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## Neuronal Blockade of Thyroid Hormone Signaling Increases Sensitivity to Diet-Induced Obesity in Adult Male Mice

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1 Neuronal blockade of thyroid hormone signaling increases sensitivity to diet-induced obesity in adult  
2 male mice

3

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45 Original data generated and analyzed during this study are included in this published article or in the  
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47

## 48 **ABSTRACT**

49

50 Thyroid hormone increases energy expenditure. Its action is mediated by TR, nuclear receptors  
51 present in peripheral tissues and in the central nervous system, particularly in hypothalamic neurons.  
52 Here, we address the importance of thyroid hormone signaling in neurons, in general for the  
53 regulation of energy expenditure.

54 We generated mice devoid of functional TR in neurons using the Cre/LoxP system. In hypothalamus,  
55 which is the center for metabolic regulation, mutations were present in 20 to 42% of the neurons.

56 Phenotyping was performed under physiological conditions that trigger adaptive thermogenesis: cold  
57 and high fat diet (HFD) feeding. Mutant mice displayed impaired thermogenic potential in brown and  
58 inguinal white adipose tissues and were more prone to diet-induced obesity. They showed a  
59 decreased energy expenditure on chow diet, and gain more weight on HFD. This higher sensitivity to  
60 obesity disappeared at thermoneutrality. Concomitantly, the AMPK pathway was activated in the  
61 ventromedial Hypothalamus (VMH) of the mutants as compared to the controls. In agreement  
62 sympathetic nervous system (SNS) output, visualized by tyrosine hydroxylase expression was lower in  
63 the brown adipose tissue (BAT) of the mutants. In contrast, absence of TR signaling in the mutants  
64 did not affect their ability to respond to cold exposure.

65 This study provides the first genetic evidence that thyroid hormone signaling exerts a significant  
66 influence in neurons to stimulate energy expenditure in some physiological context of adaptive  
67 thermogenesis. TR function in neurons to limit weight gain in response to HFD and this effect is  
68 associated with a potentiation of SNS output.

## 69 **Keywords**

70 Thyroid hormone, adaptive thermogenesis, cold exposure, diet induced obesity, neurons, brown

71 adipose tissue, sympathetic nervous system (SNS)

72

73

## 74 **1. Introduction**

75 As obesity and associated metabolic disorders became pandemic [1], the metabolic activities of  
76 thyroid hormone (3,3',5-triiodo-L-thyronine, or T3), the active metabolite of thyroxine (T4) gained  
77 renewed interest. Indeed, in humans, hyperthyroidism often results in weight loss, whereas  
78 hypothyroidism tends to favor weight gain. Most importantly, exogenous T3 increases energy  
79 expenditure (EE) both in humans and rodents [2]. As a result, an excess of T3 induces weight loss  
80 despite an increase in food intake [3].

81 T3 can increase EE either directly or indirectly. Its direct metabolic action takes place in BAT, white  
82 adipose tissue (WAT), muscle and liver, where it stimulates the mitochondrial metabolism. In BAT,  
83 T4 can be deiodinated to produce T3. This local production of T3 triggers the expression of UCP1, the  
84 protein responsible for the uncoupling between oxidative phosphorylation and ATP production,  
85 which has an important function in thermogenesis [4-5]. After hormonal stimulation, several  
86 enzymes involved in lipid synthesis and calories production are also induced in BAT [6], and EE  
87 increases in muscle [7-8]. T3 also triggers WAT browning, *i.e.* the conversion of some white  
88 adipocytes into thermogenic adipocytes producing UCP1 [9].

89 In addition to its direct influence on peripheral tissues, T3 is also involved in the central control of  
90 adaptive thermogenesis [10-11], a process that is activated to face two types of external stress: cold  
91 exposure and excess of food intake [12]. Perception of cold exposure by skin receptors is sensed by  
92 the hypothalamus and results in an increased activity of the sympathetic nervous system (SNS). The  
93 subsequent stimulation of adrenergic synapses in adipose tissues triggers immediate BAT  
94 thermogenesis, and late browning of WAT [13-14]. SNS stimulation leads to both UCP1- dependent  
95 and UCP1-independent thermogenesis in BAT [15-16-17] and muscle [18]. How a high-fat diet (HFD)  
96 provokes a thermogenic response is less documented. While the involvement of the SNS in this  
97 response is well established, the contribution of BAT seems limited [19-20]. Importantly, stimulation

98 of BAT *via* the SNS results in increased expression of the *Dio2* gene and a concomitant increase in  
99 type-2 deiodinase (D2) activity [21]. The resulting local conversion of T4 into T3 contributes to an  
100 increased thermogenic activity. *Dio2* knock-out mice are more sensitive to cold [20] and more prone  
101 to diet-induced obesity (DIO) [22].

102 Hypothalamic glial cells including astrocytes and tanycytes contain D2, which allows a local  
103 conversion of T4 into T3 [23]. The fact that this local conversion is involved in adaptative  
104 thermogenesis is supported by experiments that show that an intrahypothalamic excess of T3 is  
105 sufficient to stimulate BAT activity [10]. As all cell types in the hypothalamus express the nuclear  
106 receptors of T3, either TR $\alpha$ 1 encoded by the *Thra* gene, or TR $\beta$ 1/2 encoded by *Thrb* [24], they all  
107 have the capacity to respond to a local increase in T3. However, the link between the intra-  
108 hypothalamic T3 concentration and the increase in sympathetic tone remains unclear. Elevation of  
109 intra-hypothalamic T3 concentration is characterized by rapid activation of the AMPK-ER stress-JNK1  
110 pathway in neurons [25-10]. Although their T3 response is not documented, neuronal populations  
111 located in other areas of the brain, notably the brainstem, also participate in EE [26].

112 We address here the importance of T3 signaling in neurons for the regulation of EE under  
113 thermogenic stresses. To this end, we used the Cre/loxP methodology to produce a mouse model in  
114 which the T3 response is selectively eliminated from a significant fraction of neurons, notably in 20 to  
115 42% of the neurons in the hypothalamus depending of the nucleus. We evaluated the ability of these  
116 mice to face thermogenic stress. Mice were exposed either to an intense cold or fed for several  
117 months with an HFD. Mutant mice display an intact thermogenic response after exposure to cold at  
118 4°C, but were more prone to DIO. The decrease in SNS tone in mutants mimics what was observed  
119 after virogenetic inhibition of TR signaling in the hypothalamus and mirrors the increase triggered by  
120 ICV T3 administration [10-25]. Overall, our data show that endogenous T3 signaling exerts a  
121 significant influence on neurons involved in the regulation of energy metabolism.

122



## 123 **2. Material and Methods**

### 124 2.1 Chemicals:

125 Tri-iodothyronine (T3) and thyroxine (T4) were from Sigma-Aldrich (l'Isle D'Abeau, France).

### 126 2.2 Mice lines:

127 Mutant mice were obtained by crosses in the C57BL6/J genetic background to introduce different  
128 recombinant alleles. *Thra*<sup>AMI</sup> allows the production of the TR $\alpha$ 1<sup>L400R</sup> mutant, which has dominant-  
129 negative properties, after Cre/loxP-mediated excision of a stop cassette [27]. Despite the persistence  
130 of an intact *Thra* allele in *Cre3*<sup>Tg/+</sup>*xThra*<sup>AMI/+</sup> mice, the dominant negative action of TR $\alpha$ 1<sup>L400R</sup>  
131 eliminates the capacity of cells expressing *Thra* to respond to T3. *Thrb*<sup>lox</sup> has 2 tandem-arranged loxP  
132 sequences, allowing Cre-mediated excision of exon 3, which encodes the DNA binding domain of the  
133 TR $\beta$ 1/TR $\beta$ 2 receptor, resulting in a frameshift and a loss of function [28]. *Cre3*<sup>Tg</sup> [29] is a randomly  
134 integrated transgene that drives the expression of the Cre recombinase expression in neurons of the  
135 central nervous system, but also the peripheral nervous system. The fraction of neurons with active  
136 Cre differs in the different areas and is markedly higher in hypothalamus as compared to other areas  
137 of the brain [30]. Cre activity was not detected in astrocytes or non-neuronal cell types [29-30]. We  
138 previously verified this specific neuronal expression using the rosa YFP reporter line [31].  
139 Furthermore, in the present study, using the *ROSA tdTomato* reporter transgene [32], we verified the  
140 absence of recombination activity outside the nervous system, except for a small fraction of  
141 cardiomyocytes (Fig. S1) [33]. *Cre3*<sup>Tg/+</sup>*x ROSAtdTomato* mice were also used to highlight with red  
142 fluorescence the hypothalamic cells in which Cre-mediated recombination occurred and compare the  
143 percentage of neurons that present Cre activity in the different part of the hypothalamus (Fig. S2  
144 [33]). Mice with the *Cre3*<sup>Tg/+</sup>*xThra*<sup>AMI/+</sup>*Thrb*<sup>lox/lox</sup> genotype are called Neuron TRKO (NTRKO).  
145 *Thra*<sup>AMI/+</sup>*Thrb*<sup>lox/lox</sup> littermates were used as controls (CTRL).

146 All experiments were carried out in accordance with the European Community Council Directive of  
147 September 22, 2010 (2010/63/EU) regarding the protection of animals used for experimental and  
148 other scientific purposes. The research project was approved in the different institutions. In Lyon, it

149 was approved by a local animal care and use committee (C2EA015) and subsequently authorized by  
150 the French Ministry of Research, in Santiago by the USC Ethics Committee (Project ID 15010/14/006)  
151 and in München by the Animal Ethics Committee of the government of Upper Bavaria, Germany. A  
152 total of 180 mice have been used for this study.

153 2.3 Animal procedures and preparation of tissue samples:

154 2.3.1 Housing

155 Mice were fed an *ad libitum* LASQC Rod16 R diet (Altromin, Germany) (16.9% proteins; 4.3% fat and  
156 79,2% carbohydrates, hereafter called CHOW), housed 2 per cage when possible, or otherwise  
157 mentioned at 23°, under a 7:30 am: 8:30 pm light/dark cycle. 3- to 5- month-old male mice were  
158 used for experiments.

159 2.3.2 Cold exposure and thermography

160 The cold response was assessed in mice implanted with IPTT-300 transponders (Plexx BV, The  
161 Netherlands) by exposing them to 4°C for 58 hours. Infrared thermography was performed at  
162 different time points in awake and free running animals. To capture static dorsal thermographic  
163 images, thermal videos of 2 min duration each were recorded using an infrared camera (FLiR  
164 Systems, Inc.). Thermal images were analyzed using the FLiR Research IR program. Regions of interest  
165 (ROI) were drawn so that each type of ROI was overlaid on all thermal images at once. This allowed  
166 for identical sizing of each type of ROI across all mice. An ROI on the basis of the tail served as the  
167 temperature control, while an ROI surrounding the interscapular region served to evaluate BAT  
168 thermogenesis. The temperature difference between these two ROI was calculated and used as a  
169 proxy of BAT activation. For each time point, four images per mouse were analyzed. Four mice per  
170 genotype were included in the experiment.

171 When mentioned, the experiments were performed at thermoneutrality in thermoregulated  
172 chambers.

173 2.3.3 Induction of hypo/hyperthyroidism

174 T3 deficiency in adult animals was induced as previously described with a diet containing PTU  
175 (Propylthiouracil) (Harlan Teklad TD95125, Madison, WI) (25.3% protein, 10% fat, 64.8%  
176 carbohydrates and 0,15% PTU) and followed or not by thyroid hormones (TH) (mix of T4 and T3) daily  
177 injections [34] for five consecutive days. Mice ate between 3.75 and 5.25 mg/day of PTU (2.5 and 3.5  
178 g of diet per day). Serum concentrations of FT3 and FT4 were measured as described in paragraph  
179 2.6, and were around 1 pm/L for both FT3 and FT4 in all genotypes in the PTU treated groups and  
180 respectively above 50pm/L and 100mg/L (the maximum concentrations detectable by the assay) in  
181 all genotypes in the PTU/TH treated groups. As expected PTU leads to hypothyroidism whereas TH  
182 injection leads to hyperthyroidism.

