Expression of thyroid hormone receptor isoforms in rodents

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CHAPTER 4

Diurnal Variation in Rat Liver mRNA of TRα is Dependent on the Biological Clock in the Suprachiasmatic Nucleus (SCN), While TRβ1 is Modified by Food Intake

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Submitted for publication
Abstract

Previous studies have shown a diurnal variation of certain isoforms of thyroid hormone receptors (TR) in rat liver. The genesis of these diurnal changes is still unknown. To clarify whether the biological clock, located in the hypothalamic suprachiasmatic nuclei (SCN) is involved, we made selectively SCN-lesions (SCNx). Compared to intact animals, SCNx rats lost their circadian rhythm of plasma corticosterone and TSH. Liver TRβ1 mRNA of intact rats had no statistical significant diurnal variation. In contrast, TRα1 and TRα2 mRNA expression of control rats was higher in the light period than in the dark period; these changes were abolished in the SCNx rats. To evaluate whether these effects could be indirectly explained by a disappearance of the rhythmic feeding behavior SCNx rats, we performed a second experiment in which otherwise intact animals were subjected to a regular feeding schedule, with one meal every 4 hours. As compared to rats with free access to food, regular feeding only affected TRβ1 mRNA expression but had no effect on the diurnal changes in TRα1 and TRα2.

We conclude that liver TRβ1 expression is most clearly affected by food intake. Diurnal changes in liver TRα1 and TRα2 are controlled by the biological clock in the SCN, but not via changes in the daily rhythm of food intake. Further experiments should reveal which output pathways are used by the SCN to control TRα1 and TRα2 expression. The findings apparently may have physiological relevance for diurnal variation of T3-dependent gene expression.

Introduction

Circadian rhythms are mainly driven by the biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Input from the environment into this oscillator is primarily via the eyes (1).

The SCN regulates many rhythmic processes which may include the rhythmic expression of genes in for instance the liver. Some liver genes that show a rhythmic expression include thyroid hormone dependent genes such as cholesterol 7-α-hydroxylase (CYP7A), phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphate dehydrogenase (G6PD), glutamine synthetase (GS) and "Spot 14" (2-7).

Thyroid hormones influence the expression of these genes via thyroid hormone receptors. We have recently demonstrated that the protein expression of these receptors also shows a diurnal variation (5). A higher expression of TRβ1 proteins was observed at the beginning of the dark period when the rats are nutritionally active, whereas a higher expression of TRα2 protein was found during the light period when the animals are resting.

In our previous studies we were not able to determine the origin of the diurnal variations in TR isoform expression. In order to see whether the diurnal variation derives from a "direct" effect of the SCN or an indirect effect via SCN-controlled feeding behavior, we performed two sets of experiments. In the first experiment we
compared the diurnal variations of TR mRNA expression in livers of rats whose circadian clock was lacerated to those in intact animals. In the second experiment we examined the diurnal variations of TR mRNA expression in livers of animals that had been accustomed to a regular feeding schedule, i.e. one meal every 4 hours, whereas the control animals had free access to food.

Materials and Methods

Experimental animals
Male Wistar rats were obtained from Harlan (Horst, The Netherlands) and housed in a temperature-controlled environment (20-22°C) on a 12-h light/12-h dark schedule (lights on at 0700 h). In the first experiment, the animals were divided into intact and suprachiasmatic lesion (SCNx) groups. In the second experiment, the animals were divided in a Regular Feeding (RF) group and in a control group which had free access to food ad libitum. Before the start of the experiment animals were allowed to acclimatize to the light/dark cycle for several weeks with four animals in a cage. At least 1 week before the start of the experiments animals were placed into individual cages (38 x 26 x 16 cm). Water was always available ad libitum. In both groups there were six rats per time point. All studies were approved by the local animal welfare committee.

