Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

METABOLIC AND STEATOHEPATITIS

Molecular and biochemical modifications of liver glutamine synthetase elicited by daytime restricted feeding

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Keywords
allosteric regulation – daytime restricted feeding – food entrained oscillator – nitrogen metabolism

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Received 21 July 2013
Accepted 16 November 2013

DOI:10.1111/liv.12412
Liver Int. 2014: 34: 1391–1401

Abstract

Background & Aims: The circadian clock system in the liver plays important roles in regulating metabolism and energy homeostasis. Restricted feeding schedules (RFS) become an entraining stimulus that promotes adaptations that form part of an alternative circadian clock known as the food entrained oscillator (FEO). The aim of this study was to evaluate the daily variations of glutamine synthetase (GS) in liver under a daytime RFS. *Methods:* Hepatic GS properties were analysed at 3-h intervals over a 24-h period in adult male Wistar rats maintained in a 12:12 h light–dark cycle. RFS group: food access for 2-h in light phase, during 3 weeks. AL group: feeding *ad libitum*. Fa group: acute fast (21 h). Fa–Re group: acute fast followed by refed 2 h. mRNA expression was measured by RT-qPCR, protein presence by Western-blot and immunohistochemistry, enzyme activity by a spectrophotometric assay, and glutamine by high pressure liquid chromatography. *Results and Conclusions:* Restricted feeding schedule induced circadian rhythmicity in mRNA levels of GS and the loss of the rhythmic pattern in mitochondrial GS activity. GS activity in liver homogenates displayed a robust rhythmic pattern in AL that was not modified by RFS. The presence of GS and its zonal distribution did not show rhythmic pattern in both groups. However, acute Fa and Fa–Re diminished GS protein and activity in liver homogenates. Hepatic glutamine concentrations showed a 24-h rhythmic pattern in both groups, in an antiphasic pattern. In conclusion, daytime RFS influences the liver GS system at different levels, that could be part of rheostatic adaptations associated to the FEO, and highlight the plasticity of this system.

In the liver, glutamine synthetase (GS) (EC 6.3.1.2) is a mostly cytoplasmic enzyme that catalyses the formation of glutamine from glutamate and ammonia in an ATP-dependent reaction (1). Glutamine is in equilibrium with glutamate responding to the activities of GS and glutaminase, and in function of the roles played by the glutamate in the liver, such as anaplerotic intermediate and cellular communication acting as a signalling molecule (2). GS is a homo-oligomeric and allosteric protein formed by eight subunits (~44 kDa) that is activated by α-ketoglutarate and inhibited by several amino acids (glycine, alanine, glutamine) as well as by carbamyl phosphate (1, 3). In mammals, one functional copy of the gene is present per haploid genome; it is transcriptionally regulated by glucocorticosteroids in rats (4) and generates two different transcripts that differ in the length of their 3′-untranslated region (5).

Glutamine synthetase plays a central role in nitrogen metabolism in the liver, complementing the activity of the urea cycle in the hepatic handling of ammonia (6). GS shows a marked zonal distribution in the liver, being enriched in the hepatocytes surrounding the central vein (7), that is dependent on the Wnt pathway via beta-catenin signalling (8). In contrast, most of the urea cycle occurs in periportal hepatocytes.

Hepatic GS activity is responsive to the feeding condition, since 1–5 days of fasting produced a decrease in the enzyme activity in rats, followed by a recovery to initial levels on day 7 (9), unlike skeletal muscle where GS activity increases (10). In a protocol of protein-free meal, low GS activity recovered to normal levels after 1 month of refeeding with the standard diet, suggesting a slower adaptation of GS to new metabolic conditions in comparison with other enzymes, such as alanine aminotransferase or aspartate animotransferase (11).

Several physiological processes are under circadian control, which implies the action of complex clock machinery that involves regulation of gene expression, post-transcriptional modifications and enzymatic activity (12, 13). Circadian rhythms are endogenous daily fluctuations in molecular, biochemical, physiological and behavioural parameters. Their oscillations show a period of ~24 h, and are reset and synchronized (entrained) mainly by the light–dark cycle associated to the day–night succession, but also for non-photic signals (cues) such as cyclic food availability (14). Restricted feeding schedules (RFS) is an experimental protocol used in several species to study the adaptation of the circadian system to a mealtime schedule, out of phase of the rhythmicity imposed by the light-driven pacemaker, the suprachiasmatic nucleus (SCN) (15). RFS consists in food access to a period of 2–4 h daily for consecutive days which promotes a set of notable changes in molecular, biochemical, physiological and behavioural activities, such as the food anticipatory activity, that have been associated with the expression of a circadian oscillator, which is independent of the SCN and is known as the food entrained oscillator (FEO) (16, 17).