#### 183 2.3.4 HFD feeding

184 The response to HFD (Research Diets, New Brunswick, USA, D12331) (16.4 % proteins; 58% fat and  
185 25.5% carbohydrates) was evaluated after acute feeding periods (3.5 days) or prolonged feeding  
186 periods (3 to 7 months).

#### 187 2.3.5 Indirect calorimetry

188 A calorimetric system (LabMaster; TSE Systems; Bad Homburg, Germany) was used to assess EE and  
189 locomotor activity. Animals were placed one per cage with controlled conditions of air and  
190 temperature (24°C). Mice were in adaptation for a week before starting the measurements. After  
191 calibrating the system with the reference gases (20.9% O<sub>2</sub>, 0.05% CO<sub>2</sub> and 79.05% N<sub>2</sub>), the metabolic  
192 rate was measured for 48 hours as previously described [35]. EE, RQ (VCO<sub>2</sub>/VO<sub>2</sub>) and LA were  
193 recorded every 30 min. Mice were injected subcutaneously with 1 mg / kg of norepinephrine  
194 bitartrate salt monohydrate (Sigma, Germany) to analyse the response to norepinephrine (NE). The  
195 O<sub>2</sub> consumption was then measured for another 120-160 min every 10 min.

#### 196 2.3.6 Tissue sampling

197 At the end of the experiments, mice were anesthetized at 2pm (intraperitoneal injection of a mixture  
198 of xylazine and ketamine) to collect blood from the inferior vena cava in heparin coated tubes.  
199 Plasma was obtained after centrifugation at 3500 rpm for 15mn. Tissues were dissected and snapped  
200 frozen in liquid nitrogen and stored at -80°C for later RNA preparation or fixed in zinc formal Fixx  
201 (ThermoFisher Scientific, Waltham, MA, USA) for histology and immunohistochemistry.

202 For neuroanatomy experiments, the thorax was opened and each mouse was perfused with 4%  
203 paraformaldehyde in 0.1M phosphate buffer at room temperature and brain processed as previously  
204 described [30]

#### 205 2.4 RNA extraction and expression analyses by relative RT-qPCR

206 Total RNA was prepared using the TRI Reagent (ThermoFisher Scientific) protocol followed by a  
207 RNase free DNase treatment (Qiagen, Hilden, Germany). Concentration and purity were quantified  
208 with a Nanodrop. 1µg of RNA was used for reverse transcription with M-MLV reverse transcriptase  
209 (Promega, Madison, WI, USA).

210 For qPCR, Protocols were described before [36] Duplicates were run for each sample. The results  
211 were analyzed according to the  $\Delta\Delta Cq$  method [37]. *Hprt* was used as the reference gene, and the  
212 control group was CHOW fed, HFD fed or PTU CTRL groups. The numbers of mice used for each  
213 experiment are reported in each figure legend. All the primer sequences are listed in Table S1 [33].

214 More details on the procedure is provided in supplemental data file S11 [33].

#### 215 2.5 Western blot

216 The proteins were extracted from the hypothalamus in lysis buffer (50mM Tris-HCl, 10 mM EGTA,  
217 1mM EDTA, 16mM Triton X-100, 1 mM sodium orthovanadate, 50mM sodium fluoride, 10 mM  
218 sodium pyrophosphate and 250 mM sucrose). Western blots were transferred to PVDF membranes  
219 (PVDF; Millipore; Billerica, MA, USA) and probed with antibodies against pACC $\alpha$  (Ser79) (Cell  
220 Signaling Cat# 3661; RRID: AB\_330337; Danvers; MA, USA), pAMPK $\alpha$  (Thr172) (Cell Signaling Cat#

221 2535S; RRID: AB\_331250) or  $\beta$ -actin (Sigma-Aldrich Cat# A5316; RRID: AB\_476743; St. Louis, MO,  
222 USA) as described [8-9]. Adequate secondary antibody: anti-mouse (Agilent Cat# P0260, RRID:  
223 AB\_2636929; Santa Clara, CA, USA) or anti-rabbit (Agilent Cat# P0450, RRID: AB\_2630354) was  
224 added. Signal of autoradiographic films was quantified by densitometry using ImageJ-1.33 software  
225 (NIH; Bethesda, MD, USA). Values were expressed in relation to  $\beta$ -actin. Data are expressed as a  
226 percentage of CTRL vehicle and NTRKO vehicle. Representative images for all proteins are shown  
227 with all bands for each picture derived from the same gel, although they may be spliced for clarity  
228 (represented by vertical black lines). pACC $\alpha$  and pAMPK $\alpha$  were assayed in the same membranes,  
229 therefore, some of the  $\beta$ -actin bands are common in their representative panels; this has been  
230 specified in the appropriate figure legend.

## 231 2.6 Plasma biochemistry

232 Plasma was assayed on a Cobas 6000 automats for free T3 and free T4 with the Cobas e601 module  
233 (Roche, ECL analyzers),

## 234 2.7 D2 activity

235 Hypothalamic block or 50 mg BAT tissue was homogenized in lysis buffer (100 mM sodium  
236 phosphate, 2mM EDTA, 50mM DTT). D2 activity was measured in fresh homogenates. D2 activity was  
237 measured in duplicate using 50  $\mu$ l undiluted homogenate (50 – 100  $\mu$ g protein) that was incubated  
238 for 3h (hypothalamus) or 2h (BAT) at 37°C in a final volume of 100  $\mu$ l in the presence of 0.25 M  
239 sucrose, 1 mM PTU (to block D1 activity in the hypothalamus), 500 nM of T3 (to block D3 activity in  
240 the hypothalamus), 1 nM T4, and approximately  $1.10^5$ cpm  $^{125}$ I-T4 (in-house, single labeled tracer  
241 according to [38] Wiersinga *et al* in Phosphate-EDTA buffer (PE buffer, 100mM sodium phosphate,  
242 2nM EDTA, pH7.2). D2 activity was expressed as  $^{125}$ I- fmol released per minute per gram of protein.  
243 The assay is based on the protocol previously described by Werneck-de-Castro *et al*. [39].

## 244 2.8 Histology, immunohistochemistry

### 245 2.8.1 Adipose tissues

246 Fixed adipose tissues were embedded in paraffin. Tissue sections (4  $\mu\text{m}$  for BAT; 8  $\mu\text{m}$  inguinal WAT  
247 (iWAT) were stained with hematoxylin and eosin (H&E). Rabbit poly-clonal antibodies directed  
248 against UCP1 (Abcam Cat# ab10983, RRID: AB\_2241462, Cambridge, UK) and Tyrosine Hydroxylase  
249 (Abcam Cat# ab112, RRID: AB\_297840) were diluted in PBS/2.5% goat serum for immuno-  
250 histochemistry, (1:400 and 1:750 dilution respectively). Sections were deparaffinized and incubated  
251 overnight at 4°C with a primary antibody. Sections were then incubated with an HRP-labeled anti-  
252 rabbit (1:300) (Promega Cat# W4011, RRID: AB\_430833) for 1h at room temperature. Peroxidase  
253 activity was visualized with diaminobenzidine staining (DAB, D5905 Sigma-Aldrich) and  
254 counterstained with hematoxylin. The images were acquired using an AxioObserver Zeiss microscope  
255 at a 16x magnification. Data were collected and the average intensity of the DAB signal was  
256 quantified after color deconvolution was performed to separate the relative contributions of  
257 DAB and hematoxylin, using ImageJ software. Background correction was performed using a  
258 negative control slide, in which the primary antibody was omitted. Three slides per animal were  
259 processed.

### 260 2.8.2 Brain

261 For neuroanatomy experiments, immunohistochemistry was performed on free-floating brain sections  
262 as previously described [30]. Brain Coronal sections (50  $\mu\text{m}$ ) were cut with a vibrating microtome  
263 (Integraslice 7550 SPDS, Campden Instruments, Loughborough, UK), and stored at -20°C in  
264 cryoprotectant (30% ethylene glycol and 20% glycerol in 10 mM PBS) prior to immunohistochemistry.  
265 The following primary antibodies were used: mouse anti-NeuN (1:500) (Merck Millipore Cat#  
266 MAB377, RRID: AB\_2298772, Burlington, MA, USA), and rabbit anti-GFAP (1:2000) (Agilent Cat#  
267 Z0334, RRID: AB\_10013382). Secondary antibodies were made in donkey (ThermoFisher Scientific,  
268 anti-rabbit DyLight 488 Cat# SA5\_10038, RRID: AB\_2556618, anti-mouse DyLight 650 Cat#  
269 SA5\_10169, RRID:AB\_25556749) and used at a 1:1000 dilution. Sections were imaged using an

270 inverted confocal microscope (Zeiss LSM 780). ImageJ software was used to estimate the proportion  
271 of NeuN+ cells that were also tdTomato+ at different levels of the rostrocaudal hypothalamus.

## 272 2.9 Quantification and statistical analyzes

273 For mouse experiments, the data presented represent the average values of different animals from  
274 the same genotype given the same treatment. The number of animals (n) used is indicated in the  
275 figure legends. For RT-qPCR, each sample was run in duplicate. The error bars represent SEM, since it  
276 is more appropriate than SD when  $n \geq 6$ . Statistical relevance was determined using the one-way  
277 Anova or the T-Test method as indicated in the figure legends. \* or \$ or £ stands for  $p \leq 0,05$ , \*\* or \$\$  
278 for  $0,005 \leq p \leq 0,05$ , \*\*\* or \$\$\$ for  $0,0005 \leq p \leq 0,005$ , \*\*\*\* or \$\$\$\$ for  $p \leq 0,0005$ . This method is  
279 appropriate since the size of the compared groups is similar and the distribution of the samples  
280 normal. Given the variability of the phenotype that we observed, we used  $n \geq 6$  to obtained  
281 statistically significant results. For only two groups,  $n = 5$ , because the animals died during the  
282 experimentation procedures. ns stands for non-significant.

283

## 284 3.Results

### 285 3.1 Generation of mice with selective elimination of T3 signaling in neurons

286 Recombination with the *Cre / LoxP* system was used to generate somatic mutations in the *Thra* and  
287 *Thrb* genes. The *Cre3<sup>Tg</sup>*, *Thra<sup>AMI</sup>* and *Thrb<sup>lox</sup>* alleles were combined to obtain *Cre3<sup>Tg/+</sup>Thra<sup>AMI/+</sup>Thrb<sup>lox/lox</sup>*  
288 mice, called NTRKO mice (see methods 2.2). These mice are heterozygous for the *Thra<sup>AMI</sup>* mutation  
289 and express the TR $\alpha$ 1<sup>L400R</sup> in neurons as a result of Cre/loxP mediated recombination. This mutant  
290 receptor exerts a dominant-negative activity toward the remaining intact TR $\alpha$ 1 receptor. As mice are  
291 also homozygous for a *Thrb* floxed allele, *Cre/loxP* recombination eliminates the receptors encoded  
292 by *Thrb* [27-28]. Therefore, the T3 response is selectively eliminated from all Cre expressing cells.

293 The efficacy of Cre-dependent recombination in the *Cre3<sup>Tg</sup>* transgenic mice was first described to be  
294 pan-neuronal in both the central and peripheral nervous systems [29]. Later studies from our lab  
295 showed that the efficiency of recombination was the highest in hypothalamus as compared to other  
296 brain area [30]. Here we used a Rosa-tdTomato<sup>lox</sup> reporter [32] to show that Cre activity is merely  
297 absent in non-nervous tissues (Fig. S1 [33]) and to better evaluate the recombination pattern in the  
298 hypothalamus (Fig. S2 [33], Fig. 1). The red fluorescence systematically colocalized with the neuronal  
299 NeuN marker and did not overlap with the glial marker GFAP (Fig. 1) as previously described in other  
300 area of the brain [30]. The density of red fluorescent neurons was high in all hypothalamic nuclei (Fig.  
301 1B, Fig. S2[33]), except the suprachiasmatic nucleus (not shown), representing 20 to 42% of all  
302 neurons in the counted area (Fig. S2 [33]). Therefore, mutations take place in a large fraction of  
303 neurons but not in glial cells or other cell types of the brain. We also used TR $\beta$ 1 mRNA level as a  
304 marker for Cre-mediated recombination in NTRKO mice and found that it was only decreased in the  
305 hypothalamus but not in the peripheral tissues (Fig. 2A). As TRH is produced by the hypothalamic  
306 neurons and involved in the feed-back control of TH production by the hypothalamus-pituitary axis,  
307 we measured the expression of *TRH*. In NTRKO *TRH* levels were unchanged under either CHOW or  
308 HFD (Fig. 2B left panel) and repression of *TRH* by TH in hypothyroid animals was also normal (Fig. 2B  
309 right panel). In agreement free T3 and free T4 concentrations in serum were normal in the mutants  
310 under all physiological conditions tested in the paper (Fig. S3A [33]).