SCN lesions (experiment I)
Rats, were anesthetized with Hypnorm (Duphar, The Netherlands; 0.6 ml/kg, im), mounted with their heads in a David Kopf Stereotact (Tujunga, CA) with the toothbar set at +5.0 mm, and subjected to a bilateral SCN lesion (coordinates: 1.4 mm rostral to bregma; 1.1 mm lateral to the midline; 8.3 mm below the brain surface) using bilateral lesion electrodes, 0.2 mm in diameter, with temperature set at 85°C for 1 minute (lesion generator, Radionics, Burlington, MA). This temperature was found empirically to result in lesions large enough to eliminate the SCN bilaterally, but small enough to leave surrounding hypothalamic brain structures, such as the paraventricular (PVN) and supraoptic (SON) nuclei intact (8). A drawback of this restricted lesion size is the limited yield of animals in which the lesion is complete (i.e. 30%). To check the effectiveness of the lesion, daily water intake was measured after a rest period of two weeks to recover from anesthesia and brain trauma during the middle 8 hours of the light period. Only animals showing a daytime water intake of more than 30% of the total daily intake (in intact control animals this value is typically <10%) were assumed to have complete lesions of the SCN and were allowed to enter the experiment. After the experiments, the presence and extent of the SCN lesion was checked by immunocytochemical staining of hypothalamic sections for the presence of vasopressin (VP) and/or vasoactive intestinal polypeptide-containing cell bodies or fibers. If animals had cell bodies that stained positively for either VP or vasoactive intestinal polypeptide at the border of the lesion, or for immunoreactive fibers in SCN target areas such as the PVN, they were excluded from the analysis. The rats were killed at Zeitgeber Time (ZT)6, ZT10, ZT18, and ZT22 (ZT12 being defined as the onset of the dark period). One animal was excluded from further analysis because of a very low body weight.
Regular Feeding (experiment 2)

Rats were trained at least three weeks before the experiment on a six 10-minutes meals per day feeding schedule spaced equally over the light-dark cycle (Regular-Feeding (RF) group). Food pellets were available in metal food hoppers to which access could be prevented by a sliding door situated in front of it. Door opening and closing were activated by an electrical motor and controlled by a clock. Food became available at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22. Door opening time was determined empirically in previous experiments (9). Animals did not save food for consumption during the 4-hours intermeal period. Adaptation was considered completed when animals had learned to consume approximately 3 g at every meal. Despite the equally distributed feeding activity, general (locomotor) activity still showed a clear light-dark rhythm, with the major part of activity occurring during the dark period. During the light period animals would wake up, eat, and resume sleeping. In the experiment rats were killed at ZT 0.5, ZT6.5, ZT12.5 and ZT18.5. During final sampling three animals were excluded from further analysis because of very low body weight (RF group ZT0.5) or severe brain damage (two in ad libitum groups at ZT6.5 and ZT12.5 which showed brain thrombosis and fluid in brain ventricles).

Analysis of hormone data

Plasma concentration of triiodothyronine (T3) was determined with an in-house RIA (10), of TSH by a chemiluminescent enzyme immunoassay (Immulite, Diagnostic Products Corp, LA, California; detection limit 0.4 ng/ml) and of plasma corticosterone using a RIA (ICN Biochemicals, Inc, Costa Mesa, California; detection limit 1 ng/ml).

Analysis of mRNA expression

mRNA was isolated from each liver sample using a MagnaPure (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. Thereafter cDNA was synthesized using the First Strand cDNA synthesis kit with random primers (Roche Molecular Biochemicals). Real-time PCR reactions were performed in a LightCycler (Roche Molecular Biochemicals). TRα1 and TRα2 were simultaneously detected using sequence-specific hybridization probes and the LightCycler-FastStart DNA Master hybridization probes kit; probes, primers and program were previously described (11). To measure TRβ1 mRNA we used the DNA Master Sybergreen kit and for the housekeeping gene GAPDH the LightCycler-FastStart DNA Master Sybergreen kit. The primers used for TRβ1 were sense: 5'-TGGGCGAGCTCTATATTCCA -3; antisense: 5'-ACAGGTGATGCAGCGATAGT -3 (185 bp product according to gene bank ac. nr. gi:18855115) and for GAPDH were sense: 5'-AACCACGAGAAAATATGACAAC-3; antisense: 5'-CATCCTGGGCTACACTGAG-3 (430 bp product according to gene bank ac. nr. gi:8393417). For each mRNA assayed, a standard was generated and used in the range of 3pg to 0.3fg per 20 µl reaction mixture. All results were normalized to the amount of GAPDH for each liver.
Statistical analysis
One-way ANOVA was applied to evaluate the differences between time points within groups. Two-way ANOVA was used to analyze differences between groups and time and their interaction. ANOVA was followed by Student’s t-test (unpaired two tailed) to establish which time points differed significantly from trough values or at which time points groups differed from one another. Due to its nonuniform distribution TR mRNA values were normalized by logarithmic transformation of the relative mRNA concentrations. A difference was considered significant at $p < 0.05$. All data were evaluated using SPSS version 11 (SPSS, Inc., Chicago, IL).

