Thyroid hormone receptors are required for melatonin-dependent control of *Rfrp* gene expression in mice.

Clarisse Quignon[1], Matthew Beymer[1], Karine Gauthier[2], François Gauer[1], Valérie Simonneaux[1]

[1] Institut des Neurosciences Cellulaires et Intégratives (CNRS UPR 3212), Université de Strasbourg, Strasbourg 67000, France

[2] Univ Lyon, ENS de Lyon, INRAE, CNRS, Institut de Génomique Fonctionnelle de Lyon, 69364 Lyon, France

Running title: TRα is required for melatonin inhibition of RFRP-3

Corresponding authors :

Valérie Simonneaux,
Institut des Neurosciences Cellulaires et Intégratives,
8 allée du général Rouvillois, 67000 Strasbourg, France
Tel +(33) 6 60 64 23 78
Email: simonneaux@inci-cnrs.unistra.fr

Clarisse Quignon,
Institut des Neurosciences Cellulaires et Intégratives,
8 allée du général Rouvillois, 67000 Strasbourg, France
Tel +(33) 6 72 42 71 69
Email: c.quignon@inci-cnrs.unistra.fr

Matthew Beymer,
Institut des Neurosciences Cellulaires et Intégratives,
8 allée du général Rouvillois, 67000 Strasbourg, France
Tel : +(1) 61 75 49 43 66
Email: mjabeym@gmail.com

Karine Gauthier,
Institut de Génomique Fonctionnelle de Lyon,
32-34 avenue Tony Garnier, 69007 Lyon
Tel : +(33) 426731331
Email: karine.gauthier@ens-lyon.fr

François Gauer,
Institut des Neurosciences Cellulaires et Intégratives,
8 allée du général Rouvillois, 67000 Strasbourg, France
Tel +(33) 3 88 45 66 88
Email: gauer@inci-cnrs.unistra.fr

Abbreviations

TRα: Thyroid hormone receptor alpha

SP: Short photoperiod

LP: Long photoperiod

TSHβ: Thyroid stimulating hormone beta subunit

Dio2/3: Deiodinase 2/3

T4: thyroxine

T3: Triiodothyronine

Kp: Kisspeptin

RFRP-3: (Arg)-(Phe)-related peptide 3

DMH: Dorsomedial hypothalamus

Light/Dark: L/D

PBS: Phosphate Buffer Saline

TBS: Tris Buffer Saline

NaN3: sodium azide

PB: Phosphate Buffer

DEPC: Diethyl pyrocarbonate

SSC: Saline Sodium Citrate

TBI: Tris Imidazole Buffer

SEM: Standard error of the mean

a.u.: arbitrary unit

KO: Knock-out

Abstract

Mammals adapt to seasons using a neuroendocrine calendar defined by the photoperiodic change in the nighttime melatonin production. Under short photoperiod, melatonin inhibits the *pars tuberalis* production of TSHβ which in turn acts on tanycytes to regulate the deiodinase 2/3 balance resulting in a finely tuned seasonal control of the intra-hypothalamic thyroid hormone T3. Despite the pivotal role of this T3 signaling for synchronizing reproduction with the seasons, T3 cellular targets remain unknown. One candidate is a population of hypothalamic neurons expressing *Rfrp*, the gene encoding the RFRP-3 peptide, thought to be integral for modulating rodent's seasonal reproduction.

Here we show that nighttime melatonin supplementation in the drinking water of melatonin-deficient C57BL/6J mice mimics photoperiodic variations in expression of the genes *Tshb*, *Dio2*, *Dio3*, and *Rfrp*, as observed in melatonin-proficient mammals. Notably, we report that this melatonin regulation of *Rfrp* expression is no longer observed in mice carrying a global mutation of the T3 receptor, TRα, but is conserved in mice with a selective neuronal mutation of TRα. In line with this observation, we find that TRα is widely expressed in the tanycytes. Altogether, our data demonstrate that the melatonin-driven T3 signal regulates RFRP-3 neurons through non-neuronal, possibly tanycytic, TRα.

Key words: seasonal rhythms, deiodinase, tanycytes, neuropeptides, TSH

INTRODUCTION

Most species adjust biological and behavioral functions to align with the seasonal variations of their environment. This adaptive process is particularly critical to ensure that birth and weaning of the offspring occur around springtime when environmental conditions are optimal for the survival. Early studies, mostly performed on seasonal breeders such as Syrian or Siberian hamsters, and sheep, have highlighted the pivotal role of the hormone melatonin in synchronizing physiology with the seasons (1, 2). Indeed, under the control of the master hypothalamic clock, melatonin synthesis occurs at night only so that in long winter nights (or short photoperiod, SP) melatonin production is longer than in short summer nights (or long photoperiod, LP). Numerous experiments of pineal lesions or exogenous melatonin administration have formally demonstrated that it is the duration of nocturnal melatonin release which triggers the physiological response to seasonal change (3).

In the last fifteen years, major progresses has been made in determining how this melatonin signal is integrated (3, 4). The long SP melatonin peak inhibits expression of the β subunit of thyroid stimulating hormone gene (*Tshb)* in the pituitary *pars tuberalis* so that the melatonin signal is decoded into photoperiodic changes in TSH production with higher levels in LP compared to SP (5, 6). *Pars tuberalis* TSH in turn binds to receptors located on the tanycytes, specific glial cells lining the basal part of the third ventricle, triggering an increase in *deiodinase 2* (*Dio2*) and a decrease in *deiodinase 3* (*Dio3*) gene expression (7–14). As Dio2 converts thyroxine (T4) to the bioactive triiodothyronine (T3) and Dio3 inactivates T3, the melatonin-driven TSH-induced switch in Dio2/Dio3 leads to higher level of intra-hypothalamic T3 in LP as compared to SP. This photoperiodic change in the TSH/T3 signal is pivotal for seasonal synchronization of reproduction as along with body weight and temperature (10, 14–16). However, until now the cellular targets through which T3 synchronizes reproductive and metabolic functions have not yet been established.