311 In order to address the capacity of neurons to respond to T3, we treated NTRKO with PTU to make  
312 them hypothyroid. In response to TH the expression of the *Shh* and *Klf9* genes, previously described  
313 to be targets of T3 in neurons [36-40] was blunted in the hypothalamus of the mutants (Fig. 2C).  
314 Moreover, whereas administration of TH increased food intake in CTRL mice [41], this response was  
315 reduced in the NTRKO mice (Fig. 2D), supporting an inactivation of TH signaling in hypothalamic  
316 neurons. In contrast, a normal TH response was observed in all peripheral tissues that we tested.  
317 This was ascertained by measuring the mRNA levels for *Klf9* and *Hr* in inguinal WAT (iWAT), BAT and  
318 heart, or *Hr* and *Thrsp* in liver (Fig. S3B [33]).



319 In summary, NTRKO mice are euthyroid with functional T3 signaling in all cell types except for a  
320 fraction of neurons.

321

### 322 *3.2 T3 signaling in neurons regulates EE*

323 The NTRKO phenotype was first analyzed under standard conditions of food and temperature. Their  
324 body weight (Fig. 3A) and temperature (Fig. 3B) were normal. Their EE (Fig. 3C) measured by indirect  
325 calorimetry was lower than that of CTRL mice. Their locomotor activity (Fig. 3D) was similar but  
326 NTRKO food intake (Fig. 3E) was slightly lower. RT-qPCR analyzes did not reveal any major alteration  
327 of neither the thermogenic program in BAT (*Dio2*, *Ucp1*, *mCKB* and *mAlpl* mRNA) (Fig. S4A [33]) nor  
328 the browning program in iWAT (*Ppargc1a*, *Ucp1*, *Cidea*, *Cox7c* and *Prdm6*) (Fig. S4B [33]). However,  
329 UCP1 protein level was lower in BAT of NTRKO mice (Fig. 3F). We also assessed UCP1-independent  
330 non-shivering thermogenesis in skeletal muscle, by measuring the expression of the genes involved  
331 in Ca<sup>2+</sup> cycling (*Serca2A*, *Serca2B*, *Sln*, *Ryr1*, *Ryr2*) [18]. We found an increase in *Ryr2* expression in  
332 NTRKO muscles (Fig. 3G). Thermogenic mechanisms might be turned on in muscle to compensate  
333 defective BAT activity.

### 334 *3.3 T3 signaling in neurons is not involved in severe cold-induced thermogenesis*

335 As increased D2 activity and local production of T3 in the BAT are critical to the cold response in wild-  
336 type mice (WT) [21], the activity of D2 was checked in the hypothalamus of WT mice. It was not  
337 modified during cold exposure in the hypothalamus but, as expected, was strongly induced in the  
338 BAT (Fig. 4A).

339 To test whether despite the unchanged D2 activity in hypothalamus, impaired T3 signaling in neurons  
340 prevents the cold-induced process of adaptive thermogenesis, mice were exposed to 4°C. Their body  
341 weight (Fig 4B) and body temperature (Fig. 4C) were monitored for 2.5 days. Both NTRKO and CTRL  
342 mice managed to maintain their body temperature by increasing their food intake (Fig. 4D).

343 Thermogenesis of BAT was observed by infrared thermography after 24 hours and 48 hours at 4°C.

344 The temperature of this tissue was similar in the two genotypes at both time points (Fig. S5A [33]).  
345 Accordingly, the UCP1 protein was expressed at the same level in NTRKO and CTRL in both BAT (Fig.  
346 4E) and in iWAT (Fig. S5B [33]) at 4°C. Moreover RT-qPCR analyzes did not reveal any major alteration  
347 of either the thermogenic program in BAT (*Dio2*, *Ucp1*, *mCKB* and *mAlpl* mRNA) (Fig. 4E) or the  
348 browning program in iWAT (*Ppargc1a*, *Ucp1*, *Cidea*, *Cox7c* and *Prdm6*) at this temperature (Fig. S5B  
349 [33]).

350 The general response of BAT tissue to SNS stimulation, estimated by the level of expression of *Dio2*,  
351 *Ucp1*, and *Elovl6*, is similar in the NTRKO BAT at 4°C compared to CTRL (Fig. 4F). However, the  
352 expression of the *Adrb3* gene encoding the adrenergic receptor beta 3 the main sensor in BAT for  
353 cold-triggered SNS activation, and of one of the genes up-regulated after this activation (*Ppargc1a*)  
354 were higher in the NTRKO BAT under the same condition. A possible interpretation of these results  
355 would be that a slight increase in BAT sensitivity to SNS stimulation allows NTRKO mice to face a cold  
356 challenge.

### 357 *3.4 Inhibition of T3 signaling in neurons alters the response to DIO*

358 When fed an HFD, NTRKO mice gained more weight than CTRL (Fig. 5A). This resulted from higher  
359 body fat accumulation (Fig. 5B) without any significant changes in food intake (Fig. 5C) or locomotor  
360 activity (Fig. 5D). Circulating levels of total cholesterol and triglycerides were regulated on HFD to a  
361 similar extent in both genotypes (Fig. S3C [33]). In WT animals, D2 enzymatic activity in the  
362 hypothalamus increased slightly but significantly after 3.5 days of HFD (Fig. 5E). To characterize the  
363 altered response of mutant mice to DIO, we measured gene expression in the BAT of NTRKO and  
364 CTRL mice after an HFD. The expression of genes involved in the thermogenic program or the  
365 response to SNS stimulation and the level of UCP1 protein were not affected (Fig. S6A [33]). The gene  
366 expression analysis in muscle did not provide an indication of a defect in non-shivering  
367 thermogenesis in NTRKO mice (Fig. S6B [33]). By contrast, quantification of *Ucp1* mRNA and UCP1

368 protein (Fig. 5F) in iWAT showed that browning was less efficient in the mutants under HFD.  
369 Therefore, an impairment in iWAT browning might explain the observed sensitivity to DIO (Fig. 5A).

370 *3.5 Neuronal T3 signaling modulates SNS signaling to regulate EE*

371 Inefficient browning of iWAT under HFD, increased expression of the *Adrb3* gene in BAT at 4°C, and  
372 decreased levels of UCP1 protein in both BAT and iWAT under different conditions, all point to  
373 possible inadequate SNS signaling in NTRKO mutants. We housed mice at 30°C, to address the  
374 sensitivity of mutant mice to DIO in the absence of thermogenic SNS stimulation [42]. In this  
375 condition, obesity was more pronounced in CTRL mice than at 23°C ( $\Delta$ body weight after 12 weeks of  
376 HFD, 9 g versus 5 g at 23°C). Most importantly, the difference between mutant and control mice  
377 disappeared at 30°C (Fig. S7A [33]). The level of UCP1 protein in BAT was similar in both genotypes  
378 under the CHOW diet (Fig. S7B [33]) in contrast to what was observed at 23°C. Normalization of the  
379 NTRKO phenotype at thermoneutrality provides a strong argument suggesting that the sensitivity of  
380 NTRKO to DIO at 23°C originates from a defect in the tone of the SNS. To address the alternative  
381 possibility, which would be that mutant thermogenic organs are less sensitive to SNS stimulation, we  
382 treated NTRKO and CTRL mice with NE to activate  $\beta$  adrenergic receptor in BAT and measured the  
383 induced change in EE (Fig. 6A). Mutant and control mice responded equally well to this stimulation,  
384 reinforcing our initial interpretation. This was further reinforced by performing tyrosine hydroxylase  
385 (TH) immunostaining in BAT and iWAT, which allows us to indirectly assess the autonomic nervous  
386 input and adrenergic signaling on both CHOW (Fig. 6B) and HFD (Fig. 6C) [43]. This revealed a slight  
387 alteration in the capacity of NTRKO mice to produce catecholamines on HFD (Fig. 6C).

388 We then assessed more directly the capacity of the hypothalamus to respond to a local T3  
389 stimulation by performing intracerebroventricular (ICV) injections of T3 (Fig. 6 D, E and F). This  
390 treatment reduced AMPK and acetyl-coA carboxylase (ACC) phosphorylation levels in the  
391 hypothalamus of CTRL mice, as previously described [9-10-44] (Fig. 6D). Notably, the steady-state  
392 levels of pAMPK $\alpha$  and pACC $\alpha$  was higher in NTRKO VMH than in the VMH of CTRL mice (Fig. 6E) and

393 was not decreased after local T3 injection (Fig. 6F). This supports the inhibitory effect of T3 on AMPK  
394 signaling and the requirement for functional TR in neuron to mediate this response.

#### 395 **4. Discussion**

396 Data published in recent years provided distinct but complementary pieces of information on the  
397 mechanisms by which exogenous T3 influences energy homeostasis [9-10-11-44-45]. ICV or intra  
398 VMH injection of T3 for short term or up to 28 days increases SNS tone, BAT activity and EE [9-10-44].  
399 Despite increased UCP1 expression in BAT and iWAT, systemic hyperthyroidism increases EE also *via*  
400 the activation of thermogenic mechanisms in the muscle [46-8]. Our study provides strong genetic  
401 support to previous hypotheses drawn from these experiments and sheds light on the importance of  
402 endogenous T3 signaling in neurons in facing thermogenic stresses.

403 Here we generated NTRKO, a new mouse model in which Cre- mediated mutations of TR in neurons  
404 induce a selective blockade of T3 signaling in these neurons. In a previous study, using the same Cre  
405 ( $Cre3^{Tg/+}Thra^{AM1/+}$ ) Richard et al [30]. described that the expression of *hairless (Hr)*, a T3 target gene,  
406 was deregulated in hypothalamus but not in other cerebral areas such as the hippocampus, the  
407 cortex and the cerebellum. The hypothalamus must be one of the brain areas that is the most  
408 impacted by the mutations. Here we show that the decrease of *Thrb* expression was only observed in  
409 the hypothalamus and not in peripheral tissues. This decrease was only partial, since *Thrb* is also  
410 expressed in glia and only 20 to 42% of neurons express Cre. However, it was sufficient to observe a  
411 significant decrease of *Shh* and *Klf9* expression, two well described T3 target genes, in response to  
412 T3. Importantly, the circulating levels of TH in NTRKO mice are similar to CTRL in all physiological  
413 conditions tested and so is the regulation of *TRH* expression by TH. This suggests that TR are still  
414 functional in TRH-expressing neurons to repress *TRH* expression in response to TH and that  
415 regulation of the hypothalamo-pituitary-thyroid axis by TH remains unaltered in NTRKO mice.  
416 Altogether these mice are euthyroid with functional T3 signaling in all cell types except for a fraction  
417 of neurons mainly in hypothalamus.

418 Selective blockade of T3 signaling in NTRKO neurons triggers a decrease in EE that mirrors the  
419 increase of EE previously described after T3 injection in the hypothalamus. Our data suggest that EE  
420 is altered in NTRKO as a result of decreased uncoupling of catabolism and ATP production in BAT.  
421 Calcium futile cycling in myotubes might produce heat at 23°C to compensate for the BAT defect and  
422 to maintain normal body temperature in NTRKO mice under this mild cold stress.

423 Importantly, our data show that endogenous T3 signaling in neurons is mainly relevant for an  
424 adequate response to HFD, as NTRKO manage to maintain body temperature after a cold challenge.  
425 D2 activity is increased in the hypothalamus in response to HFD but not cold. That suggests that the  
426 local production of T3 in the hypothalamus does not change during cold exposure and that the  
427 neuronal response to T3 is not recruited for cold-induced adaptive thermogenesis. In contrast local  
428 production of T3 in the hypothalamus would increase under HFD and the inability of NTRKO to  
429 properly detect intrahypothalamic production of T3 would be responsible for their higher sensitivity  
430 to DIO.

