Influence of age, strain and season on diurnal periodicity of thyroid stimulating hormone, thyroxine, triiodothyronine and parathyroid hormone in the serum of male laboratory rats

Chun-Cheung Wong, Klaus-D. Döhler, Michael J. Atkinson, Heinz Geerlings, Rolf-Dieter Hesch and Alexander von zur Mühlen

Abstract. The influence of age, strain and season on the diurnal pattern of serum hormone levels from the pituitary-thyro-parathyroid complex was studied in male laboratory rats. Distinct 24 h periodicity in the serum levels of thyroid stimulating hormone (TSH) and triiodothyronine (T₃) was observed in all groups of rats. There was no influence of age (40, 60 and 90 days old Sprague-Dawley rats), but a significant influence of strain (Sprague-Dawley vs. BH/Ztm rats) and season (summer vs. winter) on the diurnal pattern of serum TSH and T₃ levels. Significant 24 h periodicity in serum thyroxine (T₄) levels existed during winter in BH/Ztm rats, but not in Sprague-Dawley (SD) rats of any age. Adult SD rats demonstrated 24 h periodicity in serum levels of T₄ only in summer. No diurnal periodicity in serum levels of parathyroid hormone (PTH) was observed in any group of rats.

There were significant changes in 24 h mean serum levels of TSH and T₃ throughout pubertal development. Twenty-four h mean serum levels of T₃ and T₄ were significantly higher in summer than in winter. Twenty-four h mean serum levels of T₄ were significantly lower in BH/Ztm rats than in SD rats. Significant correlation was observed between serum concentrations of T₃ and T₄. TSH and T₄, and between TSH and T₃ in some groups of rats, but not in all.

The results indicate that 24 h periodicity of serum hormone levels from the pituitary-thyroid complex of male laboratory rats may vary with age and strain of the animals and with the season of experiment performance.

In recent years increasing awareness is developing in biomedical research as to the importance of standardization, not only for assay procedures, but also for animal models and experimental designs. Experimental and pre-experimental disturbance of test animals is known to increase the variability of physiological parameters (Döhler et al. 1977; 1979) and may thus limit the significance of the conclusions. Differences in sex, strain and age of experimental animals, differences in housing conditions (light-dark schedule, temperature) and the method of blood collection may also add to variability of physiological parameters and to non-comparability of experimental results (Döhler et al. 1979). The non-comparability of experimental set-up may often be responsible for the differing results, and may subsequently lead to interpretational disagreement on cause-effect relationship in biological systems.

One such biological system, the study of which has probably yielded more confusion than clarification, is the diurnal periodicity in serum hormone levels. There have been frequent attempts to study diurnal periodicity in serum hormone concentrations in the laboratory rat. For some hormones, i.e. ACTH or corticosterone, a diurnal rhythm in serum concentration is well documented and rhythm synchronization by the daily pattern of light and dark and/or by the feeding schedule is unquestioned (Halberg 1959; Philippens et al. 1977). In regard to the hormones from the pituitary-thyroid complex, however, there is considerable disagreement on the question of the existence or non-existence of diurnal periodicity in serum
hormone levels in rats and there is disagreement on the temporal occurrence of acrophases and nadirs for those hormones, for which diurnal periodicity has been documented (Klug & Adelman 1979; Jordan et al. 1980; Leppälüoto et al. 1974; Fukuda et al. 1975; Martin et al. 1978; Singh et al. 1967; Männistö et al. 1978; Rookh et al. 1979; Jolin et al. 1976). To our knowledge no data have been reported yet on 24 h patterns of parathyroid hormone (PTH) in the rat. Many of the previously mentioned reports not only differ in the pattern of the light-dark schedule, but also in methods, frequency and times of blood sampling, in sex, strain and age of the experimental animals, and in housing conditions. Additional factors such as the stress of handling or cage, transport (Döhler et al. 1977, 1979), locomotor activity (Döhler et al. 1979) and seasonal influences (Wong et al., in press) may result in further confusion of basic hormone release patterns. We, therefore, studied under strict limitation of experimental and pre-experimental disturbance and under rigorously well controlled and standardized animal housing conditions, the influence of age, strain and season on the diurnal pattern of hormones from the pituitary-parathyroid complex in the serum of male laboratory rats.