Results

Effect of a SCN lesion on diurnal variation of hormone and TR isoform levels
One-way ANOVA indicated the existence of diurnal variation in intact animals in plasma corticosterone and TSH, but not in $T_3$. Ablation of the biological clock (SCNx) resulted in loss of the diurnal variation of both corticosterone and TSH (see table 1 and figure 1). Although the daily mean of plasma corticosterone levels did not differ between intact and SCNx rats, we found an interaction between groups and time. Post-hoc analysis indicated that plasma corticosterone levels were significantly higher at ZT10 in control animals ($p=0.02$) and significantly lower in control animals at ZT22 ($p=0.03$). The daily mean of both $T_3$ and TSH levels was higher in intact rats compared to SCNx rats. Post-hoc analysis indicated lower plasma $T_3$ levels at ZT6 ($p=0.006$) and lower plasma TSH levels at ZT10 ($p=0.04$) and ZT18 ($p=0.046$).

No statistically significant diurnal variation of liver $TR_b$1 mRNA was found, although there is a suggestion of a peak at ZT10. In contrast, $TR_\alpha$1 and $TR_\alpha$2 mRNA levels had a marked diurnal variation, which disappeared in SCNx animals. Intact rats had a higher expression of both $TR_\alpha$ isoforms than SCNx rats. $TR_\alpha$1 mRNA levels in intact animals peaked at ZT10 and were lowest at ZT18, and also differed from SCNx rats at these 2 time points. $TR_\alpha$2 mRNA levels in intact rats peaked at ZT18 and were lowest at ZT10. Liver $TR_\alpha$2 mRNA levels in intact animals differed from those in SCNx rats except at ZT18.

In conclusion, ablation of the biological clock by lesion of the SCN removes the diurnal variation in all 6 parameters studied and lowers the daily mean of $T_3$ and TSH.
### Table 1. Analysis of diurnal variations of plasma corticosterone, TSH and T₃ and relative expression of liver mRNA for TRβ1, TRα1 and TRα2 in intact and SCNx groups of rats.

<table>
<thead>
<tr>
<th></th>
<th>groups</th>
<th>Daily means ± SD</th>
<th>p-value $#$ (within group differences)</th>
<th>p-value # (between group differences)</th>
<th>p-value # (time effects of both groups)</th>
<th>p-value # (interaction group X time)</th>
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</thead>
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<tr>
<td><strong>Plasma corticosterone</strong></td>
<td><strong>ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>intact</td>
<td></td>
<td>127±75</td>
<td>0.02</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>0.003</td>
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<tr>
<td>SCNx</td>
<td></td>
<td>131±30</td>
<td>&gt;0.1</td>
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<tr>
<td><strong>Plasma TSH</strong></td>
<td><strong>ng/ml</strong></td>
<td></td>
<td></td>
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<tr>
<td>intact</td>
<td></td>
<td>2.57±1.16</td>
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<td>0.025</td>
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<td>SCNx</td>
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<tr>
<td><strong>Plasma T₃</strong></td>
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<td></td>
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<tr>
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<td><strong>mRNA</strong></td>
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<tr>
<td>intact</td>
<td></td>
<td>2.25±1.04</td>
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<tr>
<td><strong>TRα1</strong></td>
<td><strong>mRNA</strong></td>
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<tr>
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<td></td>
<td>2.48±1.10</td>
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<td><strong>TRα2</strong></td>
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<td>1.86±1.15</td>
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$\$: One-way ANOVA, # Two-way ANOVA. Daily variations of TR are expressed in Arbitrary Units of ratio TR/GAPDH. Values are expressed in means ± SD.
Figure 1. Diurnal changes of plasma corticosterone, TSH, T3 and of liver TR mRNA isoforms in intact (white circles) and SCNx (black circles) rats. ZT, Zeitgeber time. The light and dark periods are shown as white and black bars respectively on the top of graph. Data are from Table 1 and are expressed as means ± SEM.
Effect of regular feeding on the diurnal variation of hormone and TR isoform levels

A diurnal variation was found for plasma corticosterone, T₃, and TSH in the control groups (figure 2 and table 2). The diurnal variation of corticosterone remained present in the regular feeding group although associated with higher peak corticosterone levels. The daily mean of plasma TSH and T₃ was similar between control and RF rats, but RF dampened the diurnal variation. Two-way ANOVA showed a significant effect of time for plasma T₃ and TSH, but no significant group effects. For plasma TSH levels, the regular feeding regimen resulted in a shift of the peak value to another time point (in control animals at ZT18.5 versus RF animals at ZT6.5; see figure 2).