Our previous studies have demonstrated the pivotal role of two neuropeptides known to regulate the activity of the GnRH neurons: kisspeptin (Kp), expressed in neurons of the arcuate nuclei, and arginine-phenylalanine-amide-related peptide 3 (RFRP-3), expressed by neurons in the dorsomedial hypothalamus (DMH) (17, 18). Both neuropeptides exhibit robust photoperiodic variations, with a highly conserved SP-inhibition of RFRP-3 expression across seasonal species. Chronic central infusion of Kp (19, 20) or RFRP-3 (21, 22) is able to restore gonadal activity of hamsters kept in inhibitory SP conditions. Furthermore, chronic central administration of TSH in SP-adapted hamsters not only restores the LP-phenotype of gonadal activity but also that of Kp and RFRP-3 expression (14). From these observations, we hypothesized that the melatonin-controlled hypothalamic T3 could act directly on Kp or RFRP-3 neurons to regulate seasonal reproduction.

The lack of genetically modifiable seasonal models such as hamsters or sheep has strongly hampered the investigation into the cellular and molecular mechanisms of seasonality. Unfortunately, the classic mouse models using C57BL/6J mice are melatonin-deficient, and melatonin proficient strains of mice such as CBA/N and C3H do not express overt seasonal phenotype. C57BL/6J mice however express melatonin receptors and acute melatonin administration has been shown to regulate *Tshb* and *Dio2/3* expression (11). In this study, we hypothesized that genetically modified C57BL/6J mice may help us better understand the molecular mechanism underlying melatonin action on the hypothalamic circuits regulating reproduction. We first showed that expression of *Tshb* and *Dio2/3* as well as *Rfrp*, the gene encoding RFRP-3, are regulated by exogenous administration of melatonin with a nighttime (SP-like) in C57BL/6J mice, in a similar way as in CBA mice exposed to SP. Further, we demonstrated that a global, but not a neuron-specific, mutation of the thyroid-hormone receptor alpha (TRα) in C57BL/6J mice, selectively abolishes the melatonin regulation of *Rfrp expression*, but not that of *Tshb* and *Dio2/Dio3*. Finally, we found that TRα is widely co-expressed with vimentin in the tanycytes. Altogether our studies indicate that non-neuronal T3 signaling, possibly in the TRα-expressing tanycytes, is required for the melatonin regulation of *Rfrp* expressing neurons.

MATERIAL AND METHODS

Animals

Adult male mice of different strains were used in this study: CBA/N and C57BL/6J wild type (WT) (purchased from Janvier Labs, Le Genest-Saint-Isle, France), C57BL/6J with a global knock-out (KO) of *Thra*, the gene encoding TRα (*Thra*$^{0/0}$) or their control littermate (*Thra*$^{+/+}$) (23) and C57BL/6J with a neuronal-specific expression of TRα1^{L400R}, a dominant negative form of TRα (*Cre3xThra*$^{AMI/+}$) or their control littermates (*Thra*$^{AMI/+}$) (24, 25), both provided by the Institut de Génomique Fonctionnelle de Lyon. The *Cre3* mouse line (26) presents *Cre* expression and activity in neurons but not in glial cells, and the percentage of neurons expressing *Cre* depends on the brain areas with a particularly high level in the hypothalamus (25). In the *Cre3xThra*$^{AMI/+}$mice, the *Thra* modification induces the expression of the TRα1^{L400R} mutant receptor, and despite the persistence of an intact *Thra* allele, the dominant-negative influence of TRα1^{L400R} eliminates any capacity of the cells to respond to T3 (24). In order to verify the phenotype of the mutated mice, we performed dual immunohistochemistry of TRα and RFPR-3 in two Cre3xThra$^{AMI/+}$ and observed, as expected, a strikingly low expression of TRα in the mediobasal hypothalamus and no colocalisation of TRα with RFRP-3 (data not shown).

Mice were housed in approved type II cages with food and water *ad libitum*, at 20°C ± 1°C, under 12 hours white light/ 12 hours red light (12h L/12h D) photoperiod for at least one week before experimentation. The first experiment investigated the effect of photoperiod in melatonin proficient and deficient mice: CBA/N and C57BL/6J WT were kept in LP (16h L/ 8h D) or SP (8h L/16h D) for 6 weeks (n=6 for each of the 4 groups). The second experiment investigated the effect of nighttime exposure to exogenous melatonin in

mice mutated for TRα (*Thra*$^{0/0}$ and *Cre3xThra*$^{AMI/+}$) or their negative littermates (*Thra*$^{+/+}$ and *Thra*$^{AMI/+}$): mice (n=5-7 per group) were placed in SP (10h L/14h D; here SP night was reduced to 14h to obtain a melatonin peak duration similar to that observed in 8h L/16h D adapted Syrian hamsters and melatonin proficient mice (27, 28)) and were given drinking water containing either melatonin (80 mg/L in 0.1% ethanol) or vehicle (0.1% ethanol) during the night, and pure water during the day for at least 6 weeks. This protocol allowed a 4 fold higher nighttime circulating melatonin concentration in mice drinking melatonin as compared to mice drinking vehicle (337,89 pg/ml versus 85,97 pg/ml, p=0.0386, measured by radioimmunoassay (29)). Although our preliminary data confirmed those of Ono et al (11) that an acute injection of melatonin per day is sufficient to trigger a response in the PT and hypothalamus (data not shown), we decided to perform the long term melatonin administration in the drinking water for physiological and practical reasons. Experiments were conducted in accordance with the French National Law implementing the European Communities Council Directive 2010/63/EU and the French Directive 2013-118. All procedures were validated by the Animal Welfare Committee of the Animal Resource and Experimentation platform (Chronobiotron UMS 3415) of the Strasbourg Institute of Neuroscience.

Tissue sampling

At the end of the experiment, animals were euthanized in the morning (from 1 h to 4 h after lights on) by CO2 inhalation. Body weight was measured and testis were dissected and weighted, animals were then transcardiacally perfused first with 10 mL phosphate buffer saline (PBS) then 20 mL periodate-lysine-paraformaldéhyde fixative solution (30). Brains were extracted, conserved in the fixative solution for 12 h then dehydrated through an ethanol gradient and embedded in polyethylene glycol (31). Serial 12 µm-thin coronal sections were made with a microtome from the preoptic area to the posterior hypothalamus. For each animal, serial sections were dispatched on 10 SuperFrost® Plus slides (Thermo Scientific, MA, USA) in order to have 14 to 18 sections spanning the area of interest per slide. Brain sections were dried 15 min at 60°C and stored either at -80°C for *in situ* hybridization or at 4°C in Tris buffer saline (TBS) with 0.02% sodium azide (NaN3) for immunohistochemistry.