Materials and Methods

Animal housing conditions

Male Han: Sprague-Dawley rats (SD) and male Black hooded/Ztm rats (BH/Ztm) were raised by ourselves under controlled environmental conditions. In 1973 an original stock of Black hooded rats (BH) was introduced into our Animal Research Unit (Ztm). This strain had previously been inbred at the Immunology Research Unit, Department of Pathology, University of Pennsylvania, Philadelphia, USA. The SD rats derived from an original stock of 20 Han: Sprague-Dawley rats, which had been introduced into our animal compound several years ago. The animal compound is located behind semi-sterile barriers, access being strictly controlled. Hygienic precautions are observed and disposable sterile uniform clothes and face masks have to be worn when passing the barrier.

The animal rooms are air conditioned (22 ± 1°C, 55% relative humidity), light-controlled (light period from 05.00 to 19.00 h) and are enclosed by sound and pressure proof doors. All animals were born and raised under identical conditions. They were weaned at 25 days of age and were kept in groups of 4 in polycarbonate cages (43 × 27 × 15 cm) which contained sterile wooden gra-

nules. They received sterilized food (Altromin 1314 fortified) and tap water ad libitum. The animals were free from all pathogens specified in the GV-SOLAS list (1972).

Three groups of SD rats (56 animals per group) were raised to reach the age of 40 ± 2, 60 ± 2 or 90 ± 2 days within a 3 months period during the winter season 1978/79 (winter study). One group of 56 adult BH/Ztm rats (100–120 days of age) was used during the same season. An additional group of 56 SD rats (90 ± 2 days of age) was used for experiment in June 1978 (summer study).

Experimental design

Throughout a 24 h period groups of animals were decapitated either at 06.00 h, 10.00 h, 14.00 h, 18.00 h, 22.00 h, 02.00 h or 06.00 h. Red light was used for cage identification during the dark period. The animals were bleed after rapid decapitation from a specially designed funnel rack with each funnel connected to a collecting tube. This system allowed a single experimenter to decapitate 4 rats from a single cage within 30 s after removal of the cage and to exsanguinate them unattended. All decapitations were performed in an adjacent room, which was separated from the housing room by a sound proof door. In order to minimize disturbance due to general laboratory noises, blood samples were only taken on week-ends, beginning at 06.00 h on Saturdays and finishing at 06.00 h on Sundays. For the same reason of minimal disturbance, only 4 animals were sacrificed at each time interval. In order to obtain eight measurements per time interval, the 56 animals of each age group were sacrificed during two 24 h sessions on subsequent week-ends. The animal room remained sealed between sacrifice intervals.

Trunk blood was collected from each individual animal into ice-cold collecting tubes. Serum was removed after centrifugation and was stored frozen at −70°C until hormone determinations were performed.

Assay procedures

Serum levels of TSH were determined by radioimmunoassay (RIA) with the help of NIAMDD-kits, as described previously (Döhler et al. 1977). All samples were determined in duplicate in the same assay. Triiodothyronine (T3) and thyroxine (T4) levels in the serum were measured by RIA as described previously (Wong et al. 1980). The samples were measured in duplicate in two assays of each kind. Identical numbers of samples per time point and rat population were determined in each assay.