Furthermore, RFS promotes significant modifications in parameters related to liver metabolic activity and histological characteristics such as: increased oxidized redox state and energy charge (18), enhanced mitochondrial synthesis of ATP (19), reduced glycogen mobilization, increased fatty acid oxidation and changes in the dimensions of hepatocytes (20), and augmentation of peroxisomal markers (21). In addition, RFS affected the rhythmicity of urea formation in a very significant way: it promoted high levels of urea preceding food access, which decreased rapidly to very low levels in response to mealtime; some hours later, the circulating urea rose gradually until reaching a prominent peak just before the onset of feeding (22). Therefore, RFS affects the biochemical status of the liver, including energetic activity and nitrogen metabolism (23).

In the present study, we further explored the adaptations that occur in hepatic nitrogen handling by characterizing molecular, biochemical and histological properties of the GS in rats under a daytime RFS protocol. Additionally, as a feeding control condition and to eliminate the possibility that responses to RFS were due 22 h food deprivation or fasting–refeeding conditions, we compared RFS with acute fasting (Fa) and fasting/refed (Fa–Re) rats that, unlike restricted feeding rats, do not express the FEO.

Materials and methods

Animals and housing

Adult male Wistar rats weighing 200 ± 20 g at the beginning of the experiments were maintained in constant conditions of 12:12 h light–dark cycles (lights on at 08:00 hours), and constant temperature (22 ± 1°C). Rats were kept in groups of four in transparent acrylic cages (40 × 50 × 20 cm) with free access to water and balanced Purina Chow meal except during food restriction (RFS), fasting (Fa) or fasting–refeeding (Fa–Re) conditions. Illumination was provided by 40 W fluorescent bulbs, 120 lux at the cage lid. All experimental procedures were approved by the Bioethics Committee of the Instituto de Neurobiología from the Universidad Nacional Autónoma de México and conformed with international ethical standards previously recommended (24).

Experimental design

Control and experimental groups were similar to those reported previously by our group (21). Briefly, rats were randomly assigned to four groups: (i) Rats fed *ad libitum* (AL) for 3 weeks; (ii) Rats exposed to a daily RFS with access to food only between 12:00 and 14:00 hours for 3 weeks; (iii) Rats fasted for 21 h (Fa) and (iv) Rats that were fasted 22 h and refed for 2 h (Fa–Re) (from 12:00 to 14:00 hours). At the end of the third week, rats from the AL and RFS groups were sacrificed by a guillotine at 3-h intervals to complete a 24-h day–night cycle (08:00, 11:00, 14:00, 17:00, 20:00, 23:00, 02:00 and 05:00 hours). The 21-h Fa and the Fa–Re groups were sacrificed at 11:00 and 14:00 hours, respectively. These groups were used as feeding condition controls for the RFS groups corresponding to 11:00 hours (before food access and during food anticipatory activity) and 14:00 hours (after feeding), respectively. Immediately after sacrifice, livers were obtained and processed to homogenize or to be frozen in dry ice and kept at −80°C until analysis.

Subcellular fractionation

A sample of 2 g of the liver was homogenized in 15 ml of homogenization buffer (10 mM Tris–HCl, pH 7.4, 225 mM sucrose, 0.2% BSA, 0.3 M EGTA). An aliquot of liver homogenate was kept at −80°C, and the rest was processed by fractionation, as previously reported (25). Briefly, the homogenate was centrifuged at 1500*g* for 15 min, the supernatant was centrifuged at 10,000*g* for 15 min to sediment the mitochondrial fraction, which was resuspended in homogenization buffer and kept at −80°C. All centrifugations were performed at 4°C.

RT-qPCR amplifications

Glutamine synthetase gene expression was evaluated by isolating total RNA from liver tissues (20–30 mg) using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The amount and quality of RNA were estimated spectrophotometrically at 260 and 280 nm, and a constant amount of RNA (2 μg) was reverse transcribed using SuperScript™ III Reverse Transcriptase, Oligo $(dT)_{12\text{-}18}$ Primer, RNaseOUT™ recombinant ribonucle-

ase inhibitor and dNTP Set PCR Grade (Invitrogen, Carlsbad, CA, USA). Amplification was performed in triplicate in the CFX96TM real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers used for qPCR amplifications were synthesized by Sigma-Aldrich Co. (St. Louis, MO, USA), and the corresponding sequences were: for GS, forward 5′-GACCCTATTACT GCGGTGTGG-3′, reverse 5′-TAAAGTTGGTGTGGCA GCCTG-3′ and for ribosomal protein S18 (Rps18) used as housekeeping gene, forward 5′-TTCAGCACATCC TGCGAGTA-3′, reverse 5′-TTGGTGAGGTCAATGTC TGC-3′. Amplifications were carried out with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA) in a 10 μl final reaction volume containing cDNA (1/100) and 0.5 μM of each of the primer pairs in SYBR Green Master Mix, according to the following protocol: activation of Taq DNA polymerase and DNA denaturation at 95°C for 10 min, followed by 40 amplification cycles consisting of 10 s at 95°C, 30 s at 62°C and 30 s at 72°C. The PCR data were analysed by the $2^{-\Delta\Delta C}{}_{T}$ method, and cycle thresholds (C_T) normalized to the housekeeping gene Rps18 was used to calculate the mRNA levels of GS.