Non-radioactive in situ hybridization

In situ hybridization was performed with 300 to 400 nucleotide-long antisense riboprobes selective for mouse *Tshb* (NM_009432.2), *Dio2* (NM_010050.4), *Dio3* (NM_172119.2), *Rfrp* (NM_021892.1) and rat *kiss1* (NM_181692.1) validated on mouse tissue and labeled with digoxigenin (DIG RNA labeling kit, Roche, Meylan, France) according to Klosen et al, 2013 (14). Briefly, brain sections were post-fixed in formaldehyde 4% in phosphate buffer (PB) for 10 min at room temperature, washed with PBS, treated by 0.5 µg/mL proteinase K (Roche) for 30 min at 37°C, washed with cold PBS, post-fixed in formaldehyde 2% in PB, washed in PBS + 0.01% diethyl pyrocarbonate (DEPC), acetylated twice for 10 min in 100 mM triethanolamine with respectively 0.25% and 0.5% acetic anhydride, washed with PBS + 0.01% DEPC,

equilibrated in 5X saline sodium citrate (SSC) + 0.05% tween 20 + 0.01% DEPC twice for 5 min at room temperature, and finally incubated for 48 h at 60°C with 1% (about 200ng/ml) riboprobes in a hybridization buffer containing 50% formamide + 5X SSC + 5X denhart solution + 1 mg/ml salmon DNA + 0.1% tween 20 + 0.04% DEPC. Slides were then washed once in 5X SCC + 0.05% tween 20 + 0.01% DEPC buffer, and post-hybridization was performed at high stringency to reduce non-specific labeling in a 0.01X SSC + 0.05% tween 20 solution at 72°C for 6 x 10 min. Slides were washed once in A-DIG + 0.05% tween 20 solution, incubated with blocking buffer (Roche) + 0.02% azide (BBT) for 1 h, and riboprobe labelling was detected by an overnight incubation with an anti-digoxigenin antibody coupled to alkaline phosphatase (AP, Roche; 1/5000 in BBT). Thereafter, slides were washed once in A-DIG + 0.05% tween 20 solution then equilibrated in AP buffer. AP activity was visualized with a mixture of nitro blue tetrazolium (NBT)/ bromo-chloro-indolyl phosphate (BCIP) in AP buffer for 1 to 7 h depending on the probes and stopped with pure water before the staining intensity reached saturation. Slides were then covered with cristal mount premounting media (Sigma Aldrich, MO, USA) and coverslipped with Eukit (Sigma Aldrich).

Immunohistochemistry

The *Tshb* mRNA level being very low in the C57BL/6J mice *pars tuberalis,* we performed an immunochemical detection of the TSHβ protein to study melatonin-dependent variations of TSHβ expression in these mouse strains, given the tight correlation between *Tshb* mRNA and protein levels (6). Immunolabelling of TSHβ was done at room temperature using an avidine-biotin system of detection. Brain sections were pretreated with a blocking reagent (TBS + 3% powder milk + 0.02% NaN3) for 1 h, incubated overnight with the primary TSHβ antibody (rabbit anti-TSHβ antibody; National Hormone and Pituitary Program AFP-12747891; diluted at 1/65 000 in TBS + 0.05% tween 20 + 1% donkey serum), washed in TBS + 0.02% tween 20, and finally incubated with a biotinylated secondary antibody (donkey anti-rabbit bio; Jackson ImmunoResearch, PA, USA; diluted at 1/2 000 in TBS + 0.05% tween 20 + 1% donkey serum) for 1 h. After washing in TBS + 0.02% tween 20, the signal was amplified by a 1 h incubation of neutravidine-horseradish peroxydase solution (HRP; Thermo Scientific; 1/2 000 in TBS + 0.02% tween 20 + 0.2% cold water fish gelatin). Sections were washed twice in TBS + 0.02% tween 20, then once in Tris-Imidazole buffer (TBI) buffer and then HRP activity was detected with a 30 min incubation in 1% diaminobenzidine (DAB) + 0.003% H_2O_2 in TBI buffer. Finally, brain sections were dehydrated by 5 min incubation in solutions of increasing alcohol concentration (from 70 to 100%), and coverslipped with Eukit.

In order to look for TRα localization in tanycytes, we performed dual immunochemical labelling of TRα with the tanycyte marker vimentin in C57BL/6J mice. Brain sections were first treated for an antigen reactivation in citrate buffer (pH 6.0) for 1 h at 95°C, washed twice in PBS, pretreated with the blocking reagent for 1 h, and incubated with the primary TRα antibody (rabbit anti-TRα antibody; tested (32) and kindly provided by Dr L. Dufourny (INRA, Nouzilly); diluted at 1/250 in TBS + 0.20% triton X100 + 1%

donkey serum) for 48 h at 4°C, washed in TBS + 0.02% tween 20, and finally incubated with an Alexa 594 secondary antibody (donkey anti-rabbit Alexa594; Invitrogen, CA, USA; diluted at 1/1 000 in TBS + 0.05% tween 20 + 1% donkey serum) for 1 h. After two washing with TBS, the vimentin labelling was performed following the same protocol than for TSHβ immunolabelling using a vimentin primary antibody (chicken anti-vimentin antibody; Merck, Darmstadt, Germany; diluted at 1/5 000), a biotinylated anti-chicken secondary antibody (anti-chicken bio; Jackson ImmunoResearch; diluted at 1/2 000), and HRP activity detection was done by using hapten-conjugated tyramides (diluted at 1/500 in PBS + 100 mM imidazole + 0.001% H_2O_2). Slides were washed with PBS, covered with cristal mount, and coverslipped with Eukit.