Serum levels of PTH were determined by the use of a sequence specific RIA. We have developed such a system using synthetic human h44–68 PTH as standard and tracer (Atkinson et al. 1981). This system, which is oriented toward the mid-region of the human PTH molecule, overcomes the problem of heterogeneity of
circulating PTH peptides and available tracer and standard preparations, and thereby aids the development of defined PTH immuneassay conditions. It not only reduces assay variability and non-specific effects of serum, but the highly purified components also result in an increase in assay sensitivity (0.5 fmol/tube h44–68 PTH). The assay utilizes the sheep anti-PTH antiserum Giselle III (Hehrmann et al. 1977), which has a single apparent binding site for the h44–68 PTH peptide. The tyrosinated h44–68 Tyr derivate of the target sequence was radioiodinated to serve as the tracer, whilst the h44–68 peptide was employed as standard. The assay has been described in detail elsewhere (Atkinson et al. 1981) and its application to the measurement of rat PTH has been discussed (Atkinson et al., submitted). The serum samples were measured in duplicate in two different assays. Identical numbers of samples per time group and rat population were determined in each assay.

Statistical analyses

Statistical analyses were performed by one way and two way analysis of variance (ANOVA) when data from more than two groups were compared. The Statistical Package for the Social Sciences (SPSS), Vogelbeck Computing Center, Northwestern University was used for computation. Student's t-test was performed when data from two individual groups were compared. In addition coefficients of correlation between different hormones in individual serum samples were calculated.

Results

Thyroid stimulating hormone (TSH)

Effects of age. There was a significant influence of age on the 24 h means (P < 0.001, Table 1), but not on the circadian pattern of serum TSH levels. The 24 h means increased between 40 and 90 days by 35%. A significant 24 h rhythm with highest TSH levels at 10.00 h and 14.00 h and lowest levels at 06.00 h was observed at all ages (Fig. 1).

Effects of strain. Rats of both strains demonstrated significant 24 h periodicity in the pattern of serum TSH levels (Fig. 1). There was, however, significant interaction between strains and diurnal pattern (P < 0.02; 2-way ANOVA). In winter serum TSH levels increased rapidly after the onset of the light period and reached peak levels already at 10.00 h in both strains of rats (Fig. 1). Whereas in BH/Ztm rats serum TSH levels decreased rapidly between 10.00 h and 14.00 h, they remained elevated in SD rats until 14.00 h and decreased thereafter. In both strains there was a slight but statistically not significant increase in serum TSH levels during the first part of the dark period. There was no significant influence of strain on 24 h mean serum TSH levels (Table 1).

Table 1.
Mean (± SEM) serum levels of TSH, triiodothyronine (T3), thyroxine (T4), and parathyroid hormone (PTH) during a 24 h period in different groups of male rats. SD = Sprague-Dawley rats, BH = BH/Ztm rats, W = study performed during winter, S = study performed during summer. Ages of rats in days are numerically indicated (i.e. 40, 60, 90 or 100–120).

<table>
<thead>
<tr>
<th>Group numbers</th>
<th>Group</th>
<th>N</th>
<th>TSH ng/ml</th>
<th>T3 ng/100 ml</th>
<th>T4 µg/100 ml</th>
<th>PTH pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SD/40/W</td>
<td>48*</td>
<td>233 ± 14</td>
<td>74.5 ± 1.6</td>
<td>3.97 ± 0.09</td>
<td>157 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>SD/60/W</td>
<td>48</td>
<td>337 ± 25</td>
<td>52.5 ± 1.6</td>
<td>4.19 ± 0.11</td>
<td>166 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>SD/90/W</td>
<td>48</td>
<td>314 ± 22</td>
<td>55.1 ± 2.4</td>
<td>4.15 ± 0.12</td>
<td>156 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>SD/90/S</td>
<td>48</td>
<td>267 ± 18</td>
<td>69.1 ± 3.1</td>
<td>4.93 ± 0.14</td>
<td>164 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>BH/100-120/W</td>
<td>48</td>
<td>300 ± 28</td>
<td>54.9 ± 2.9</td>
<td>3.18 ± 0.11</td>
<td>148 ± 5</td>
</tr>
</tbody>
</table>