Western-blot analysis

Liver homogenates and mitochondrial fractions were subjected to denaturing SDS-PAGE under reducing conditions. Total protein concentrations were determined by the Bradford method, and equal amounts (40 μg) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blocked for 1 h in TBST buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.5% Tween 20) containing 5% non-fat milk. Membranes were then washed and incubated overnight at 4°C in the presence of mouse anti-GS antibody (clone GS-6, MAB302; Millipore, Billerica, MA, USA) diluted 1/1000 in TBST. As controls, in the case of homogenate, membranes were incubated in the presence of mouse anti-tubulin antibody (ab56676; Abcam, Cambridge, UK) diluted 1/1000, or in the case of mitochondrial fractions in the presence of rabbit anti-VDAC1/Porin antibody (ab15895; Abcam) diluted 1/1000. After washing, membranes were incubated with secondary antibodies conjugated to alkaline phosphatase (1/5000). Bands were revealed using the AP conjugate substrate kit (Bio-Rad). Densitometric analysis was performed using the Image Lab Software (v 3.0; Bio-Rad).

Immunohistochemical staining

Liver tissues were fixed and dehydrated for paraffin embedding. Briefly, the obtained liver tissue was fixed for 1 week in 10% formalin at 4°C, with formalin changes every 2 days. After fixation, the tissue was embedded in paraffin and sectioned in 7 μm slices. Liver sections were deparaffinized for 2 h at 60°C in a dry heat oven, then rehydrated in a series of solvents: Xylol 100% (10 min), ethanol 100% (5 min), ethanol 96% (5 min), ethanol 80% (5 min) and deionized water (10 min), and then bathed in permeabilization buffer (3.9 mM sodium citrate, 0.1% Tween 20) for 8 min followed by a 60 s incubation in boiling sodium citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). The sections were then blocked with 5% non-fat milk for 1 h. After three washes with 0.05% TBST, sections were incubated overnight at 4°C with anti-GS antibody diluted 1/300. Afterwards, sections were washed three times with 0.05% TBST and incubated for 2 h with Alexa Fluor®-488 secondary antibody (Invitrogen) diluted 1/400. The fluorescence was visualized using microscopy (Microscope Olympus CX31, Center Valley, PA, USA) and quantified by the image analysis system Image-Pro Plus 6.0 software.

Glutamine synthetase activity

Glutamine synthetase activity was measured in both, liver homogenate and mitochondrial fraction by measuring NADH oxidation in a coupled-enzymatic reaction according to the procedure reported by Kingdon (26). Briefly, the ATP consumed during the glutamine synthesis from glutamate and NH_4^+ is regenerated by including pyruvate kinase and phosphoenolpyruvate in the incubations; the resulting pyruvate is reduced to lactate by added lactate dehydrogenase. This step is coupled to the oxidation of NADH, which is recorded spectrophotometrically at 340 nm. The GS activity was assayed in a final volume of 3 ml using 0.2 mg of total homogenate protein or 0.5 mg of total mitochondrial protein. The reaction was followed for 3 min (to ensure linearity), and the results were expressed as μmol/min/mg using a value of 6.22 as the mM extinction coefficient for β-NADH at 340 nm.

Measurement of hepatic glutamine by high pressure liquid chromatography

Glutamine concentrations within the liver were assayed by high pressure liquid chromatography (HPLC) using OPA/sulphite derivatization (stock OPA/sulphite solution, 20 mM OPA in 1 M sodium sulphite and 0.1 M sodium tetraborate, pH 9.3; working OPA/sulphite solution, 1/3 stock solution) and recorded by electrochemical detection. The liver samples were homogenized 1:5 (w/v) in 0.1 M perchloric acid containing 0.2 mM EDTA. The HPLC system consisted of a delivery pump (Solvent Delivery System PM-80; Bioanalytical Systems, Inc., West Lafayette, IN, USA), a simple injector (BASi Liquid Chromatography CC-SE, 20 μl loop; Bioanalytical Systems, Inc.), a C18 reverse phase column (BASi ODS C18, 100 × 3 mm, 3 μm particle size; Bioanalytical Systems, Inc.) and an electrochemical detector (Epsilon; Bioanalytical Systems, Inc.) with a carbon electrode; potential was adjusted to +700 mV vs. the reference electrode (Ag/AgCl). Glutamine concentrations were determined from peak areas using an external standard of L-Glutamine (Sigma-Aldrich Co.). The mobile phase contained 0.1 M