Labeling of both TSHβ and TRα/vimentin was observed with an optic microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with a fluorescent lamp (Leica Microsystems) to visualize fluorescent staining.

Data analysis and statistics

For each gene/protein investigated, brain sections of all animals of a given experimental group were processed similarly (treatments for labeling, optical microscope setting and analysis). Signal quantification was done by a person unaware of the animal's experimental condition.

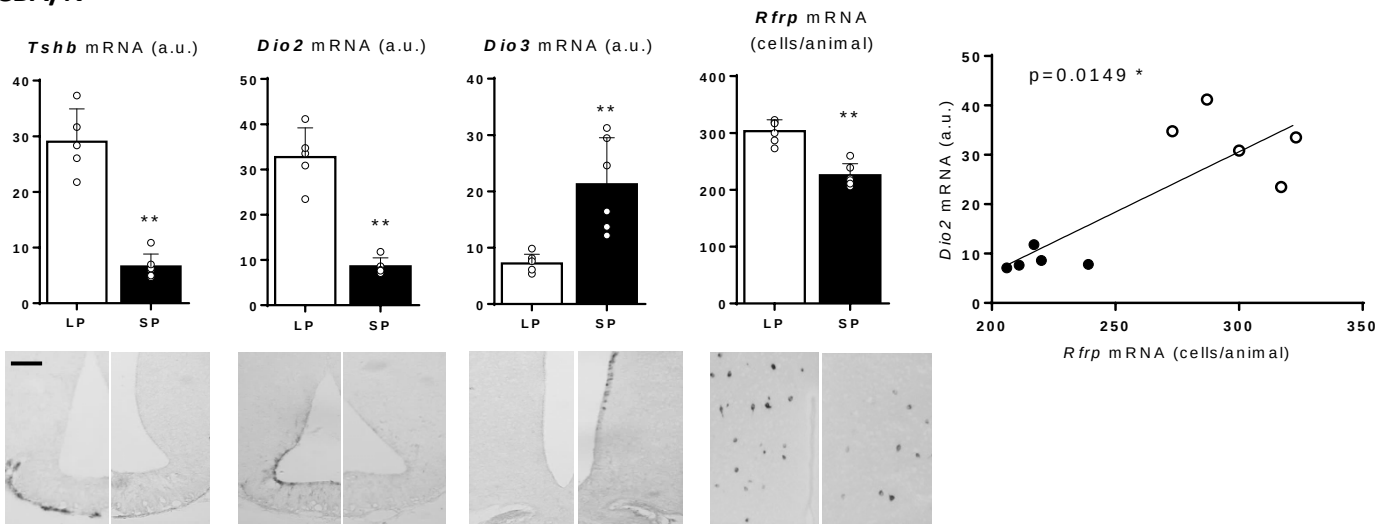
For the quantification of *Kiss1* and *Rfrp* expression, the total number of labeled neurons in all sections of an individual hypothalamus was counted manually with an optic microscope (Leica Microsystems), equipped with an Olympus camera (Olympus France, Rungis, France, x20). For each animal, data is given as the total number of counted labelled neurons. For the quantification of *Tshb*, *Dio2* and *Dio3*, the intensity of the signal was quantified with the ImageJ software (W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD, USA), on pictures taken with an optic microscope equipped with camera (Leica Microsystems, x10). All pictures for a given gene were taken with the same microscope and camera setting parameters and a background image without section was taken for each slide and subtracted to overcome any irregularities of illumination of the picture field. Signal intensity was measured by calculating the mean pixel grey value (arbitrary unit) of a line covering the entire *pars tuberalis* for *Tshb*, and the β and α tanycytes for *Dio2* and *Dio3* respectively. For each animal, data results from the mean of signal intensity measured on 3 selected sections of the area of interest.

All data are presented as mean ± SEM of 4 to 7 animals per experimental group. Graphs and statistical analysis were done with GraphPad 6.0 (GraphPad software Inc., San Diego, CA, USA). Variance homogeneity and normality were tested and an adapted Mann-Whitney non-parametric test was performed to analyze the mean differences of expression for each gene and a non-parametric Spearman test was performed to analyze the correlation between *Dio2* and *Rfrp* expression.

RESULTS

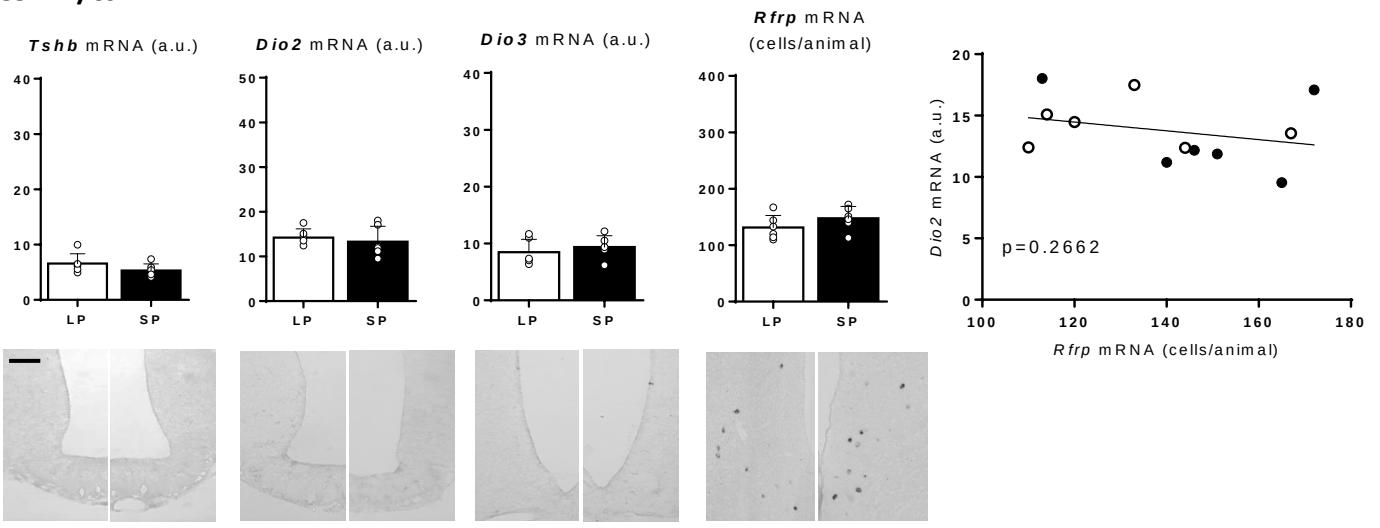
A

CBA/N



B

C57BL/6J



Differential photoperiodic gene regulation between melatonin-proficient (CBA/N) and melatonin-deficient (C57BL/6J) mice