431 Interaction with the SNS is involved in the NTRKO sensitivity to DIO, as it is normalized at  
432 thermoneutrality. Modulation of the hypothalamic AMPK pathway plays an important role in the  
433 central action of T3 on both BAT and liver metabolism [9-10-44]. Our data show that while central T3  
434 decreases pAMPK $\alpha$  and pACC $\alpha$  protein levels, the absence of T3 signaling in neurons increases the  
435 basal level of these two phosphoproteins and blunts their regulation by T3. The expected result is a  
436 reduced SNS tone to BAT in NTRKO mice at 23°C on CHOW diet. Consequently, under these  
437 conditions the level of UCP1 protein in BAT is reduced. The associated decreased in BAT activity  
438 might be responsible for the decrease in EE and could be compensated by alternative thermogenic  
439 mechanisms, which include the activation of the Ca<sup>2+</sup> futile cycle in the skeletal muscle to maintain  
440 body temperature of NTRKO at 23°C. In any case, these results confirm that T3-induced  
441 thermogenesis in the brain mostly relies on stimulation of the BAT, while peripheral T3  
442 administration also stimulates not only BAT [6] but also muscle thermogenesis [8-46].

443 Thus, T3 signaling in neurons is an important player in the regulation of EE. The interference with the  
444 AMPK regulation for SNS activation, previously described after central administration of T3, appears  
445 to be also relevant when a physiological thermogenic stress stimulates the hypothalamic production  
446 of T3. Importantly NTRKO mice efficiently maintained their body temperature when exposed to an  
447 intense cold, displaying normal BAT and WAT response, despite a suboptimal SNS tone. We propose  
448 a model for the role played by T3 in neurons for the regulation of EE in response to cold and HFD in  
449 Figure 7.

450 This contrasts with data obtained in a mouse model that ubiquitously expressed TR $\alpha$ 1<sup>R384C</sup> another  
451 dominant negative TR $\alpha$ 1 [47]. Unlike NTRKO, these mice are hypermetabolic and hypothermic, with  
452 a theoretical defended temperature of 38°C [48]. In depth phenotyping led to the conclusions that  
453 this phenotype is primarily due to a chronic vasodilatation, which increases heat dissipation. The  
454 defect in vasoconstriction was found to reflect a reduced sensitivity of blood vessels to SNS  
455 stimulation [49] that should not take place in NTRKO mice.

456

#### 457 *Conclusions*

458 We show that T3 does control EE under physiological stresses *via* its action in neurons. Moreover,  
459 although selective inhibition of T3 signaling in neurons leads to a less severe metabolic phenotype  
460 than hypothyroidism, modulation of T3 concentration and signaling in neurons definitively  
461 represents a significant fraction of metabolic regulation by T3. Targeting T3 to neurons in adult might  
462 help fight obesity.

463

#### 464 *Limitations to our study*

465 First, the Cre-induced mutations occur in juveniles and are not restricted to adult life. Therefore, we  
466 cannot rule out that the post-natal maturation of neuronal circuits is altered in mutant mice even so  
467 we did not observe any obvious behavioral abnormality. Similarly, while the hypothalamus is the area  
468 of the brain that displays the highest percentage of Cre-mediated recombination and that is known

469 to control EE, the intervention of other brain areas in the determination of the metabolic phenotype  
470 of NTRKO is possible. Finally, only male mice were included in the study. Since sex specificity can  
471 occur in metabolic responses, similar experiments should be run on females.  
472 Further analyzes using models with selective inhibition of T3 signaling in other metabolic tissues,  
473 specific neuronal populations, and other brain cell types would clarify the mechanism by which T3  
474 regulates energy balance.

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488

## 489 **Author contributions**

490 KG, FF and ML conceived the study. KG, ER-P, CC ran the *in vivo* experiments. TDM participated in  
491 study design and interpretation of data. LC ran the thermography experiments, and performed the  
492 histology/immune histology studies, SW performed some immune histology studies, JW and AB

493 measured D2 activity. ER-P, KG, RG and SR performed *in vitro* experiments. KG, FF and ML wrote the  
494 paper. All authors helped analyze the data, reviewed and commented on the final manuscript.

495

## 496 **Declaration of competing interest**

497 We declare no competing interest.

## 498 **Figure titles and legends**

499 *Fig. 1: Neuron-specific Cre-mediated recombination in the hypothalamus in mice that carry the Cre3*  
500 *transgene (Cre3<sup>Tg/+</sup>/ RosatdTomato<sup>+</sup> mice).*

501 All cells expressing *tdTomato* (cre-mediated recombination reporter) also expressed NeuN (neuronal  
502 marker), while none of them expressed GFAP (glial cell marker). For each field, 4 images of confocal  
503 fluorescence microscopy are shown: tdTomato, combination of tdTomato and GFAP, NeuN,  
504 combination of tdTomato and NeuN. (A) In the rostral hypothalamus, about 20% of NeuN+ cells also  
505 expressed tdTomato. (B) In images with higher magnification, which were taken in the dorsomedial  
506 hypothalamus, arrows have been added to help verify that all tdTomato+ cells were also NeuN+.

507 *Fig. 2: Characterization of the NTRKO mice: a model of selective blockade of T3 signaling in neurons.*

508 The selective activity of Cre recombinase in the *Cre3<sup>Tg</sup>* transgenic line was evaluated by  
509 immunohistochemistry using the *Cre3<sup>Tg/+</sup>/ RosatdTomato<sup>+</sup>* reporter line (Fig. 1, Fig. S1). Different  
510 experiments were done to demonstrate neuronal blockade of T3 signaling in NTRKO mice. (A) *Thrb*  
511 expression was analyzed by relative RT-qPCR in different tissues of 3- to 4-month-old NTRKO (black  
512 n=6) and CTRL (white n=6) males fed a CHOW diet. (B) 6-month-old male mice CTRL or NTRKO fed a  
513 CHOW (n=5 and n=7) or an HFD for 3 months (n=5 and n=7) (left panel), or 3- to 4-month old males  
514 from NTRKO and CTRL rendered hypothyroid by propyl-thio-uracil (PTU) and treated (n=6 per  
515 genotype) or not by T3/T4 (TH) (n=6 per genotype) (right panel) were sacrificed and hypothalami  
516 sampled for RNA preparation. The white and black columns, respectively, represent CTRL and



517 NTRKO. Expression of *TRH* was measured by relative RT-qPCR in the hypothalamus. (C) Expression of  
518 well characterized T3 target genes (*Klf9*, *Shh*) was also measured in the hypothalamus of the same  
519 PTU/PTUTH treated animals by relative RT-qPCR. CTRL PTU, NTRKO PTU, CTRL PTUTH, and NTRKO  
520 PTUTH are, respectively, represented by white, black, light gray, and dark gray bars. (D) 3- to 4-  
521 month old CTRL (white bars n=10) and NTRKO (black bars n=9) male mice were on CHOW diet and  
522 injected daily with T3/T4 (TH) for 5 days from D0. Food intake was measured every day. CTRL animals  
523 CHOW fed (A, B left panel and D) or PTU fed (B right panel and C) were taken as the reference group.  
524 Statistical ANOVA tests were used for pairwise comparisons of CTRL and NTRKO mice. Stars indicated  
525 a significant difference between CTRL and mutants in a given condition, dollars a significant  
526 difference in a given genotype between two conditions. ns stands for non-significant.

527 *Fig. 3: T3 signaling in neurons regulates EE*

528 The different metabolic parameters were determined in 4-month-old CTRL (n=12) and NTRKO (n=12)  
529 male mice housed at 23°C on a CHOW diet. We measured (A) Body weight (B) body temperature (C)  
530 EE measured by indirect calorimetry (D), locomotor activity and (E) daily food intake. The gray-  
531 shaded areas represent night time. (F) Six-month-old male mice CTRL and NTRKO fed a CHOW diet  
532 (n=5 and n=7) at 23°C were sacrificed to sample BAT, iWAT. UCP1 immunostaining was performed  
533 on BAT and iWAT slices and quantified by image analysis. (G). The expression of genes involved in  
534 non-shivering thermogenesis was measured in the skeletal muscle of the same mice for RT-qPCR. The  
535 white and black columns, respectively, represent CTRL and NTRKO. Statistical T- tests were used for  
536 pairwise comparisons of CTRL and NTRKO mice in (A, B, C and D), statistical ANOVA tests for pairwise  
537 comparisons of CTRL and NTRKO mice. ns stands for non-significant.

538

539 *Fig. 4: Blocking T3 signaling in neurons does not affect cold-induced thermogenesis*

540 (A) D2 activity was assayed in hypothalamus (Hy) and BAT of 2-month-old C57bl6 males maintained  
541 for 58h at 4°C (n=6) (grey bars) or 23°C (n=6) (white bars). Five 4-month-old CTRL (n=6) and NTRKO

542 (n=6) male mice were housed at 4°C for 58 h. White and black squares/columns respectively  
543 represent CTRL and NTRKO. (B) Body weight, (C) temperature as measured by telemetry and (D) food  
544 intake were measured during cold exposure. BAT was sampled after cold-exposure for histology and  
545 RT-qPCR analyzes. (E) (left panel) UCP1 immunostaining was performed on BAT slices and quantified  
546 by image analysis. Markers of  $\beta$  adrenergic response/BAT activity in the BAT were analyzed by  
547 relative RT-qPCR (right panel). Statistical ANOVA tests were used for pairwise comparisons of two  
548 temperature in a given organ in (A), of CTRL and NTRKO mice in (B, C, D, E). ns stands for non-  
549 significant.

550

551 *Fig.5: Blocking T3 signaling in neurons prevents adequate response to Diet Induced Obesity (DIO)*

552 2- to 3- month-old CTRL (n=9) and NTRKO (n=9) male mice were fed a CHOW diet and then switched  
553 to an HFD (time 0). Their body weight was measured once a week. (A) Body weight gain ( $\Delta$  body  
554 weight) was calculated between any time point and the day diet was switched to HFD. (B) Body fat  
555 mass was evaluated by echoMRI at 3 time points. (C) Daily food intake was measured and locomotion  
556 (D) was studied for 4 consecutive days on HFD. (E) D2 activity was measured in the BAT and  
557 hypothalamus (Hy) of 2-month-old C57bl6 mice fed either a CHOW diet (n=5) or an HFD (n=6) for 3.5  
558 days. Tissues were sampled from 6-month-old male mice either CTRL or NTRKO fed an HFD for 3  
559 months (n=5 and n=7). (F) iWAT were used for histology or RNA preparation. UCP1 immunostaining  
560 was performed on iWAT slices (left panel). The expression of markers of browning in iWAT (right  
561 panel) were assessed by relative RT-qPCR. The white and black columns, respectively, represent CTRL  
562 and NTRKO. CTRL animals were taken as the reference group. Statistical T- tests were used for  
563 pairwise comparisons of CTRL and NTRKO mice in (A, B, C and D). Statistical ANOVA tests were used  
564 for pairwise comparisons of two temperature in a given organ in (E), of CTRL and NTRKO mice in (F).  
565 C, D, E). ns stands for non-significant.