Statistics

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (groups 1–3) ANOVA</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Season (groups 3 vs. 4) t-test</td>
<td>NS</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Strain (groups 3 vs. 5) t-test</td>
<td>NS</td>
<td>NS</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

* For calculation of 24 h mean hormone levels the last (overlapping) group of animals was excluded. NS = not significant.
24 h patterns of serum thyroid stimulating hormone (TSH) levels in male rats. Top left: Sprague-Dawley rats, 40 days of age, winter study (SD/40/W). Top right: Sprague-Dawley rats, 60 days of age, winter study (SD/60/W). Center left: Sprague-Dawley rats, 90 days of age, winter study (SD/90/W). Center right: Sprague-Dawley rats, 90 days of age, summer study (SD/90/S). Bottom: BH/Ztm rats, 100–120 days of age, winter study (BH/100–120/W). Numbers on the abscissa represent the time of the day. The dark period (from 19.00 to 05.00 h) is denoted by the dark horizontal bar. Group means ± SEM (n = 8) and statistical significance (ANOVA) are listed.

**Effects of season.** During both seasons SD rats demonstrated significant 24 h periodicity in the pattern of serum TSH levels (Fig. 1). The pattern of this periodicity was, however, significantly different in summer and winter ($P < 0.02$). In summer serum TSH levels increased continuously during the light phase, reaching peak levels at 18.00 h, and decreased continuously during the dark phase. In
winter serum TSH levels increased rapidly after the onset of the light period and reached peak levels at 10.00 h (Fig. 1). There was no significant influence of season on 24 h mean serum TSH levels (Table 1).

Triiodothyronine (T3)

Effects of age. There was a significant influence of age on the 24 h means (P < 0.001, Table 1) of serum T3 levels. The 24 h means decreased between 40 and 90 days by 26%. A significant 24 h
rhythm with peak levels at 10.00 h or 14.00 h was observed in all age groups (Fig. 2). Serum levels of T₃ decreased toward the end of the light period. In 90-day-old animals a second peak was observed at the middle of the dark period. This peak was missing at 40 and 60 days. There was a serial effect (Halberg 1959) in 60-day-old animals, serum levels of T₃ being significantly lower (P < 0.05) at 06.00 h at the end of the experimental period, than at 06.00 h at the beginning of the experimental period.

Effects of strain. Rats of both strains demonstrated significant 24 h periodicity in the pattern of serum T₃ levels (Fig. 2). This pattern was, however, significantly different (P < 0.001) in the two strains. In both strains the serum levels of T₃ increased during the first few hours of the light period and reached peak levels at 10.00 h. The amplitude of this increase was higher in BH/Ztm rats than in SD rats (in winter). Subsequently serum levels of T₃ decreased in both strains and reached lowest levels

Fig. 3.

24 h patterns of serum thyroxine (T₄) levels in male rats of different ages (top and center), different strains (center left and bottom) and during different seasons (center). NS = not significant. For further details see Fig. 1.
at 18.00 h. There was an additional increase in serum levels of T₃ during the first part of the dark period. This increase occurred faster in the SD strain than in the BH/Ztm strain (Fig. 2). There was no strain difference in 24 h mean serum T₃ levels (Table 1).

**Effects of season.** During both seasons SD rats demonstrated significant 24 h periodicity in the pattern of serum T₃ levels (Fig. 2). Both patterns were, however, significantly different (P < 0.001). Serum concentrations of T₃ reached peak values during both seasons at 10.00 h. The amplitude of this peak was, however, higher in summer than in winter. In summer serum T₃ levels decreased slowly, reaching lowest levels at 02.00 h, in winter they decreased faster, reaching lowest levels at 18.00 h. Twenty-four h mean serum T₃ levels were significantly higher during summer than during winter (Table 1).