In order to assess how photoperiod regulates expression of *Tshb* in the *pars tuberalis*, *Dio2* and *Dio3* in the tanycytes, and *Rfrp* and *Kiss1* in the hypothalamus of both melatonin proficient and melatonin deficient mice, both CBA/N and C57BL/6J were exposed to either LP (16L/8D) or SP (8L/16D) conditions for 6 weeks. SP exposed CBA/N mice exhibited robust inhibition of *Tshb*, *Dio2*, and *Rfrp* expression, and induction of *Dio3* expression as compared to LP exposed CBA/N mice (Figure 1A). In contrast, in the C57BL/6J mice, none of these genes showed photoperiodic change in their expression (Figure 1B). Moreover, in CBA/N mice *Dio2* expression is strongly correlated to the number of *Rfrp* expressing neurons whereas there is no such correlation in C57BL/6J mice (Figure 1A and B). No photoperiodic change in *Kiss1* expression and the weight of body, testis, and seminal vesicles has been observed in the two mouse strains (data not shown).

Exogenous melatonin restores SP inhibition of Rfrp expression in control Thra$^{+/+}$ but not Thra$^{0/0}$ C57BL/6J *mice*

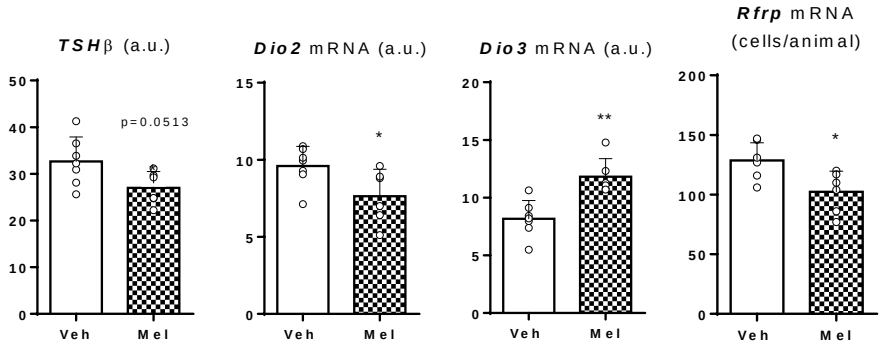
In order to investigate whether melatonin is able to regulate pituitary and hypothalamic gene expression in *C57BL/6J* mice, exogenous melatonin or vehicle were provided in the drinking water during the dark period. Nighttime melatonin induced a significant decrease in TSHβ, *Dio2* and *Rfrp* expression and a significant increase in *Dio3* expression (Figure 2A) therefore mimicking the effect of SP in melatonin proficient CBA/N mice (Figure 1A). To assess whether this melatonin signal integration requires functional TRα, a similar melatonin supplementation was given to mice bearing a complete mutation of TRα. In *Thra$^{0/0}$* mice, melatonin still reduced TSHβ and *Dio2* and increased *Dio3* expression but was no longer able to inhibit *Rfrp* expression (Figure 2B). Moreover, while there was a significant correlation between *Dio2* and the number of *Rfrp* expressing neurons in the C57BL/6J WT mice, this correlation no longer existed in the *Thra*$^{0/0}$ mice (Figures 2A and B). This result demonstrates the key role of TRα in mediating the melatonin-dependent thyroid hormone effects on *Rfrp* expression. As previously observed for the photoperiod experiment, melatonin administration had no effect on *Kiss1* expression and on the weight of body, testis, and seminal vesicles in the two mouse strains (data not shown). Of note however, and in agreement with earlier observations (33), *Thra$^{0/0}$* mice appeared more anxious and slightly smaller (body weight = 24.6 ± 2.7g, n = 13) than their control littermate (body weight = 26.2 ± 4.1 g, n = 13; Student t-test p = 0.0409).

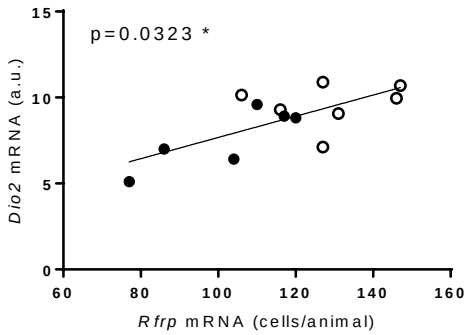
Melatonin inhibits Rfrp expression through non-neuronal TRα

As TRα is expressed by various cell types in the nervous system (34), we further investigated whether TRα-expressing neurons mediate the inhibitory effect of melatonin on *Rfrp* expression in the DMH RFRP-3 neurons. Nighttime melatonin supplementation in drinking water was thus provided in C57BL/6J mice