dibasic sodium phosphate pH 5.3, 20% methanol (v/v). The flow rate was maintained at 0.6 ml/min at a pressure of 2400 psi. All chromatograms were recorded and analysed using the ChromGraph Report software 2.3 (Bioanalytical Systems, Inc.). The protein concentration in each liver homogenate was determined to normalize the data and the results are expressed as μmol/μg protein.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). All data were replicated in two independent experiments and with three or four rats for each sampling time. The statistical analyses were performed using Sigma Stat software (v 3.5; Systat Software Inc., Richmond, CA, USA) and GraphPad Prism Software (v 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Normality distribution was determined by the Kolmogorov–Smirnov test. The data showed a parametric distribution or equal variances; therefore, statistical differences between different points in the time curves were determined by one-way ANOVA followed by Bonferroni's post hoc comparison test. All pairwise multiple comparisons were performed by the Student's *t*-test. Differences among groups were considered statistically significant at $P \leq 0.05$. Rhythm analyses were performed by CHRONOS-FIT (v 1.06; (27)) and COSANA (v 3.1 developed by AA Benedito-Silva, GMDRB, ICB/USP, Brazil).

Parameters of circadian rhythmicity

A given oscillation is defined by the next parameters (see Fig. 1): period, the time elapsed for one complete cycle; phase, each instantaneous state of an oscillation; acrophase, the time in which the peak of the rhythm occurs; MESOR (midline estimating statistic of rhythm), the average value around which the variable oscillates; amplitude, the difference between the peak and the mean value of a cycle.

Calculations for daily fluctuations

For chronobiological analysis, first, a one-way ANOVA was performed in each group and then a 24-h period single-cosinor method was used as previously described (29). For rhythmic interpretation of the results, the following parameters were considered: acrophase, MESOR, amplitude and rhythmicity, which correspond to a *P* value (<0.01) of an *F* test of fitting the original results to an expected sinusoidal curve with a 24-h period.

Results

Effect of time-restricted feeding on mRNA expression of hepatic glutamine synthetase

Starvation for 24 h has been reported to result in a decrease in GS mRNA (4), while prolonged starvation (72 h) induces absence of mRNA expression in pericentral hepatocytes (30). Since restricting food availability during daytime can regulate oscillations at the transcript level in the liver, we evaluated whether RFS modified the mRNA expression of GS by measuring it over a 24-h period by RT-qPCR. Our analysis showed that liver mRNA expression of GS from AL rats did not change over the 24 h, and neither the Fa group nor the Fa–Re group showed modified expression levels compared with the AL group (Fig. 2). In rats under RFS, hepatic mRNA expression of GS displayed a circadian variation ($P < 0.05$ 14:00 hours vs. 2:00 hours by one-way ANOVA) (Table 1). As determined by Cosinor analysis, acrophase appeared at 13:53 hours, in coincidence with the end of the mealtime. However, the MESOR was not significant different comparing the RFS to the AL group (Table 1). Controls of food condition, both acute Fa and acute Fa with a subsequent refeeding for 2 h (Fa–Re), showed mRNA expression levels significantly lower (~80%) than RFS and not different from AL at 11:00 and 14:00 hours, respectively (Fig. 2). These results indicate that daytime RFS induced changes in GS at the molecular level that are not observed in acute Fa or Fa–Re conditions.

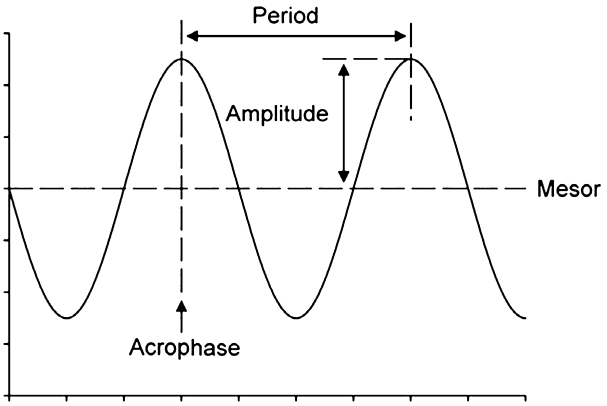

Fig. 1. Demonstrative oscillatory trace depicting the principal chronobiological parameters: period, acrophase, amplitude and MESOR. For details, see corresponding section in Methods. Taken from Refinetti (28).