The TRβ1 mRNA expression in the RF group showed a peak at ZT12.5 (figure 2). Two-way ANOVA revealed significant effects of group, time and interaction for TRβ1 mRNA levels. Post-hoc analysis showed a lower expression of TRβ1 mRNA at ZT18.5 in the RF group. A diurnal variation for TRα1 was found in the control group. Two-way ANOVA analysis indicated a significant time effect due to a lower TRα1 mRNA expression at ZT18.5 in RF groups.

For TRα2 a diurnal variation was observed in both control and RF groups. Two-way ANOVA revealed significant effects of time and a significant interaction. Post-hoc analysis showed that TRα2 mRNA expression was higher at ZT6.5 (p=0.005) during dark period and lower at ZT12.5 (p=0.043) during light period.

In conclusion, regular feeding does not abolish diurnal variation in corticosterone, TSH or T₃, although it does affect the amplitude of the diurnal variations in TRα1 and TRα2 and, interestingly, the diurnal variation in the TRβ1 mRNA appears to be reinforced.
Table 2: Analysis of diurnal variations of plasma corticosterone, TSH and T₃ and relative expression of liver mRNA for TRβ1, TRα1 and TRα2 in control and regular feeding groups of rats.

<table>
<thead>
<tr>
<th>groups</th>
<th>groups</th>
<th>Daily means ± SD</th>
<th>p-value $ (within group differences)</th>
<th>p-value # (between group differences)</th>
<th>p-value # (time effects of both groups)</th>
<th>p-value # (interaction group X time)</th>
</tr>
</thead>
<tbody>
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<td><strong>Plasma corticosterone ng/ml</strong></td>
<td>Control</td>
<td>68±63</td>
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<td>0.04</td>
<td>0.0001</td>
<td>&gt;0.1</td>
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<td></td>
<td>RF</td>
<td>100±78</td>
<td>0.0001</td>
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<td><strong>Plasma TSH ng/ml</strong></td>
<td>Control</td>
<td>1.90±1.06</td>
<td>0.009</td>
<td>&gt;0.1</td>
<td>0.02</td>
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<td>RF</td>
<td>1.84±1.28</td>
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<tr>
<td><strong>Plasma T₃ mol/l</strong></td>
<td>Control</td>
<td>0.99±0.18</td>
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<td>&gt;0.1</td>
<td>0.005</td>
<td>&gt;0.1</td>
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<tr>
<td></td>
<td>RF</td>
<td>1.07±0.23</td>
<td>&gt;0.1</td>
<td></td>
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<tr>
<td><strong>TRβ1 mRNA</strong></td>
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<td>1.19±0.41</td>
<td>&gt;0.1</td>
<td>0.03</td>
<td>0.0001</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>RF</td>
<td>1.06±0.46</td>
<td>0.001</td>
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<tr>
<td><strong>TRα1 mRNA</strong></td>
<td>Control</td>
<td>3.61±0.82</td>
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<td>&gt;0.1</td>
<td>0.03</td>
<td>0.07</td>
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<td>RF</td>
<td>3.79±0.39</td>
<td>&gt;0.1</td>
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<td></td>
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<tr>
<td><strong>TRα2 mRNA</strong></td>
<td>Control</td>
<td>3.13±1.02</td>
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<tr>
<td></td>
<td>RF</td>
<td>3.26±0.44</td>
<td>0.001</td>
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$; One-way ANOVA, #; Two-way ANOVA. Daily variations of TR are expressed in Arbitrary Units of ratio TR/GAPDH. Values are expressed in means ± SD.
Figure 2. Diurnal changes of plasma corticosterone, TSH, T3 and of liver TR mRNA isoforms in control (white circles) and regular fed (black circles) rats. ZT: Zeitgeber time. The light and dark periods are shown as white and black bars respectively on the top of graph. Data are from table 2 and are expressed as means ± SEM.
Discussion