A

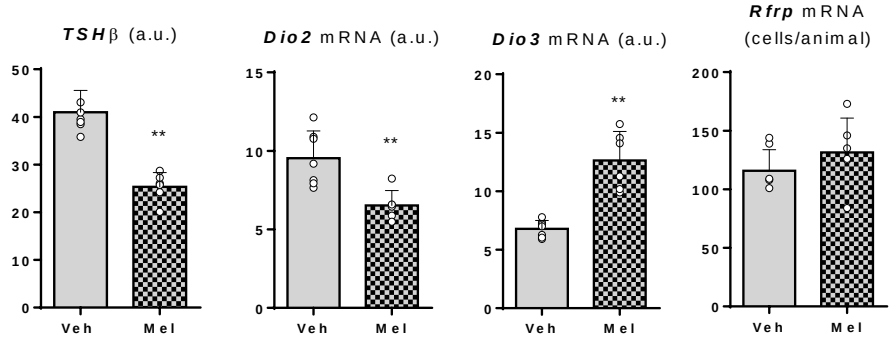
C57BL/6J *Thra*$^{+/+}$ control

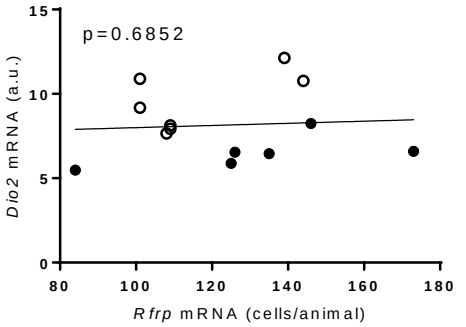




B

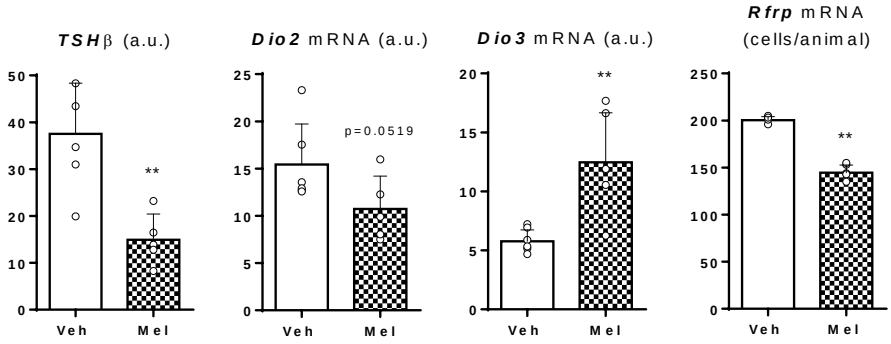
C57BL/6J *Thra*$^{0/0}$

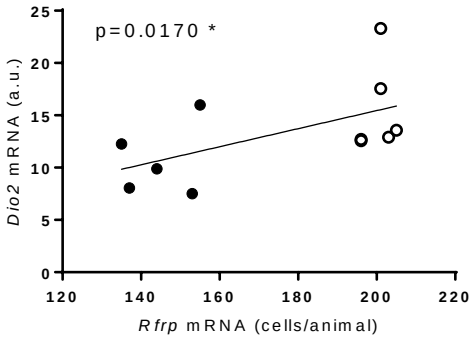




A

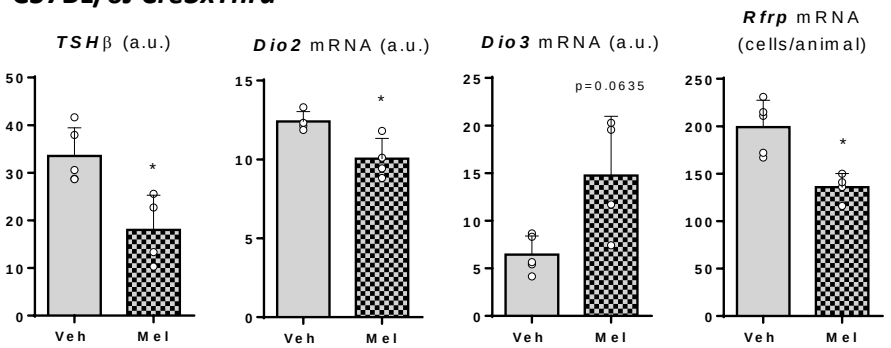
C57BL/6J *Thra*$^{AMI/+}$ control

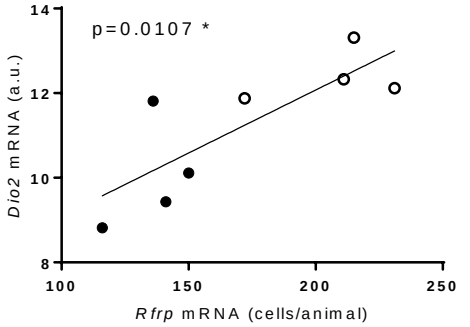




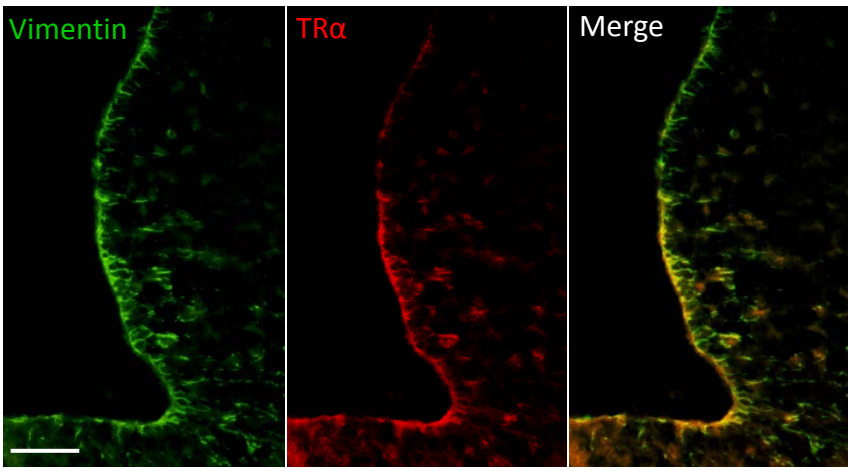
B

C57BL/6J *Cre3xThra*$^{AMI/+}$





C



carrying a selective mutation of *Thra* in neurons (*Cre3xThra*$^{AMI/+}$) or their control littermate (*Thra*$^{AMI/+}$). In both groups, melatonin similarly regulated TSHβ, *Dio2/3* but also *Rfrp* expression, independently of the presence (Figure 3A) or absence (Figure 3B) of neuronal TRα. Moreover, both groups kept a significant correlation between the expression of *Dio2* and the number of *Rfrp* expressing neurons (Figure 3 A and B) therefore indicating a conserved link between *Dio2* and *Rfrp* expression with or without neuronal TRα

Considering the non-neuronal effect of TRα on *Rfrp* expression, we investigated whether TRα could be expressed in the tanycytes, visualized by a specific vimentin labelling. The dual labelling of TRα and vimentin showed a strong co-localization of both proteins, suggesting that tanycytes could integrate the T3 signal through TRα receptors (Figure 3C).

