pSG5 was used to keep DNA concentration constant. Animals were treated with T<sub>3</sub> (2.5  $\mu$ g/g of b.w.) or saline solution as vehicle (-T<sub>3</sub>). The experiment was repeated three times providing similar results. Data are expressed in RLU as means  $\pm$  SEM of triplicates;  $n \geq 10$  per point. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  vs control mice.

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