Presence of hepatic glutamine synthetase protein under the time-restricted feeding protocol

Glutamine synthetase in mammalian liver is generally considered to be a cytosolic enzyme which is mostly bound to the outer membrane of endoplasmic reticulum and Golgi complex (1). It has been reported that GS is also found in mitochondrial fraction. To investigate if RFS influenced the amount of GS protein, the presence of GS (Fig. 3) was studied in liver homogenates (panel A) and the mitochondrial fraction (panel B) by Western-blot analysis. A specific band of ~44 kDa corresponding to GS was detected in all cases. As determined by densitometry (Fig. 3, lower panels), the levels

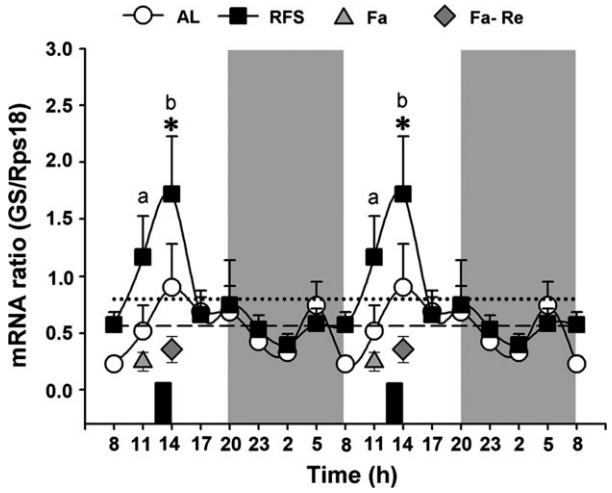

Fig. 2. Analysis of 24-h profile of relative mRNA expression of GS in the liver of rats under *ad libitum* (AL) and restricted feeding schedules (RFS) conditions. mRNA levels over a 24-h period are shown in a double plot. The shaded zone represents the dark phase. Livers from AL and RFS rats were collected at 3-h intervals over a 24-h period, and from controls of feeding condition Fa at 11:00 hours and Fa–Re at 14:00 hours. Food availability for the RFS group is indicated by dark boxes (from 12:00 to 14:00 hours). Relative mRNA levels were determined by RT-qPCR and normalized to Rps18 expression. Graphs represent the mean ± SEM of six to eight rats per time point. MESOR values are represented as a dashed line for AL group and dotted line for RFS group. *$P < 0.05$ vs. 02:00 hours by one-way ANOVA and post hoc Bonferroni test, a = $P < 0.05$ vs. Fa and b = $P < 0.05$ vs. Re–Fa by the Student's *t*-test.

of hepatic GS in both homogenate and mitochondrial fraction of RFS were not significantly different from the Al group over a 24-h period in terms of rhythmicity and mesor (Table 1). GS abundance in liver homogenate in the control groups of feeding condition, Fa and Fa–Re, was reduced (~70%) in comparison with AL and RFS at 11:00 hours and RFS at 14:00 hours, respectively (panel A). Fa–Re group was not different from AL at 14:00 hours (panel A). In the mitochondrial fraction, GS was not altered by the acute Fa or Fa–Re condition. Hence, in the liver homogenate where GS is enriched, the amount of GS protein diminished in the acute Fa and Fa–Re conditions, but it is maintained during daytime RFS (Fig. 3).

Presence of glutamine synthetase protein in pericentral hepatocytes under the time-restricted feeding protocol

Different metabolic pathways are carried out in hepatocytes according to their position within the hepatic parenchyma and related to the vascular architecture of the liver, known as hepatic zonation. In particular, GS is expressed only in a subpopulation of hepatocytes surrounding the central veins. Starvation for 3 days was reported to reduce the GS-positive zone, while a 10 consecutive repetitions of a 48-h starvation /24-h refeeding protocol resulted in expansion of the expression from the pericentral to the middle zone (31). To assess if RFS could modify the signal intensity of GS within the hepatic parenchyma, we evaluated the protein by immunofluorescence staining in rats under RFS at two critical times, before and after food access when the highest GS mRNA expression was observed (11:00 and 14:00 hours, respectively), as well as at a time during the dark phase (02:00 hours). Fluorescent signal appeared over the cytoplasm, in a particulate pattern and was restricted to one to three rows of hepatocytes surrounding the central veins (Fig. 4, panel A). The main signal was in the first layer, with no interruption in the continuity of the lane. Quantification of positive cells (Fig. 4, panel B) and fluorescence intensity (Fig. 4, panel C), as well as in a panoramic view at 2× magnification (Fig. 4, panel D), did not show significant differences among groups or related to the time. These observations indicate that GS zonation in the liver is not influenced by RFS, neither in the amount of GS protein nor in the number of cells of expressing it.