To our knowledge, we are the first to show diurnal variation of TRα1, TRα2 and TRβ1 in rat liver in relation to biological clock and feeding behavior. We reported earlier that a diurnal variation in TRβ1 protein expression exists in rat liver (5) which peaks at ZT12.5, a time point when the rodents become nutritionally active. The present study was undertaken to determine whether this variation in TR isoform expression is the result of a "direct" action of the biological clock. To find out whether the biological clock in the hypothalamus is involved, we destroyed the suprachiasmatic nucleus (SCN) by thermoablation. As a control for the effectiveness, we determined the hormone levels for corticosterone representing the HPA-axis and those for TSH and T3, representing the HPT-axis. In accordance with previous studies, SCN ablation removed diurnal variations in corticosterone, TSH and T3. SCN ablation did not affect mean daily levels of corticosterone, but had a lowering effect on mean TSH and T3, as previously reported (8). The mean daily levels of the TR isoforms were not affected, but the clearly present diurnal variation in TRα1 and TRα2 was abolished by the SCN lesion. Thus the SCN seems to be the major driving force in regulating the diurnal variation of TRα isoforms in liver, which therefore can be viewed as a real circadian rhythm.

To evaluate whether these effects could be indirectly explained by alterations in feeding behavior in the SCNx rats, we performed a second experiment in which, otherwise intact, animals were subjected to regular feeding every four hours. Regular feeding resulted in a higher daily mean in corticosterone levels which is in accordance with previous studies which shown that (mild) food deprivation enhances the activity of the HPA-axis (9,12-15). Regular feeding caused a shift in the daily peak for TSH which is in accordance with observations in rats receiving their food spread over several meals showed no diurnal variation of T3 (16). In this study, regular feeding regimen resulted in differential expression of TR mRNA. Regular feeding enhanced the rhythm in TRβ1 mRNA expression, but the diurnal variation in the expression of TRα2 and TRα1 mRNAs was reduced.

In summary, we show that diurnal variations of TRα1 and TRα2 mRNA levels are dependent on the biological clock in the SCN, although feeding activity may modify the amplitude. In this study we looked at TR expression at the mRNA level since circadian regulation of gene expression is primarily a result of a rapid increase of mRNA levels (17-19). However, it is important to mention two limitations of the present study which are due to the experimental conditions, namely the limited sample size and the difference in time points between the two experiments. The limited sample size is presumably responsible for the absence of statistically significant diurnal variation in TRβ1 mRNA in our control rats due to the high variability at ZT0.5. We did demonstrate however that the highest expression of TRβ1 is observed at ZT12.5 in both control and RF rats, which is in accordance with the results we obtained on the TRβ1 protein in our previous study. An additional explanation may be that TR mRNA and protein levels do not always correlate (20,21). Furthermore, due to the limited number of sampling points, we may have missed the highest and lowest expression of TRβ1 mRNA. Therefore, we cannot
completely rule out a central regulation of TRβ1 mRNA in addition to regulation by food intake.

It is interesting to speculate on the physiological relevance of our study. Since the TRα gene was probably present earlier during the evolution than the TRβ gene (22), it can be postulated that TRα, being the first, is involved in the main overall - thyroid hormone dependent - daily housekeeping tasks in the organ and therefore under regulation of the central biological clock. The TRβ1 gene, having developed later, could be thought to be more involved in fine-tuning of hepatic gene expression, for instance in response to feeding activity. It has been shown recently that restricted feeding induces shifts in the circadian rhythm of liver gene expression independent of the SCN mediated clock (23,24), which agrees with our finding that the rhythm of the TRβ1 changes as a result of RF. Although no data are available on the effect of SCN lesions on the expression of T3-responsive genes in liver, it can be hypothesized on the basis of this speculation that mRNA levels of for instance the TRβ1-regulated CYP7A gene, which is involved in cholesterol metabolism (25), will be unaffected by input from the light-dark cycle. That this may indeed be the case can be inferred from experiments which show that restricted feeding does shift the pattern of CYP7A expression (24) independent of the central clock (26). Conversely, the diurnal expression of "Spot 14", another TRβ1 dependent gene (27), is mainly dependent on the SCN since adaptation to feeding restricted to either light-light or dark-dark cycles for 15 days did not greatly affect the diurnal rhythm in "Spot 14". In the same experiments photo reversal resulted in a 180 degrees phase shift, whereas the rhythm persisted in the presence of constant light which suggests involvement of SCN as important determinant of rhythmic changes of "Spot 14" (7). Recent studies show a diurnal variation for several transcription factors and nuclear receptors which indicates that the diurnal variation we report here are not restricted to the TR isoforms (28-30). In conclusion, our study shows that the biological clock in the SCN has a predominant role in regulation of TRα gene expression as opposed to the feeding regimen.

Acknowledgements:
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