566

567 Fig. 6: *Neuron T3 signaling interferes with SNS signaling to regulate EE*  
568 4-month-old CTRL and NTRKO male mice were injected (6 per genotype) (squares) or not with  
569 norepinephrine (NE) (n=6 per genotype) (circles) at the time marked by an arrow. (A) EE was  
570 recorded by indirect calorimetry before and after injection Tyrosine Hydroxylase (TH) is expressed in  
571 nerve endings and catalyzes the production of NE. TH immunostaining was performed and quantified  
572 on BAT (left panels) and iWAT (right panels) isolated from (B) CHOW-fed (n=5 for CTRL and n=7 for  
573 NTRKO) or (C) HFD-fed (n=9 of each genotype) CTRL or NTRKO 6-month-old male mice housed at  
574 23°C. 4-month-old CTRL and NTRKO male mice were ICV injected with T3 (n=6 per genotype) or  
575 vehicle (VEH) (n=6 per genotype). After dissection, proteins were extracted from VMH and the  
576 quantity of pAMPK $\alpha$  and pACC $\alpha$  estimated by western blot.  $\beta$ -actin is used for normalization. (D/E/F)  
577 Blots are provided on the left, (D/E/F) their quantification on the right. The previously described  
578 effect of T3 in CTRL is depicted in (D), the effect of the mutation is in (E), and the lack of effect of T3  
579 in NTRKO in (F). In the western blot analyses, values were expressed in relation to  $\beta$ -actin.  
580 Representative images for all proteins are shown; all the bands for each picture are derived from the  
581 same gel, although they may be spliced for clarity (represented by vertical black lines). pAMPK $\alpha$  and  
582 pACC $\alpha$  were assayed in the same membranes, therefore, some of the  $\beta$ -actin bands are common in  
583 their representative panels, this has been specified in Figures 6D-F. (A, D, E and F) Statistical T- tests  
584 or (B,C ) Statistical ANOVA tests were used for pairwise comparisons in (B, C, E) of CTRL and NTRKO  
585 mice , in (A) of CTRL NE and CTRL (S) and of NTRKO NE and NTRKO , in (D) of CTRL T3 and CTRL VEH  
586 and in (F) of NTRKO T3 and NTRKO VEH. ns stands for non-significant.

587 Fig. 7: *Proposed model for the role of T3 in neurone for regulation of energy expenditure in response*  
588 *to cold and HFD*

589 On the left panel, a scheme of regulation of energy expenditure in response to cold and HFD in CTRL  
590 mice. Black arrows picture the regulation observed under either COLD or HFD stimuli as compared  
591 respectively to 23°C and CHOW diet (the amplitude of the response might be different under cold

592 and HFD). The Blue arrows the regulation observed only at 4°C, the red the regulation observed only  
593 under HFD. The lightening sign stands for activation.  
594 On the right panel are summarized three situation in the NTRKO. In green it shows, the difference of  
595 energy regulation pathways in the NTRKO as compared to CTRL on regular conditions (CHOW and  
596 23°C). Black arrows picture the regulation observed in NTRKO under either COLD or HFD as  
597 compared respectively to 23°C and CHOW diet. Red arrows picture the regulation observed only  
598 under HFD as compared to CHOW in the NTRKO and the Blue arrows the regulation only observed at  
599 4°C as compared to 23°C in NTRKO as well.

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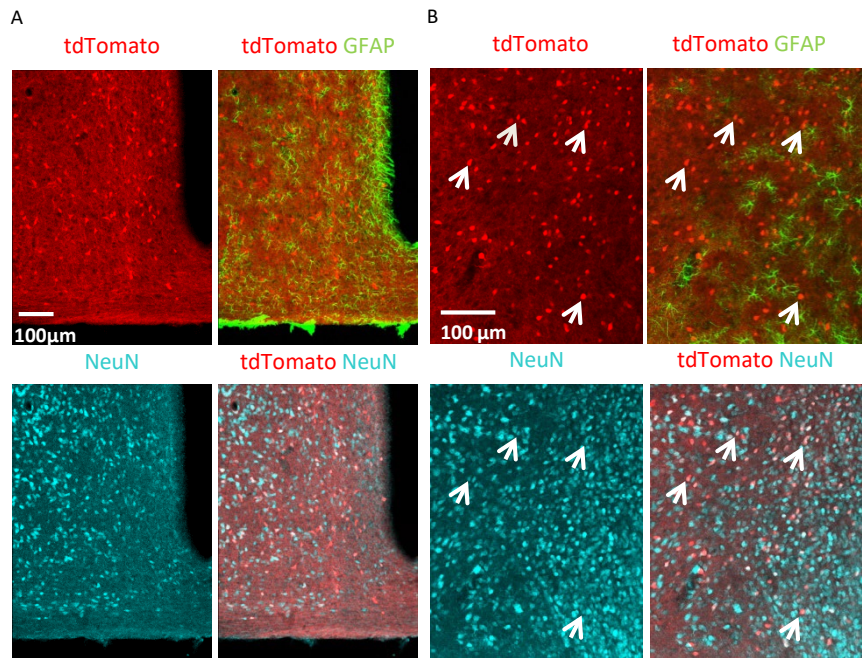
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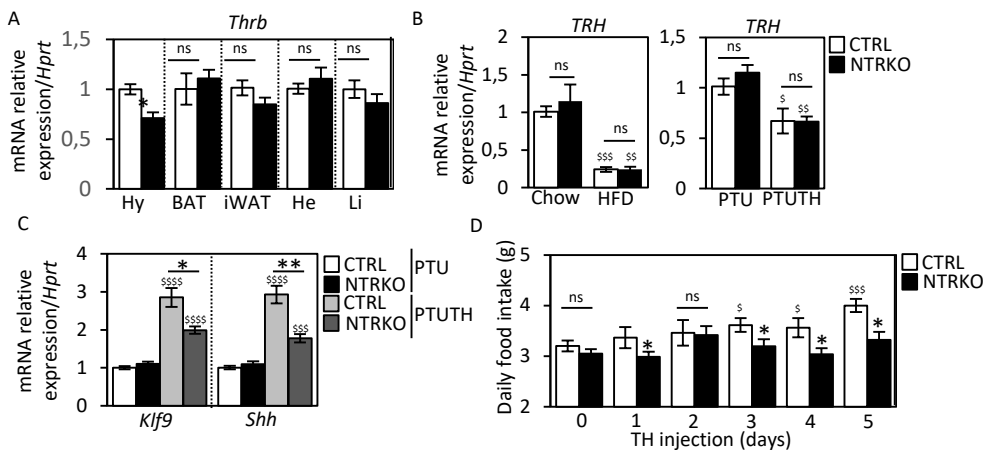
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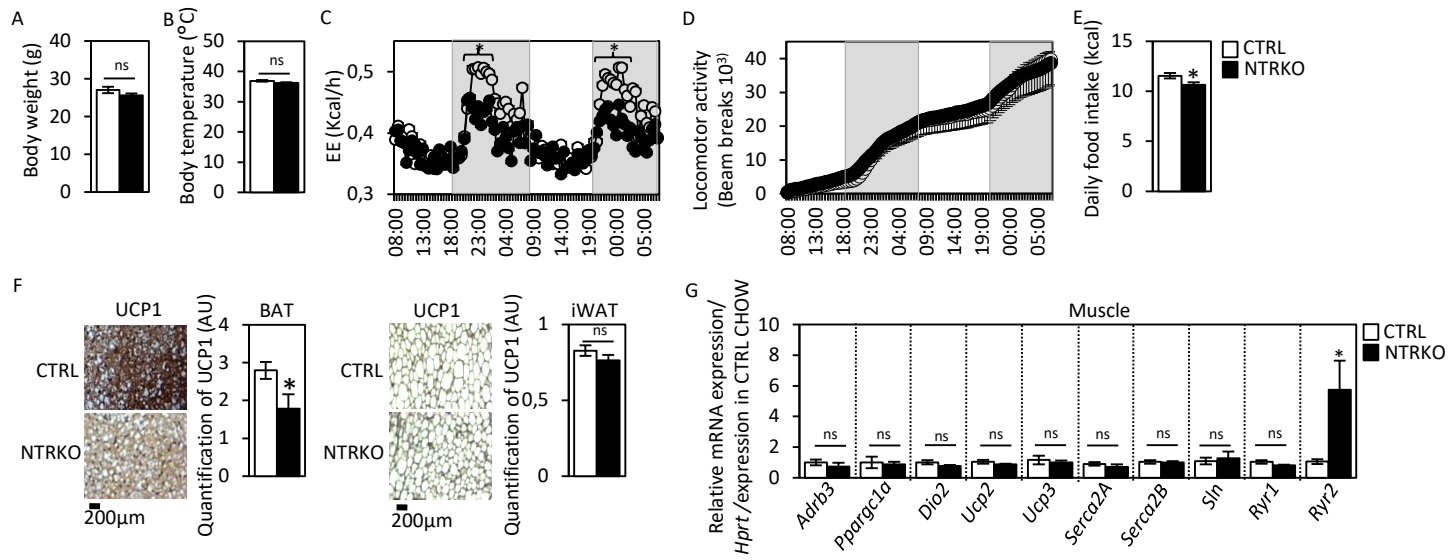
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**Fig. 1: color**