Discussion

Since the discovery of the fundamental role of hypothalamic T3 in relaying seasonal cues to the central nervous system (7), numerous studies have sought to understand the neuroendocrine pathways linking the annual changes in photoperiod with the central reproductive network. In mammals, melatonin is key to this adaptive process since it drives the seasonal changes in the *pars tuberalis* TSH (13, 35) which in turn regulates hypothalamic T3 via a switch in the tanycytic Dio2/Dio3 ratio (36–38). Further studies then demonstrated that the photoperiodic change in the TSH/T3 signal drive seasonal reproduction through a specific action on Kp and/or RFRP-3 neurons known to regulate GnRH neuronal activity (14, 18, 39–41). Despite these major advances in seasonal reproductive physiology, our understanding of the underlying molecular mechanisms involved has been hampered by the lack of relevant genetically modified seasonal animal models.

In this study we show that mice, although showing no overt seasonal phenotype, can be used to investigate the molecular pathway involved in the melatonin signal integration into the central nervous system. Thus, we reveal that melatonin-proficient CBA/N mice, but not melatonin-deficient C57BL/6J mice, exhibit a significant photoperiodic variations in the pars tuberalis *Tshb*, tanycytic *Dio2* and *Dio3* gene expression (as already reported (11)), and also a significant SP-induced reduction in the number of neurons expressing *Rfrp* gene. By contrast, however, *Kiss1* expression does not show photoperiodic variation in CBA/N mice which is in line with the absence of overt photoperiodic changes in their reproductive activity. Altogether, these data indicate that in the melatonin-proficient CBA/N mice, the photoperiodic signal carried by melatonin is integrated down to the hypothalamic *Rfrp* expressing neurons, as reported in all other seasonal species investigated so far (18), while *Kiss1* expression appears unaffected by the environmental photoperiod in mice. Although C57BL/6J mice are deficient in melatonin, they have functional melatonin (42) and TSH (11) receptors and an acute injection of exogenous melatonin regulates *Tshb* and *Dio2/Dio3* gene

expression. Here, using a protocol mimicking a SP-melatonin pattern (28, 43) in C57BL/6J mice, we found that the melatonin-induced switch in the *Dio2/Dio3* ratio is associated with a significant reduction in the number of *Rfrp* expressing neurons, as observed in CBA/N mice. These results demonstrate that nighttime melatonin supplementation in C57BL/6J mice is able to restore photoperiodic like variations in the genes involved in seasonal timing, indicating that this animal model is suitable to tackle the molecular mechanisms underlying photoperiodic integration into the rodent hypothalamus. Of note, in C57BL/6J and CBA/N mice, *Rfrp* response to melatonin appears smaller than that of the other melatonin-driven genes. This might be related to species-dependent seasonal phenotype as *Rfrp*'s inhibition by melatonin (or SP) is larger in hamsters (44) and MSM mice (45).

Earlier studies reported that central administration of TSH in SP-adapted hamsters restores the LP level of *Rfrp* expression, suggesting that the number of RFRP-3 expressing neurons is regulated by T3 (14). In order to address this hypothesis, we investigated the effect of nighttime melatonin supplementation in C57BL/6J mice carrying a global *Thra* knock-out. Melatonin in $Thra^{0/0}$ mice is no longer able to inhibit the number of *Rfrp* expressing neurons while it still regulates *Tshb*, and *Dio2/Dio3* expression. Furthermore, the significant correlation between *Dio*2 and *Rfrp* expression observed in melatonin-proficient CBA/N and in melatonin-supplemented C57BL/6J mice is lost in mice lacking TRα. Therefore, the melatonin inhibition of *Rfrp* expression appears to involve T3 signaling on TRα, whereas the seasonal regulation of *Tshb* in the *pars tuberalis* and *Dio2/Dio3* in the tanycytes is independent of this T3-TRα pathway.

TRα is widely distributed in the central nervous system (34, 46–48), especially in the hypothalamic region (32, 49) suggesting that T3 may act directly on RFRP-3 neurons. However, we observed that nighttime melatonin supplementation in C57BL/6J mice lacking TRα signaling in neurons only (24) is still able to reduce the number of *Rfrp* expressing neurons, with a conserved correlation between *Dio2* and *Rfrp* expression, as it does in their control littermates. Preservation of the melatonin inhibition of *Rfrp* expression in absence of TRα signaling in neurons suggests that T3 is acting through non-neuronal cells expressing TRα. Earlier studies reported that astrocytes express thyroid hormones transporters such as MCT-8 and OATPs (50) but not TRα (34, 51), suggesting that astrocytes do not mediate the T3 regulation of RFRP-3 expressing-neurons. Here, we show that TRα is widely expressed in the vimentin-expressing tanycytes, as already reported by Wallis and collaborators who found that tanycytes are the only GFAP-labelled cells expressing the TRα1 receptor in the mouse hypothalamus (34).

Tanycytes are ependymal-like glial cells which play a crucial role as hypothalamic integrators of reproduction and energy metabolism (52) and exhibit cellular extensions in the mediobasal hypothalamus, and notably in the area where RFRP-3 neurons are located (52, unpublished observations). Those non-neuronal TRα-expressing cells could therefore indirectly mediate the melatonin-driven regulation of *Rfrp* expression by delivering a messenger molecule regulated by T3 to the RFRP-3 neurons. Tanycytes are also

recognized as a putative niche of stem cells able to divide and create newborn neurons and glial cells invading the neighboring hypothalamus during development but also in adult (53–55). A photoperiodic/seasonal regulation of hypothalamic neurogenesis has been reported in adult Syrian hamster (56) and sheep (57, 58), which could originate in the tanycytes (52, 59, 60). Notably, blocking seasonal neurogenesis in sheep has been demonstrated to alter the timing of annual reproduction (59). Since T3 is involved in adult neurogenesis (61, 62), we can hypothesize that disruption of the tanycytic TRα signaling could impair hypothalamic neurogenesis, which in turn would prevent photoperiodic regulation of the number of RFRP-3 neurons.