Effect of time-restricted feeding on hepatic glutamine synthetase activity

Besides the mRNA expression and protein presence, enzymes are also regulated at the level of activity; specifically, GS activity is allosterically modulated by divalent cations and some aminoacids (3). In addition,

Table 1. Chronobiological analysis of liver GS-related parameters: mRNA, protein, and activity and hepatic glutamate concentration

	GS mRNA		GS protein		GS activity				Glutamine	
					Hg		Mit			
	AL	RFS	AL	RFS	AL	RFS	AL	RFS	AL	RFS
Rhythm (%) by cosinor	–	16.4	–	–	68.3	72.7	33.6	–	63.8	56.6
Mesor	0.56	0.79	1.74	2.01	93.11	83.03	**52.95**	**103.50***	6.73	7.19
Amplitude	–	0.45	–	–	60.75	43.2	13.56	–	1.31	1.92
Acrophase (hours:min)	–	13:53	–	–	12:14	11:48	16:07	–	**11:56**	**23:34**

CHRONOS-FIT analysis was performed to evaluate the 24-h rhythmicity of different parameters in liver. Bold characters represent significative changes between AL and RFS groups. Hg, homogenate; Mit, mitochondrial fraction.

*$P < 0.05$ vs. AL in the same parameter by the Student's *t*-test.

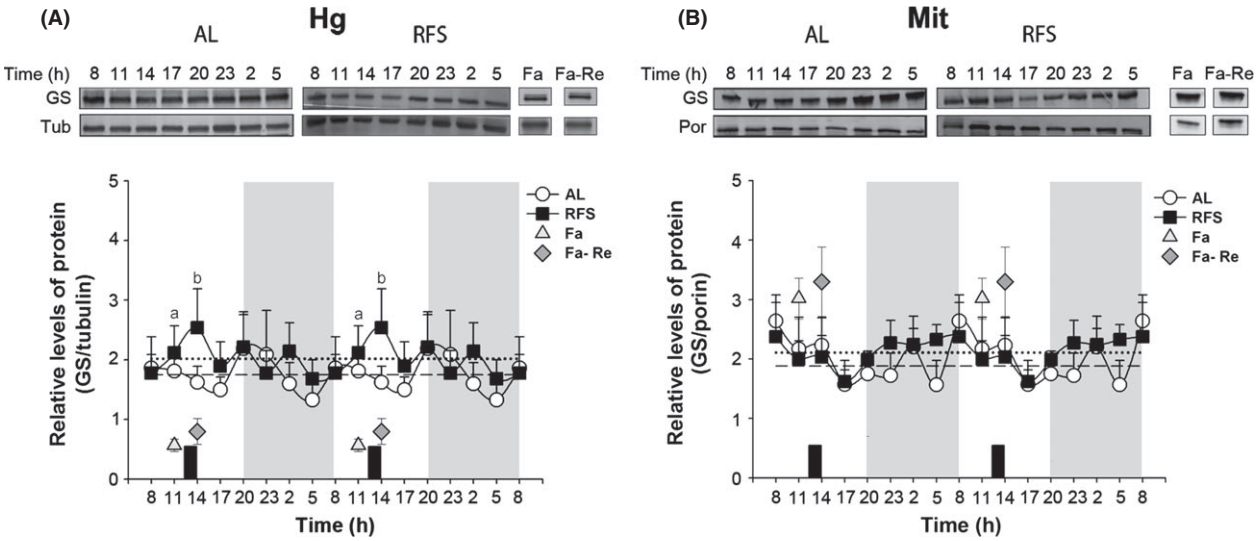

Fig. 3. Western-blot analysis of the 24-h profile of GS in liver of RFS and AL rats. Representative images of liver homogenates (Hg) (upper panel A) and the mitochondrial fraction (Mit) (upper panel B) loaded onto 10% SDS-polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. Tubulin and VDAC1/porin were used as loading controls for the homogenates and mitochondrial fraction, respectively. Densitometry of the bands over the 24-h period is shown in the graphs for the homogenates (panel A) and mitochondrial fractions (panel B) from the AL, RFS, and the food condition control groups, Fa and Fa–Re. Food availability for the RFS group is indicated by dark boxes (from 12:00 to 14:00 hours). MESOR values are represented as a dashed line for the AL group and as a dotted line for the RFS group. Graphs represent the mean ± SEM of seven to eight rats per time point. a = $P < 0.05$ vs. Fa and b = $P < 0.05$ vs. Fa–Re by Student's *t*-test.