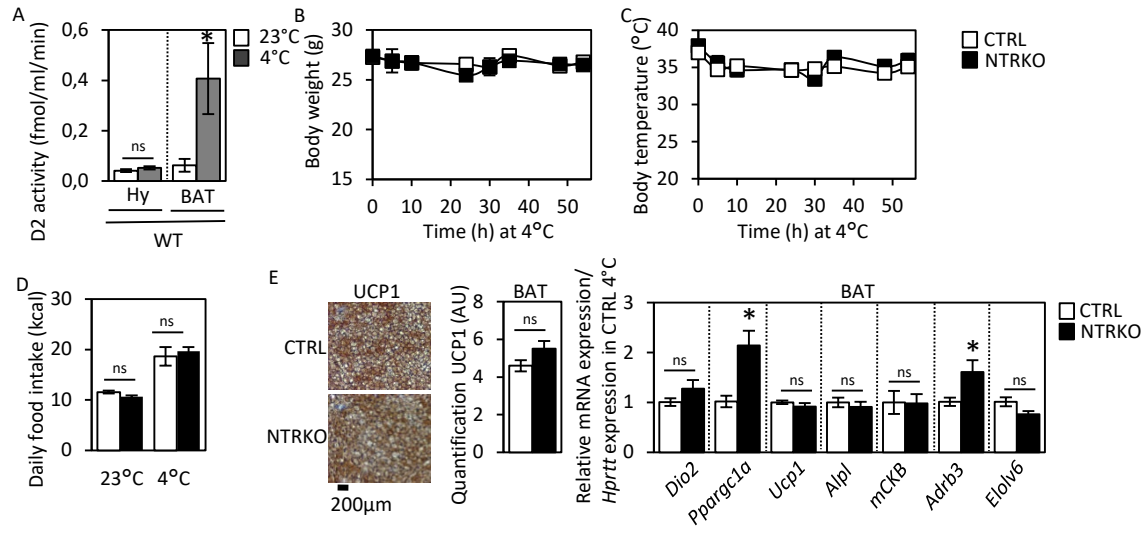


**Fig. 2**

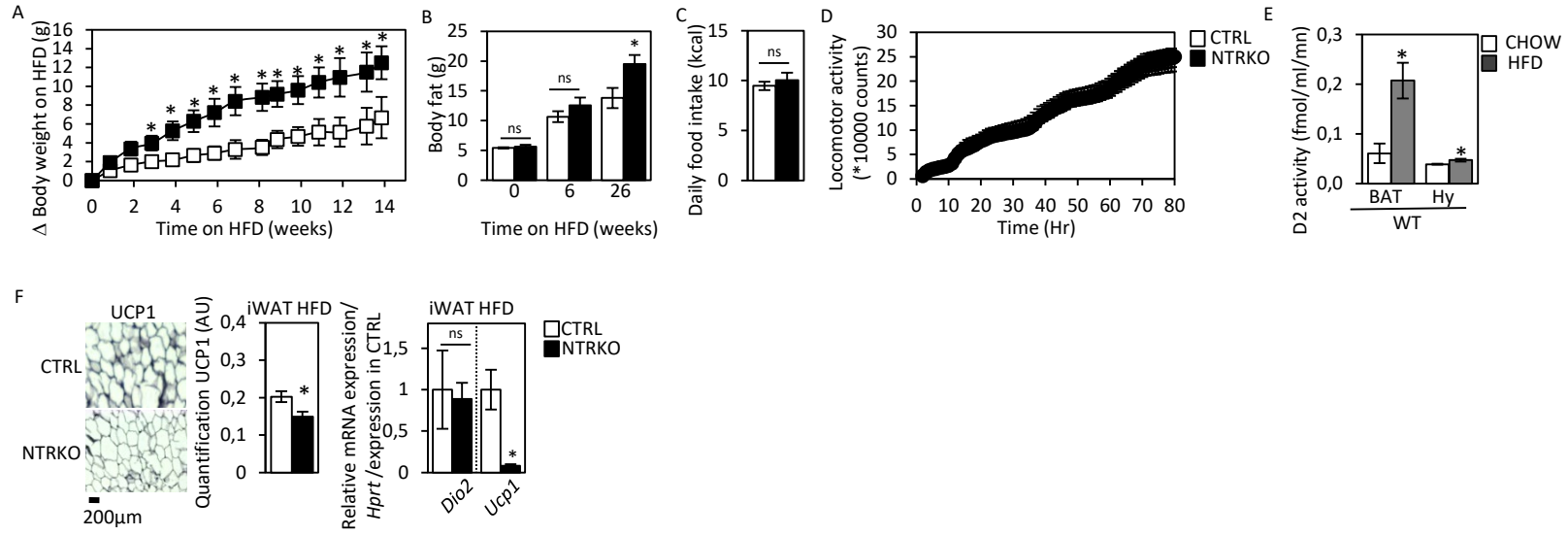
**Fig. 3: color**



**Fig. 4: color**

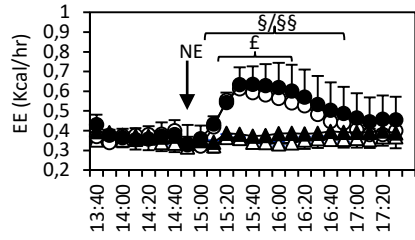


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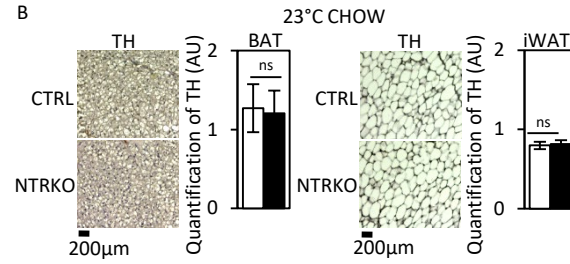


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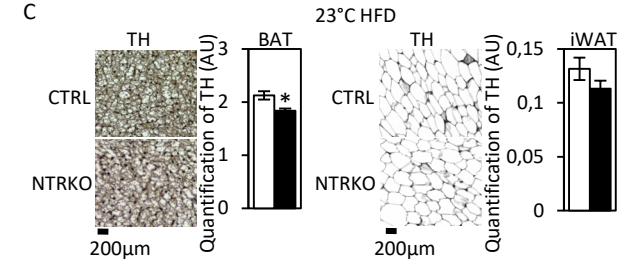
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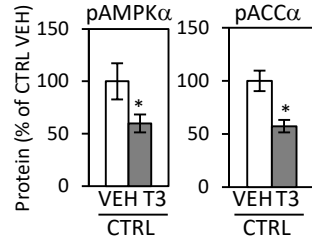
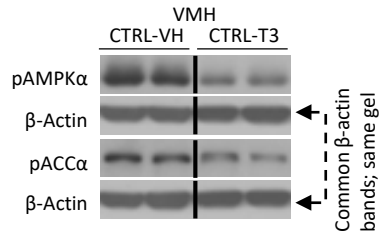
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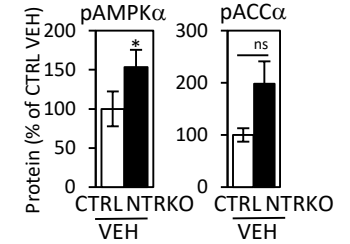
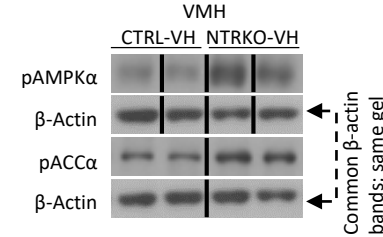
C



D



E



F

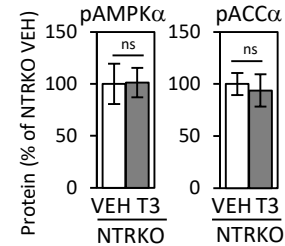
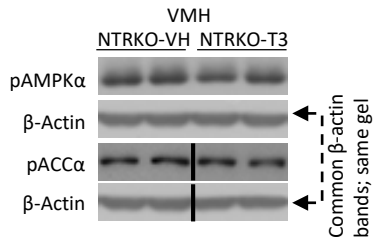
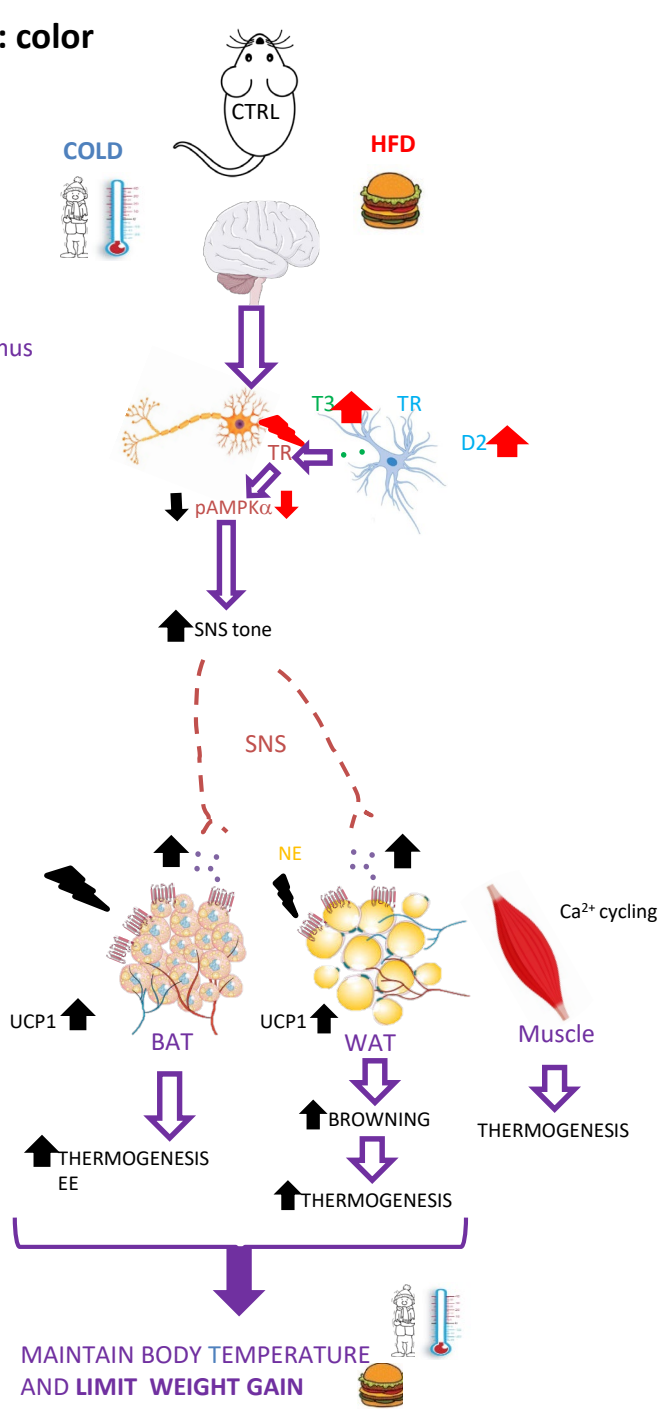
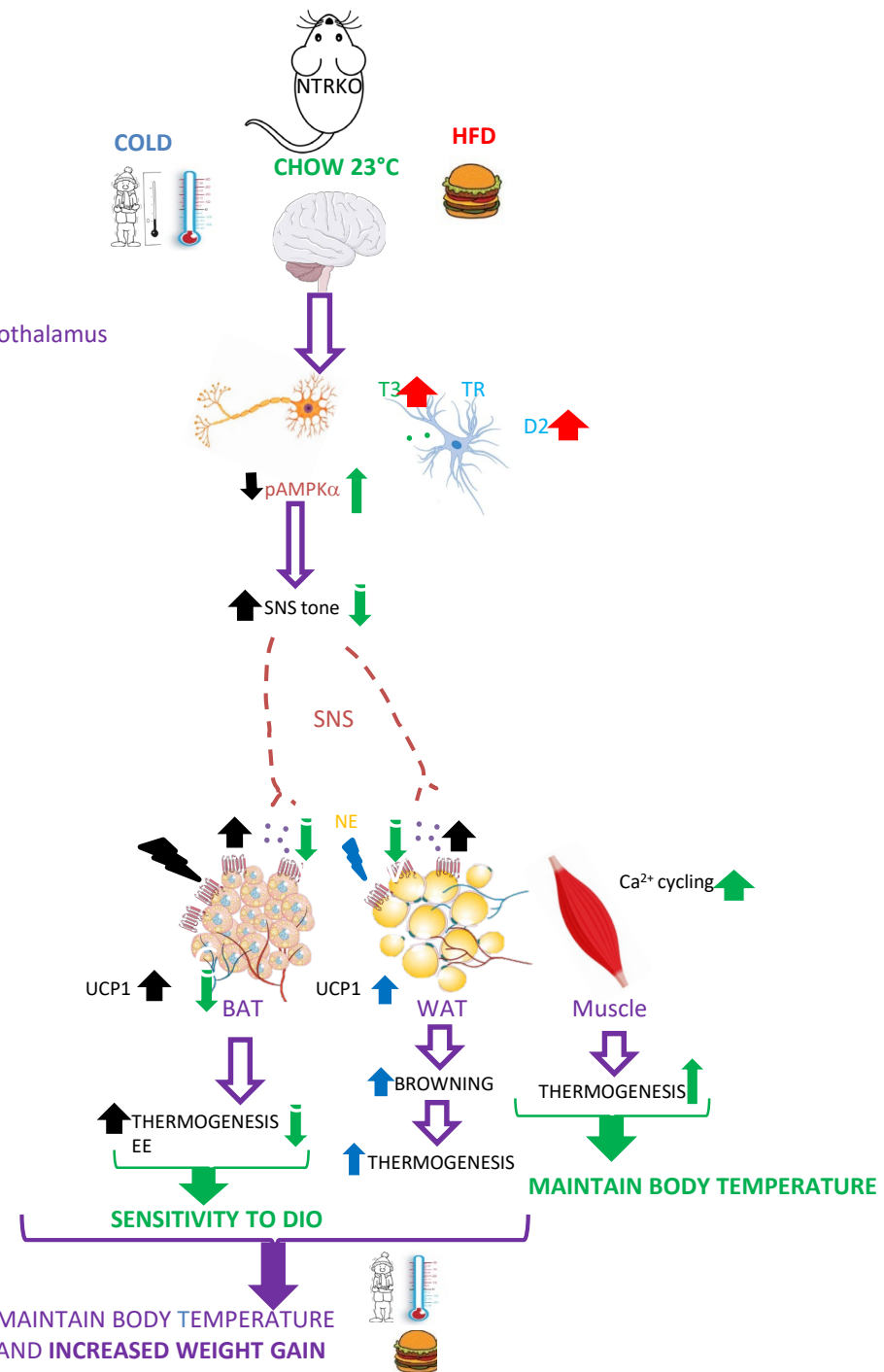


Fig. 7: color

Hypothalamus



Hypothalamus





## Supplemental information

### SI1

RNA extraction and expression analyzes by relative RT-qPCR: detailed procedure

#### RNA extraction

Total RNA was prepared using the TRI Reagent (ThermoFisher Scientific) protocol followed by a RNase free DNase treatment (Qiagen). Reaction was stopped by EDTA. Concentration and purity was quantified with a Nanodrop.

#### Reverse transcription:

- Heat 1 µg RNA in 9,5 µl H<sub>2</sub>O + 1 µl Random Hexamer Primer 100ng/µl, 70°C 5 min. Put on ice after the 5mn to avoid re-hybridization of RNAs.

- Add 9,5µl of MIX (4µl 5X MLV buffer Promega, 2 µl dNTP (5mM each), 0,5 µl RNasin Promega, 1 µl RT Promega (MMLV), 2 µl H<sub>2</sub>O)

Reaction is performed for 1h at 37°C and RT is inactivated by 10 min at 70°C on a BIORAD T100 thermal cycler.

cDNAs are diluted 1:10, aliquote by 10µl and stored at -20°C

As we design our primers to span different exons, we do not use « no RT » control for each sample.