**Thyroxine (T₄)**

**Effects of age.** The 24 h means of serum T₄ levels did not change significantly with age (Table 1). Serum T₄ levels did not undergo diurnal variations in 40- and 90-day-old rats (Fig. 3). At 60 days of age the variability in the pattern of serum T₄ levels was statistically significant (Fig. 3). In this particular group of rats, however, a serial effect was observed, serum levels of T₄ being significantly (P < 0.02) higher at 06.00 h at the beginning of the experiment, than at 06.00 h 24 h later. The variability in the pattern of serum T₄ levels throughout the observation period in this group of rats seems to be due to this serial effect, rather than due to true diurnal periodicity.

**Effects of strain.** The 24 h means of serum T₄ levels were significantly higher in SD rats than in BH/Ztm rats (Table 1). Whereas BH/Ztm rats demonstrated distinct 24 h periodicity in serum T₄ concentrations with increasing levels from 06.00 h to 10.00 h and decreasing levels thereafter, SD rats did not show significant diurnal periodicity during winter (Fig. 3).

**Effects of season.** The 24 h mean of serum T₄ levels in SD rats was significantly higher in summer than in winter (Table 1). In summer SD rats demonstrated significant 24 h periodicity with higher T₄ levels from 10.00 h to 18.00 h than during the rest of the day. In winter no significant 24 h periodicity was observed in SD rats (Fig. 3).

**Parathyroid hormone (PTH)**

Serum levels of PTH did not show circadian fluctuations in any of the five investigated groups of rats. There was no significant influence of age, strain or season on the 24 h mean (Table 1) or on the circadian pattern of serum PTH levels.

**Correlation of hormone concentrations**

There was significant correlation between serum concentrations of TSH and T₃ in adult BH/Ztm rats (P = 0.002) and in 40-day-old SD rats (P = 0.013). Serum levels of TSH and T₄ correlated significantly in adult BH/Ztm rats (P = 0.003), in 40-day-old SD rats (P = 0.011) and in 90-day-old SD rats during winter (P = 0.02). Serum levels of T₃ and T₄ correlated significantly in adult BH/Ztm rats (P < 0.001), in 40- (P = 0.005) and 60-day-old SD rats (P < 0.001) and in 90-day-old SD rats during summer (P < 0.001).

**Discussion**

Under strict limitation of experimental and pre-experimental disturbance, and under well controlled and standardized housing conditions we have observed significant 24 h periodicity in the serum levels of T₃ and TSH in five, and for serum levels of T₄ in two groups of male rats. We also observed significant influences of age, strain and season on 24 h patterns and/or on 24 h means of serum hormone levels.

In regard to serum levels of TSH distinct diurnal periodicity has been observed in most studies (Fukuda et al. 1975; Jordan et al. 1980; Jolin et al. 1976; Klug & Adelman 1979; Leppaluoto et al. 1974; Männistö et al. 1978; Martin et al. 1978; Rookh et al. 1979; Singh et al. 1967). There is a disagreement, however, as to the precise phase relationship between the light-dark cycle and the occurrence of peaks and troughs. Our results indicate that age and strain differences in experimental animals are not likely to be responsible for the different diurnal patterns of serum TSH levels, but rather differences in the season of experiment performance. With the exception of one study, in which no diurnal periodicity in serum TSH levels had been observed (Hostetter & Piacsek 1977) all other studies have yielded principally two different types of pattern. One type of pattern, showing rapidly increasing TSH levels at the beginning of the light phase and peak values in the late morning
or early afternoon, was observed by Fukuda et al. (1975), Jordan et al. (1980), Klug & Adelman (1979), Leppäluoto et al. (1974), Männistö et al. (1978), Martin et al. (1978), Rookh et al. (1979) and in all our studies, which we had performed during the winter season. The other type of pattern, showing rather slowly increasing TSH levels with peak values toward the end of light period was observed by Jolin et al. (1976), Singh et al. (1967) and in one of our studies, which we had performed during summer. Unfortunately the dates of experimental performance were not mentioned in previous studies. We can, therefore, only cautiously suggest that the variability of the circadian pattern in serum TSH levels might be controlled by seasonal changes. We had previously also observed seasonal influences on basal serum levels of TSH in rats which had lived for many generations in constant laboratory conditions (Wong et al., in press).