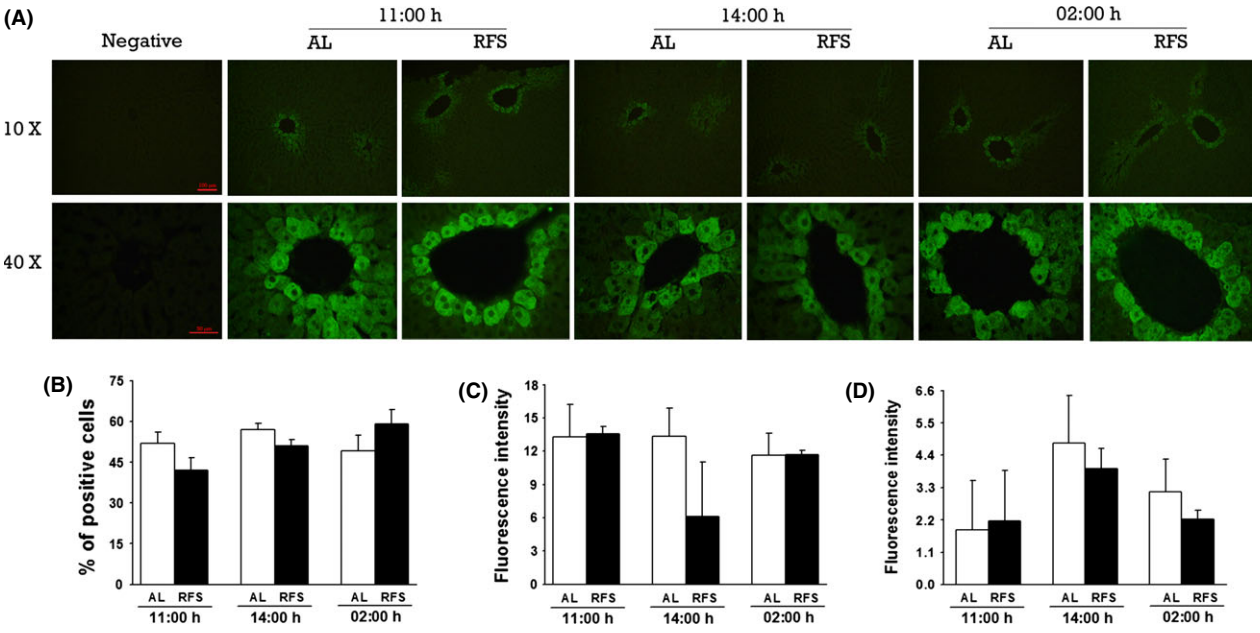

Fig. 4. Immunohistochemical localization of GS in hepatocytes surrounding the central lobular vein in rats under AL and RFS conditions. (A) Representative images of rat liver lobules of AL and RFS rats at 11:00, 14:00 and 02:00 hours visualized under fluorescent microscopy at two magnifications: 10× and 40×. Negative staining controls are shown. Quantifications in terms of percent positive hepatocytes and signal intensity are represented in the graphs (panels B and C, respectively). In a panoramic view (2×) fluorescence intensity was quantified (panel D). Two slides per rat and three rats per time were analysed. Bars represent the mean ± SEM. Differences were not significant as analysed by the Student's *t*-test.

calorie restriction has been shown to reduce the activity of the hepatic GS (32). Therefore, to investigate whether daytime RFS modified the activity of hepatic GS, this parameter was measured over a 24-h period. Since GS is considered as a cytosolic enzyme in mammalian liver because mostly is bound to the outer membranes of diverse organelles (80% in microsomes), we evaluate enzymatic activity in liver homogenate and in mitochondrial fraction.

In liver homogenate, AL and RFS groups displayed a strong rhythmicity in GS activity, with a daytime peak (acrophase) at ~12:00 hours (Table 1) followed by a significant decline towards the beginning of the dark phase (Fig. 5, panel A). Both groups showed an overall comparable GS activity (similar mesor) (Table 1). The Fa group (control of food condition) had significantly lower GS activity (~60%) than AL and was not different from RFS at the same time; however, the liver GS activity in Fa–Re was significantly lower than both RFS and AL at 14:00 hours (Fig. 5, panel A).

Glutamine synthetase activity in the mitochondrial fraction displayed significant differences between AL and RFS groups. The AL group showed 24-h rhythmicity, and according to cosinor analysis, the acrophase was at 16:09 hours, while daytime RFS led to constitutively elevated activity and the absence of a rhythmic pattern (Table 1). A significant increase (~2-fold) in the mitochondrial GS activity in the RFS group compared with AL group was observed over the entire period of 24 h (Fig. 5, panel B). GS activity in controls of food condition (Fa and Fa–Re) was lower (~60%) than in the RFS groups at 11:00 and 14:00 hours, respectively, and did not differ from the AL group (Fig. 5, panel B). These results suggest that RFS reprograms the GS activity, depending on the source of the enzyme, promoting significant changes related its regulation and rhythmicity.

Effect of time-restricted feeding on hepatic glutamine

Glutamine is synthesized from glutamate and NH_4^+ in an ATP-dependent reaction catalysed by GS. It is considered a non-toxic carrier of ammonia and is one of the amino acids whose concentration is highest in the blood plasma of vertebrates (33). In rat plasma its concentration is in the mM range, while within the liver is about μmol/mg of total protein. Since the contribution from blood to liver is less than 30%, we assumed that glutamine concentrations in the liver represent the intrahepatic content of glutamine.