#### qPCR :

Triplicates are always better than duplicates, but more expensive. As qPCR is very reproducible in our hands we use duplicates. Should the CV of a sample >4%, this sample is discarded and should be analyzed again . It is however much more important to have biological repeats.

cDNA are diluted 1 :10 and 2µl is used for each qPCR. Add 8µl of the reaction mix (5µl of 2X syber IQ supermix (Biorad), 330nM final concentration of each Forward and Reverse primers).

For each gene tested we use one “no template” control (no cDNA in QPCR) to check for contaminants in mixes and that is important for the melting curve (detection of primer dimers). Primers have been verified for efficiency on a comparable sample (same tissue, cell type...) → see primer design.

We use HPRT as our reference gene that display a similar abundance to our genes of interest (usually HPRT for Cq around 24).

Samples are run on a BioRad CFX96 qPCR machin. The program used is universal, and the same for all the primers. The program end with a « melting curve » step to verify that only one amplicon is produced with neither water contamination nor primer dimers.

Program : 95°C 3'essential for release of the Taq chemical inhibition, (95°C 10" denaturation, 64°C 30" annealing / elongation, plate read out) x40, from 65 to 95°C, 5" every 0,5°C, read-out to determine the melting curve.

#### Primer design and validation :

We use primer blast (primer3 + blast) on [http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK\\_LOC=BlastHome](http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The most important criteria are :

-primers must span an exon-exon junction” or “Primer pair must be separated by at least one intron on the corresponding genomic DNA” (to avoid amplification of contaminating genomic DNA).

-Amplified fragment should be between 80bp and 150bp. Tm around 60-62-64°C.

-%GC between 40% and 60%.

To test the efficiency of each primer pair, we performed a standard curve on cDNA dilutions spanning at least 4 or 5 log, and also a negative control. cDNA should be from the same tissue as our samples. Efficiency is calculated, by the following formula :  $Eff = 10^{-1/slope} - 1$ . It should be as close as possible to 100%, we qualify primers between 90 and 110% ( $3.6 > slope > 3.1$ ) This experiment also checks for the unicity and size of the amplicon and the absence of primer dimerization.

### Data analysis.

We are using the Software provided with the CFX, id BioRad CFX Maestro, use CFX Manager. Cq is automatically determined by the software. We consider a Cq of 35 as the upper limit of sensitivity. Melting curve is provided for each sample, and amplification efficiency can be measured.

The  $2^{-\Delta\Delta Cq}$  method is used for standardization using HPRT as the reference gene and control group was CHOW fed, HFD fed or PTU CTRL groups. We verify duplicate reproducibility and use the average value. Statistics (average, standard deviation, etc...) are made with biological repeats, not technical repetitions. The numbers of mice used for each experiment are reported in each figure legend, but never lower than 5.

## **Supplemental information titles and legends**

Fig. S1: *CRE activity in the peripheral metabolic organs*

$Cre3^{Tg/+}RosatdTomato^+$  and  $RosatdTomato^+$  mice (n=3 per genotype), were dissected and their tissues observed under the Leica M205FA fluorescent stereomicroscope. The green line delimits the  $Cre3^{Tg/+}RosatdTomato^+$  (upper) from the  $RosatdTomato^+$  (lower) organs. Results are shown from one mouse of each genotype that is representative of the 3.

Fig. S2: *Neuron-specific Cre-mediated recombination in different hypothalamic nuclei in mice bearing the Cre3 transgene ( $Cre3^{Tg/+}/RosatdTomato^+$  mice).*

All cells expressing tdTomato (reporter for Cre recombination) also expressed NeuN (neuronal marker), whereas none of them expressed GFAP (glial cell marker). In the rostral hypothalamus, at the level of the (A) paraventricular nucleus of the hypothalamus, about 20% of NeuN+ cells also expressed tdTomato. At the level of the (B) rostral ventromedial hypothalamus, about 40% of NeuN+ cells also expressed tdTomato. At the level of the (C) caudal ventromedial hypothalamus, about 20% of NeuN+

cells also expressed tdTomato. For each brain level, the red squares in DAPI images help locate the fields shown with higher magnification for tdTomato, NeuN and GFAP labellings. Arrows have been added to help checking that all tdTomato+ cells were also NeuN+.

*Fig. S3: Characterization of the NTRKO mice: a model of selective blockade of T3 signaling in neurons.*

(A) Concentration of free T3 and free T4 were measured in the plasma of the different groups of mice used in the study. The white and black marks/columns, respectively, represent CTRL and NTRKO. (B) 3- to 4-month old males from NTRKO and CTRL rendered hypothyroid by propyl-thio-uracil (PTU) and treated (n=6 per genotype) or not by T3/T4 (TH) (n=6 per genotype) were sacrificed and BAT, iWAT, Heart (He) and Liver (Li) sampled for RNA preparation. Expression of well characterized T3 target genes measured in BAT, iWAT, Heart (He) (*Klf9* and *Hr*) and Liver (Li) (*Klf9* and *Spot14*) was analyzed by relative RT-qPCR. CTRL PTU, NTRKO PTU, CTRL PTUTH, and NTRKO PTUTH are, respectively, represented by white, black, light gray, and dark gray bars. (C) Concentration of total cholesterol and triglycerides were measured in the plasma of the different groups of mice used in the study (Chow same groups as in Figure 3, HFD same groups as in Figure 5. White and Black marks/columns respectively represent CTRL and NTRKO. Ns stands as non-significant.

*Fig. S4: T3 signaling in neurons regulates EE but the expression of BAT thermogenesis and I WAT browning are not altered in CHOW fed NTRKO.*

6-month-old male mice CTRL and NTRKO fed a CHOW diet (n=5 and n=7) at 23°C were also sacrificed to sample BAT and iWAT for relative RT-qPCR analyzes. Markers of adrenergic response/BAT activity in (A) BAT and browning in the (B) iWAT were analyzed by relative RT-qPCR. The white and black columns, respectively, represent CTRL and NTRKO. Significance was calculated for CTRL/NTRKO. ns stands for non-significant.

Fig. S5: *Blocking T3 signaling in neurons does not affect cold-induced thermogenesis*

Five 4-month-old CTRL (n=6) and NTRKO (n=6) male mice were housed at 4°C for 58 h. (A) BAT activation was evaluated daily by thermography, average values were calculated from the data obtained for all mice before and after 24h or 48h at 4°C. (B) iWAT were sampled after cold-exposure for histology and RT-qPCR analyzes. (left panel) UCP1 immunostaining was performed on iWAT slices and quantified by image analysis. (right panel) Browning markers in iWAT were analyzed by relative RT-qPCR. White and black squares/columns respectively represent CTRL and NTRKO. Significance was calculated for a given organ between the two temperatures in A and between CTRL/NTRKO in B. ns stands for non-significant.

Fig.S6: *Blocking T3 signaling in neurons did not modify the expression of thermogenic markers, neither in BAT nor in skeletal muscle under an HFD.*

BAT and Skeletal muscles were sampled from 6-month-old male mice either CTRL or NTRKO fed a HFD for 3 months (n=5 and n=7). (A) BAT was used for histology or RNA preparation. (left panel) UCP1 immunostaining was performed and quantified on BAT slices. (right panel) The expression of *thermogenic markers* in BAT were assessed by relative RT-qPCR. Skeletal muscles were used for RNA preparation. (B) Expression of *thermogenic markers* in muscles were assessed by relative RT-qPCR. The white and black columns, respectively, represent CTRL and NTRKO. CTRL animals were taken as the reference group. Significance was calculated for CTRL/NTRKO. ns stands for non-significant.

Figure. S7: *Blocking T3 signaling in neurons in NTRKO mice did not alter their response to Diet Induced Obesity (DIO) at 30°C*

3-month-old male mice either CTRL (n=6) or NTRKO (n=5) were housed at 30°C fed a CHOW diet and then switched to a HFD (D0). (A) Body weight gain ( $\Delta$  body weight) was calculated between any time point and the day diet was switched to HFD. UCP1 immunostaining was performed and quantified in

(left panel) BAT and (right panel) iWAT isolated from CHOW-fed male mice housed at 30°C. The white and black columns, respectively, represent CTRL and NTRKO. Significance was calculated for CTRL / NTRKO. ns stands for non-significant.

**Fig. S1: color**

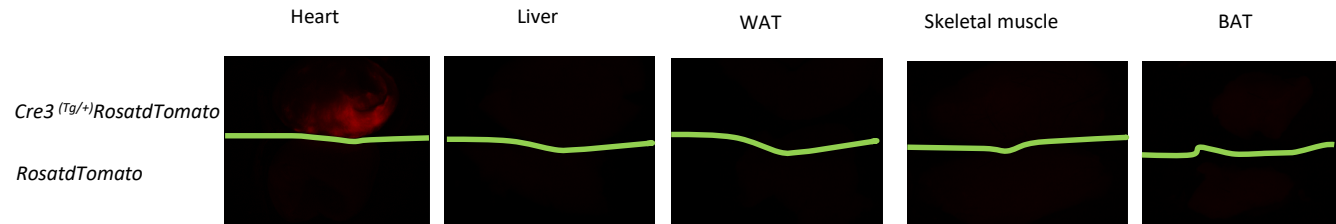
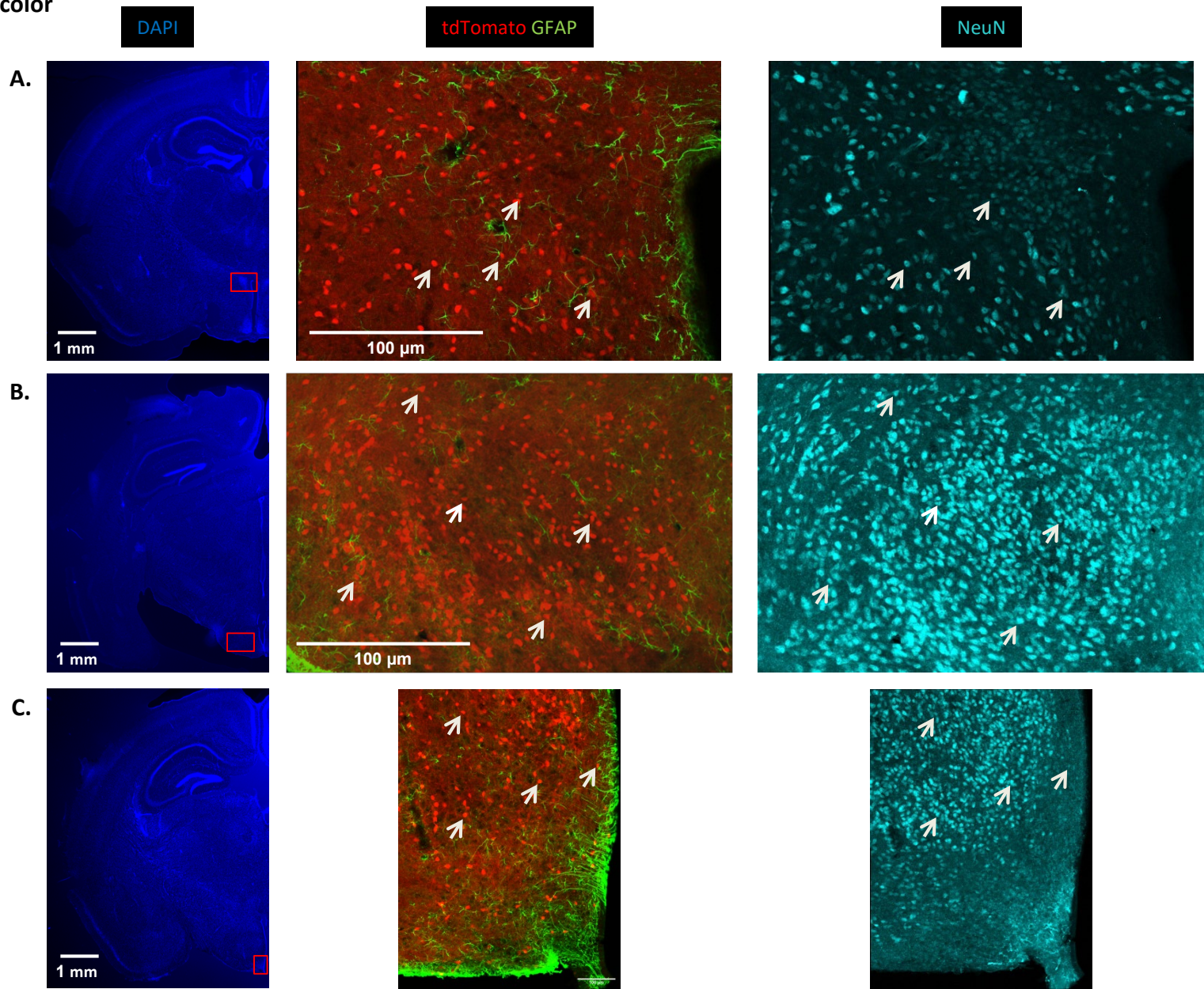