In contrast to the many reports on diurnal periodicity of serum TSH levels in the rat, there are only a few reports on diurnal patterns of thyroid hormone levels (Fukuda et al. 1975; Jordan et al. 1980; Martin et al. 1978; Rookh et al. 1979). Nevertheless, the available data are no less confusing. Jordan et al. (1980) and Martin et al. (1978) reported significant diurnal periodicity for serum levels of T3 and T4, whereas Fukuda et al. (1975) and Rookh et al. (1979) did not observe diurnal periodicity for either hormone.

In regard to serum levels of T4 we observed significant diurnal periodicity in BH/Ztm rats and in adult SD rats during summer, but no periodicity in SD rats of any age group during winter. Our results indicate that the differences in the diurnal pattern of serum T4 levels in previous studies may be a result of the use of animals from different strains (Fukuda et al. 1975 and Rookh et al. 1979 as compared to Martin et al. 1978) or from experiment performance during different seasons. Differences in age of the animals used by Fukuda et al. (1975), Rookh et al. (1979) and Jordan et al. (1980) do not seem to be responsible for the different results.

The disagreement on existence or non-existence of a 24 h rhythm in serum levels of T3 cannot be explained by strain differences or by seasonality. Although we observed a somewhat different 24 h pattern of serum T3 levels in summer than in winter, both patterns showed significant periodicity. Significant periodicity in serum T3 levels was not only observed during different seasons and in different strains, but also in rats of different ages. The patterns were reasonably similar to the ones observed by Jordan et al. (1980) and by Martin et al. (1978). Although differences in housing condition (high temperature of 28°C) and blood sampling method (serial sampling via intraatrial catheter) may explain the different results obtained by Rookh et al. (1979), we have no explanation for the fundamentally different results obtained by Fukuda et al. (1975).

Whereas serum levels of calcium (Hunt & Perris 1974; Milhaud et al. 1972; Staub et al. 1979) and calcitonin (Talmage et al. 1975) were shown to undergo significant diurnal periodicity in rats, our results did not indicate diurnal variations in serum levels of PTH regardless of age or strain of the animals and regardless of the season of experiment performance. Under different physiological conditions, however, our h44–68 PTH RIA system responded very well to fluctuations in calcium metabolism. We observed high levels of PTH in newborn rats, in which Thomas et al. (1981) had previously shown that serum calcium levels are low. As calcium levels in the serum increased with post-natal age (Thomas et al. 1981), PTH levels decreased (Atkinson & Wong, unpublished).

In conclusion, our results indicate that some but not all of the disagreements on existence or non-existence of circadian rhythms in serum concentrations of hormones from the rat pituitary-thyroid and parathyroid complex may be explained by the fact that animals of different strains or ages had been used or that experiments were performed at different times of the year. A similar conclusion may be drawn in regard to statistical correlation of serum hormone levels. We observed significant correlation between serum concentrations of T3 and T4, TSH and T4, and between TSH and T3 in some groups of rats, but not in all.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft, the Stiftung Volkswagenwerk, the Heisenberg-Program and the German Academic Exchange Service. We are grateful to the NIAMDD Rat Pituitary Hormone Distribution Program for providing us generously with RIA-kits.

We thank Dr. R. Ködding (Hannover) for his supply of T3 and T4 antiserum, Miss B. Tyrasa, Miss B. Lenz, Mrs.

384
S. Hanssen and Miss M. König for invaluable technical assistance, and Miss E. Ryssel for manuscript preparation.

We are especially grateful to Prof. K. Gärtner (Hannover) for providing us with optimal animal housing conditions.

References


Received on August 18th, 1982.