N-Phthaloyl gamma-aminobutyric acid affects biochemical circadian rhythms in Wistar rats

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N-Phthaloyl gamma-aminobutyric acid (P-GABA) was administered to Wistar rats and 24 hr rhythms of glucose, cholesterol, total protein and lactic acid levels in blood were studied under semi-natural light dark conditions. P-GABA administration caused desynchronisation of the rhythms; while glucose and lactic acid rhythms were advanced, cholesterol and total protein rhythms were delayed. Since GABA is being involved in conveying dark information to the clock, exogenous administration of P-GABA may reduce the photic information received by the clock. The results could be explained by slightly less than 1 hr daily delays (or) advances respectively which would bring the peak times to the points 21 days after the start of administration.

Circadian rhythms of plasma glucose and muscle glycogen in domestic fowls and cholesterol synthesis in animals and humans have been reported. In rats, hepatic hydroxymethylglutaryl coenzyme A (HMGCoA) reductase, the rate limiting enzyme in cholesterol synthesis pathway and the precursors of cholesterol synthesis are known to be circadian in nature. Circadian rhythms in total plasma proteins have also been documented in humans and mice. Gamma-aminobutyric acid (GABA) is reported to be the principal neurotransmitter of the circadian system. Agents which affect GABA transmission have been shown to affect circadian rhythms. Exogenously administered GABA does not cross the blood brain barrier. To overcome this, derivatives of GABA like N-Phthaloyl GABA (P-GABA) is synthesized.

Although studies have been carried out on the influence of GABA agonists and antagonists on various circadian rhythms, the influence of GABA on biochemical circadian rhythms has not been investigated intensively. In the present study, GABA is administered as P-GABA to Wistar rats and its influence on biochemical circadian rhythms has not been investigated intensively. In the present study, GABA is administered as P-GABA to Wistar rats and its influence on circadian rhythms of blood variables (glucose, cholesterol, total protein and lactic acid) are monitored.

Materials and Methods

Adult male Wistar rats (160-180 g) obtained from Faculty of Medicine, Annamalai University were used. The rats were housed in polypropylene cages at room temperature (30° ± 2°C) under semi-natural conditions (L:D-12:12). The animals were randomized and divided into 3 groups of 6 each: normal, control and experimental. Food pellets (Hindustan Lever Ltd., Mumbai, India) and water were available ad libitum to the animals and replenished daily.

GABA (6.18 g) and phthalic anhydride (6.85 g) were heated for 30 min with stirring in an oil bath at 145°-150°C. After cooling the solid material was dissolved in hot methanol. The filtrate was diluted with 20 ml water and the product was allowed to crystallize. The product was identified by (i) measuring melting point, (ii) UV absorption and (iii) IR spectroscopy. Thin layer chromatography of the product was also performed.

P-GABA and ethanol were administered to the animals through ip injections every day between 1000 and 1100 hrs. P-GABA (10 mg/kg body weight in 2 ml of 20% ethanol) and ethanol (2 ml; 20%) were administered to experimental and control animals respectively. LD₅₀ of P-GABA was found to be 327±8.2 mg/kg, ip. After 21 days of P-GABA/ethanol treatment, estimations of biochemical variables were done.

Minimal amount of blood (0.5 ml) was collected from orbital sinus using heparinized tubes from normal, control and P-GABA treated groups every 3 hr (0300, 0600, 0900, 1200, 1500, 1800, 2100 and 2400 hrs) throughout the 24 hr period for 3 days (3 cycles) The chemicals were from S.D. Fine chemicals, India (analytical grade). Blood glucose, serum choles
terol\textsuperscript{11} and protein\textsuperscript{22} and blood lactic acid\textsuperscript{23} were estimated immediately after blood collection. At the end of the study, normal, control and P-GABA treated animals were killed by decapitation. Brain tissues were taken in ice cooled trichloroacetic acid. GABA levels were measured by spectrofluorimetry\textsuperscript{24}.

Peak time (the time at which the level of the variable is highest over a 24 hr period), range (half of the difference between maximum and minimum values of the variable over a 24 hr period) and the 24 hr mean (mean value of the variable for equidistant data covering a 24 hr period) were calculated\textsuperscript{13-18}. The range and 24 hr mean are expressed with the same unit as the documented variable. The peak time is expressed in hr. Range of the rhythm is given in ± SD while 24 hr mean is given in ± SE\textsuperscript{13-18}. Student’s t-test was performed to detect the significant changes between control and P-GABA treated groups.

Results and Discussion

P-GABA prepared by the method of Bhowmick et al.\textsuperscript{13} showed melting point of 116\textdegree -117\textdegree C, absorption

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Fig. 1—Circadian rhythms of (a) glucose, (b) cholesterol, (c) total protein and (d) lactic acid levels at 3 hr intervals for 3 days in rats (•••• normal, + + + + control and - - - - P-GABA treated rats). Note advances (a and d) and delays (b and c) of peak times in P-GABA treated rats.
peak at 215 nm and presence of functional groups were confirmed by IR spectra\textsuperscript{13}. The \( R_f \) value of the sample was found to be 0.21\textsuperscript{13}. Brain GABA concentration was increased significantly (\( P < 0.001 \)) in P-GABA treated animals (5.18 ± 0.57 \( \mu \)g/g wet tissue) when compared with normal (1.30 ± 0.21) and control animals (1.40 ± 0.15).