Fig. S2: color





**Fig. S3:**

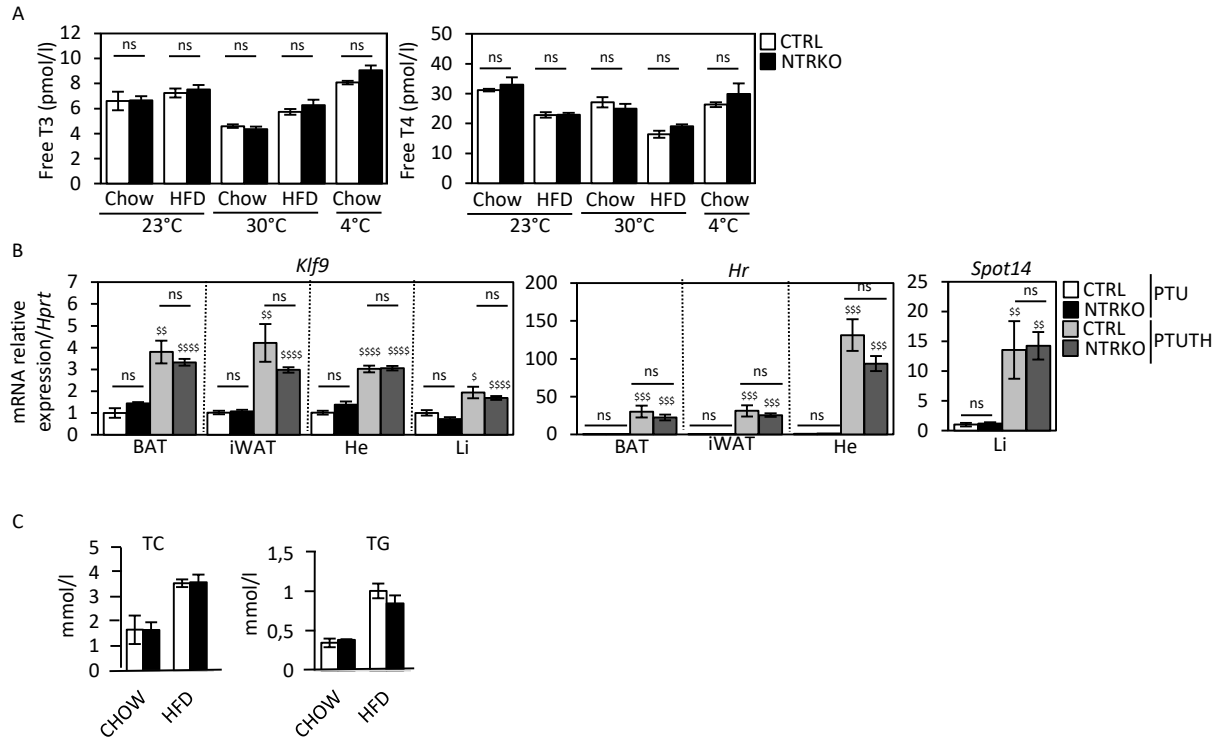


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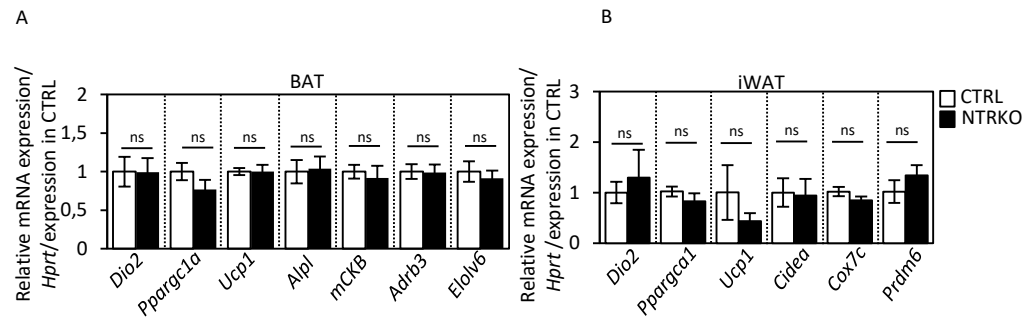
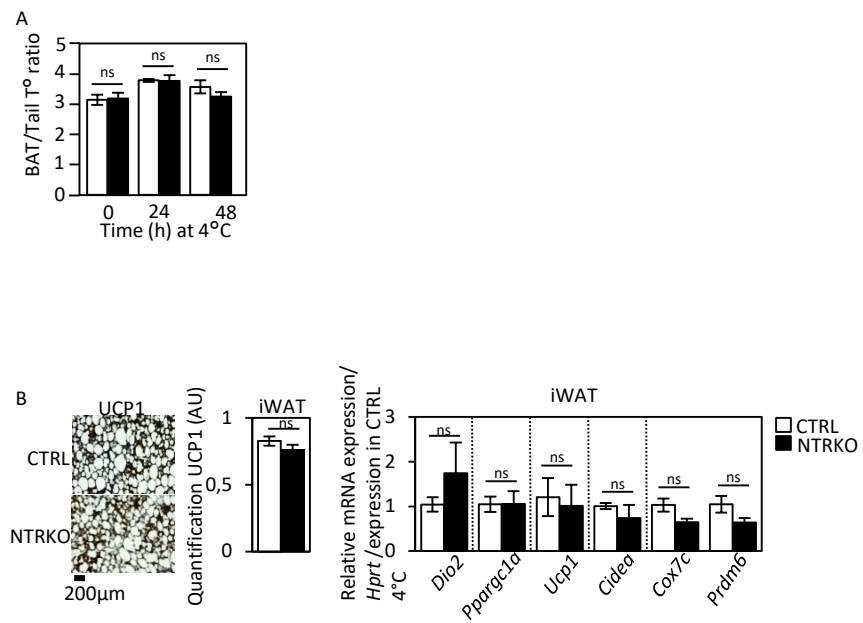
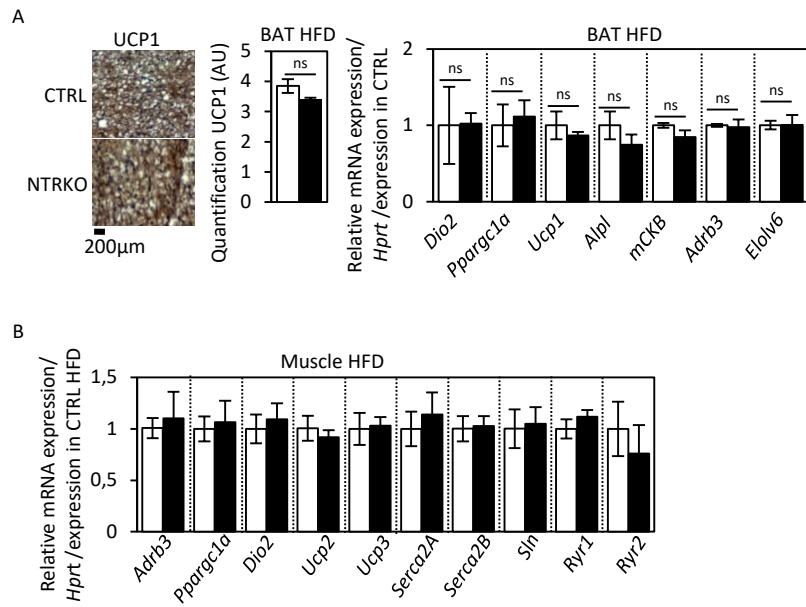


Fig. S5:



**Fig. S6:**



**Fig. S7:**

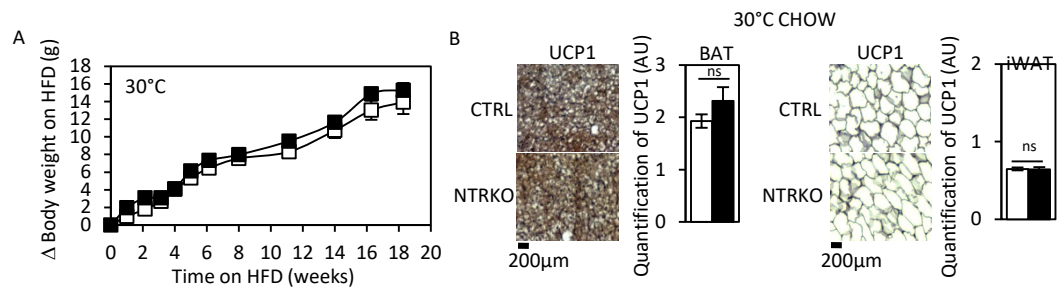


Table S1

	Forward primer	Reverse primer
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Cidea	TGACATTCATGGGATTGCAGAC	GGCCAGTTGTGATGACTAAGAC
Cox7c	CCA GAG TAT CCG GAG GTT CA	GAA AGG TGC GGC AAA CC
Dio2	CCT AGA TGC CTA CAA ACA GGT TAA A	TGT TAC CTG ATT CAG GAT TGG A
Elovl6	ACAATGGACCTGTCTAGCAAA	GTA CCA GTG CAG GAA GAT CAG T
Hprt	CAGCGTCGTGATTAGCGATG	CGAGCAAGTCTTTCAGTCCTGTCC
Hr	AGAGGTCCAAGGAGCATCAAGG	GCCTTGCTTCTATGATTGTCTCC
Klf9	CACGCCTCCGAAAAGAGGCACAA	CTTTTCCCAGTGTGGGTCCGGTA
mCBK	TGACCTGGACCCCAACTACG	AGGCTGGACAGAGCTTCTACTG
Ppargc1a	GAT GGC ACG CAG CCC TAT	CTC GAC ACG GAG TTA AAG GAA
Prdm6	CCCACATTCCGCTGTGAT	CTCGCAATCCTTGCACTCA
Ryr1	GAGTCTAAGCGCCAGTTCATCTTC	GCTCACGAACAGCTCCATCTTC
Ryr2	ATGGCTTTAAGGCACAGCG	CAGAGCCCGAATCATCCAGC
Serca2a	GTCATTTTCCAGATCACACCG	GTTACTCCAGTATTGCGGGTTG
Serca2b	ACCTTTGCCGCTCATTTTCCAG	AGGCTGCACACACTCTTTACC
Shh	AGCAGGTTTCGACTGGGTCTAC	ATTTGGCCGCCACGGAGTTC
Sln	GTCCTCTTCAGGAAGTGAAG	TGGCCCCTCAGTATTGGTAGG
Spot14	CTC GGA GGA GCT GGA CCT A	GTG ATG GAG GCT GCA GAA GT
Thrb	AGCCAGAACCCACGGATGAGGA	TGCCACCTTCTGGGGCATTAC
TRH	CTTGGTGCTGCCTTAGATTCCTG	CCAGGGTGCTGTCGTTTGTG
Ucp1	AAG CTG TGC GAT GTC CAT GT	AAG CCA CAAA CCC TTT GAA AA
Ucp2	GCCTCTGGAAGGGGAGTTCTC	ACCAGCTCAGCACAGTTGACA
Ucp3	TTTCTGCGTCTGGGAGCTT	GGCCCTCTTCAGTTGCTCAT