In conclusion, our results have established that endogenous or exogenous nighttime melatonin not only regulates TSHβ and Dio2/Dio3, but also the number of *Rfrp* expressing neurons in mice. Further, using genetically modified C57BL/6J mice, we have demonstrated the pivotal role of T3 signaling through TRα in the melatonin-driven regulation of *Rfrp* neurons. Finally, because the selective inactivation of TRα signaling in neurons does not impair *Rfrp* regulation by melatonin, we hypothesized that T3 may act through the tanycytes, which express a high level of TRα. Considering the role of hypothalamic neurogenesis in the seasonal reproduction (55), future studies should now investigate whether the melatonin-driven regulation of RFRP-3 neurons involves neurogenesis processes initiated by a T3 action on the tanycytes.

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Author contributions

C. Quignon designed, performed research and wrote the manuscript, M. Beymer provided probes and CBA/N tissue, K. Gauthier provided the C57BL/6J *Thra*$^{0/0}$, *Thra*$^{+/+}$, *Cre3xThra*$^{AMI/+}$and *Thra*$^{AMI/+}$, F. Gauer discussed data significance, and V. Simonneaux designed the experimental studies, discussed data significance, and corrected the manuscript. All co-authors have read and approved the manuscript.

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Figure Legends

Figure 1: Effect of photoperiod on *Tshb* in the *pars tuberalis, Dio2* and *Dio3* in the tanycytes, and *Rfrp* in the dorsomedial hypothalamus of melatonin proficient CBA/N and melatonin deficient C57BL/6J mice. CBA/N (A) and C57BL/6J (B) adult male mice were exposed to either long (LP: 16h light/8h dark, white bars) or short (SP: 8h light/16h dark, black bars) photoperiod for 6 weeks. Expression of *TSHβ*, *Dio2*, *Dio3* and *Rfrp* were assessed by non-radioactive *in situ* hybridization. *TSHβ, Dio2 and Dio3* values are given as mean level of grey value in arbitrary unit (a.u.), and *Rfrp* value is given as total number of labelled cells counted per animal. Data are given as individual values and mean values ±SEM of 5-6 mice; * p< 0,05 ; ** p<0,01. Right panels are scatterplot showing the positive correlation between *Dio2* expression and the number of *Rfrp* neurons in CBA/N mice (r = 0.7576; r^2 = 0.5740; p = 0.0149) and lack of correlation in C57BL/6J mice (r = -0.3497; r^2 = 0.1223; p = 0.2662). Representative photomicrographs of *in situ* hybridization for *Tshb*, *Dio2*, *Dio3* and *Rfrp* in SP an LP CBA/N and C57BL/6J are presented under each corresponding plot. Scale bars = 75µm.

Figure 2: Effect of exogenous melatonin on TSHβ in the *pars tuberalis, Dio2* and *Dio3* in the tanycytes and *Rfrp* in the dorsomedial hypothalamus in C57BL/6J mice with (*Thra*$^{0/0}$) or without (*Thra*$^{+/+}$) global TRα mutation. C57BL/6J *Thra*$^{+/+}$ control (A) and *Thra*$^{0/0}$ (B) adult male mice, kept in short photoperiod (SP: 10h light/14h dark), were given either vehicle (0.1% ethanol in water, plain bars) or melatonin (80 mg/L melatonin, 0.1% ethanol in water, gridded bars) in the drinking water during the dark period and normal water during the light period for at least 6 weeks. Expression of TSHβ was assessed by immunohistochemistry and *Dio2*, *Dio3* and *Rfrp* by non-radioactive *in situ* hybridization. TSHβ, *Dio2 and Dio3* values are given as mean level of grey value in arbitrary unit (a.u.), and *Rfrp* value is given as total number of labelled cells counted per animal. Data are given as individual values and mean values ±SEM of 6-7 mice; * p< 0.05 ; ** p<0.01. Right panels are scatterplot showing the positive correlation between *Dio2* expression and the number of *Rfrp* neurons in C57BL/6J *Thra*$^{+/+}$ control mice (r = 0.6025; r^2 =0.3630; p = 0.0323) and lack of correlation in C57BL/6J *Thra*$^{0/0}$ mice (r = 0.1240; r^2 = 00154; p = 0.,6852).

Figure 3: Effect of exogenous melatonin on TSHβ in the *pars tuberalis, Dio2* and *Dio3* in the tanycytes and *Rfrp* in the dorsomedial hypothalamus in C57BL/6J mice with (*Cre3xThra*$^{AMI/+}$) or without (*Thra*$^{AMI/+}$ control) selective neuronal TRα mutation, and dual immunolabelling of TRα with the tanycytic marker vimentin in C57BL/6J mice. C57BL/6J *Thra*$^{AMI/+}$ control (A) and *Cre3xThra*$^{AMI/+}$ (B), adult male mice kept in short (SP: 10h light/14h dark) photoperiod, were given either vehicle (0.1% ethanol in water, plain bars) or melatonin (80 mg/L melatonin, 0.1% ethanol in water, gridded bars) in the drinking water during the dark period and normal water during the light period for at least 6 weeks. Expression of TSHβ was assessed by immunochemistry and expression of *Dio2*, *Dio3* and *Rfrp* by non-radioactive *in situ* hybridization. TSHβ, *Dio2 and Dio3* values are given as level of labelling intensity in the region of interest

in arbitrary unit (a.u.), and *Rfrp* value is given as total number of labelled cells counted per animal. Values are given as mean ±SEM of 4-7 mice; * $p< 0.05$; ** $p<0.01$. Right panels are scatterplot showing the positive correlation between *Dio2* expression and the number of *Rfrp* neurons in C57BL/6J $Thra^{AMI/+}$ control mice ($r = 0.7123$; $r^2 =0.5074$; $p = 0.0323$) and in C57BL/6J $Cre3xThra^{AMI/+}$ mice ($r = 0.8571$; $r^2 = 0.7346$; $p = 0.6852$). (C) Photomicrographs of an immunohistochemical detection of vimentin detected with tyramide (green) and TRα detected with Alexa594 (red) in the tanycyte with the co-labelling shown on the merge image; Scale bar = 50µm.