The patterns of temporal variations of glucose (Fig. 1a), cholesterol (Fig. 1b), total protein (Fig. 1c) and lactic acid levels (Fig. 1d) in normal, control (20% ethanol treated) and P-GABA treated animals are shown. The variations of range and 24 hr mean values in P-GABA treated animals when compared with normal and control animals are shown in Table 1.

The biochemical variables exhibited marked fluctuations over 24 hr period. From the results, it can be concluded that environmental light-dark cycles may be the most effective synchronizers for the biochemical circadian rhythms in Wistar rats. Presence of GABA in retina\textsuperscript{25}, suprachiasmatic nuclei (SCN – the biological clock in mammals)\textsuperscript{8,26} and lateral geniculate nuclei strongly suggests the role of GABA in regulating circadian rhythms\textsuperscript{7,8}.

In the present study, administration of P-GABA increased the brain GABA levels corroborating the previous results\textsuperscript{13}. P-GABA acts like a non-specific agonist of GABA and more lipophilic than GABA\textsuperscript{13}. The volume of ethanol (20\%) was reduced while administering GABA since ethanol may influence circadian oscillations\textsuperscript{17}. However, no significant differences were detected in peak time, range and 24 hr mean values between normal and control (ethanol treated) animals suggesting that ethanol has no additive/interactive/synergistic action with P-GABA in the present study. After 21 days of administration of P-GABA, desynchronisation of biochemical circadian rhythms was observed; the peak time advances/delays of variables could be explained by slightly less or more than 1 hr daily delays/advances respectively, which would bring the peak times to the points after the start of administration.

Agonists of GABA are known to evoke phase shifts similar to dark pulses\textsuperscript{10,11,28}. Hence, it is suspected that GABA may be involved in conveying dark information to the clock (SCN) via the afferent pathways\textsuperscript{28}. Exogenous administration of P-GABA could reduce the photic information received by the clock or it could mimic dark conditions which would probably cause the loss of synchronization of the rhythms when compared to controls in the present study.

<table>
<thead>
<tr>
<th>Rhythm studied</th>
<th>Control (20% ethanol treated)</th>
<th>P-GABA (20% ethanol treated)</th>
<th>P-GABA (20% ethanol treated)</th>
<th>P-GABA (20% ethanol treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
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<td>2.40</td>
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<td>2.40</td>
</tr>
<tr>
<td>Cholesterol</td>
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<tr>
<td>Total Protein</td>
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<td>0.00</td>
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<tr>
<td>Lactic Acid</td>
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<td>24.00</td>
<td>24.00</td>
</tr>
<tr>
<td>Peak time (hr)</td>
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<tr>
<td>Magnitude of charge</td>
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</tr>
</tbody>
</table>

Table 1: Changes in the characteristics of biochemical circadian rhythms during P-GABA treatment (n=6)
Several biochemical circadian rhythms are synchronized by meal timing\(^{29}\). In the present experiment, peak time of glucose at 0300 hrs could be attributed to the food intake, digestion and accumulation of glucose in blood. The animals showed low level of glucose at afternoon corroborating the previous results\(^{30}\). The levels were lowest at 1800 hrs; this can be attributed to the continuous utilization of glucose during light period\(^1\).

In rats, eating is most pronounced during the first few hours of dark period and it virtually ceases when the lights are on\(^{31}\); rats consume 80% diet during the dark period\(^{32}\). The peak times of enzymes involved in glycolysis and TCA cycle are found to lie at early dark phase in rodents\(^{32}\). Hence, peak times of glucose (at late dark phase) and occurrence of nadir values (at the onset of dark phase) over a 24 hr period can be well correlated with food intake, digestion and accumulation of glucose in blood.

Cholesterol levels in rats in the present study were increased at night as reported previously\(^{33}\). The whole body free and total cholesterol synthesis oscillate periodically and predictably. Further, the cholesterol content in serum was clearly influenced by activity-rest cycles\(^{34}\). In rats, the rate limiting enzyme (HMGCoA reductase) in the cholesterol synthesis pathway peaks its activity at midnight\(^{35}\). In addition, free cholesterol of plasma low density lipoprotein and high density lipoprotein of the rats were high during dark period\(^{36}\). The peak level of transcription of cholesterol-7α-hydroxylase (7αH) gene was reported to occur in the evening\(^{37}\). All these factors may contribute to the night time increase of cholesterol.

Peak levels of total protein occurred at 0900 hrs in normal and control rats in the present study. Circadian rhythms in protein levels have been reported in mice, rats and humans\(^{38}\). The positive or negative balance between synthesis and degradation of proteins may be responsible for circadian rhythmic pattern of protein levels. Circadian rhythm in plasma lactic acid was reported in rats\(^{32}\). In the present study, peak time of lactic acid rhythm lied at 2400 hrs. This conspicuous night time increases of lactic acid level could be correlated with the activity rest status of the animal.

Agonists of GABA are known to alter the activity status of the animal as soon as after administration; while triazolam is known to induce hyperactivity for few hours after administration\(^{39}\), diazepam is known to cause inactivity for 1-2 cycles after administration\(^{40}\). The changes in range and 24 hr mean values of glucose levels could be due to altered status of activity after P-GABA treatment. Altered glucose levels could very well alter the range and 24 hr mean values of cholesterol and lactic acid levels. The changes in range and 24 hr mean values of total protein levels could be due to an altered balance between synthesis and degradation of proteins during P-GABA treatment.

References

11. Subramanian P & Subbaraj R, Diazepam modulates the period of locomotor rhythm in mice (Mus boodiga) and attenuates light induced phase advances, Pharmacol Biochem Behav, 54 (1996) 393.