Hepatic glutamine concentrations, quantified by HPLC, exhibited significant rhythmicity in the AL and RFS groups, according to one-way ANOVA and Cosinor analysis (Fig. 6, Table 1). Although the mesor and amplitude did not show significant differences (Table 1), the peak concentration of glutamine was shifted by the daytime RFS, with the highest value occurring during the dark phase (23:32 hours) in contrast with the peak in the AL group in the light phase (11:56 hours). Significant differences were also observed between the control Fa rats and RFS rats at 11:00 hours. The glutamine concentration was 1.6 times higher in Fa than in RFS. Moreover, the serum

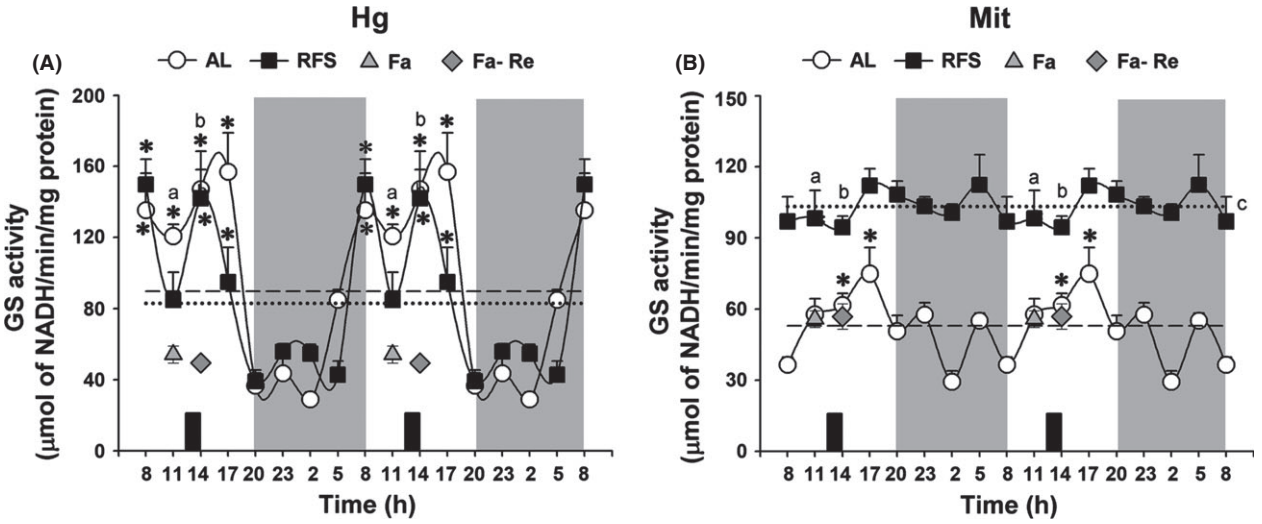

Fig. 5. Analysis of 24-h profile of hepatic GS activity in AL and RFS rats. GS activity was measured in both the liver homogenate (panel A) and mithocondrial fraction (panel B) of AL and RFS rats by a spectrophotometric assay. GS activity over a 24-h period is shown in a double plot. Rats were subjected to a regime of light:dark = 12:12 h. The shaded zone represents the dark phase. Food availability for the RFS group is indicated by dark boxes (from 12:00 to 14:00 hours). Controls of food condition are shown (Fa and Fa–Re). MESOR values are represented as a dashed line for AL group and a dotted line for RFS group. Graphs represent the mean ± SEM of eight rats per time point. *$P < 0.05$ vs. 02:00 hours by one-way ANOVA and post hoc Bonferroni test. a = $P < 0.05$ vs. Fa, b = $P < 0.05$ vs. Re–Fa, c = $P < 0.05$ vs. AL, all by Student's *t*-test.

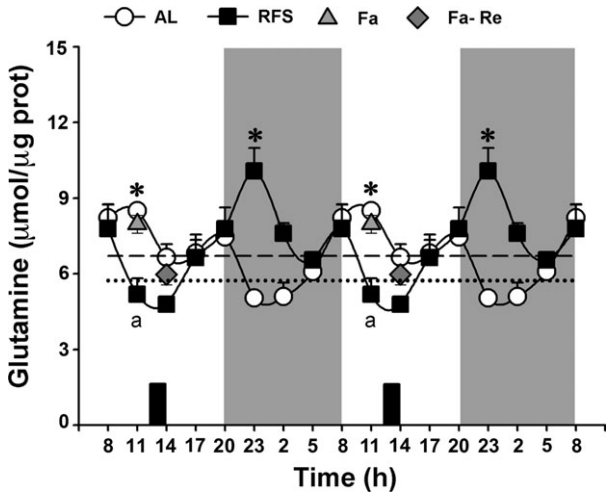

Fig. 6. Analysis of 24-h profile of glutamine in liver homogenate in AL and RFS rats. Glutamine concentrations in liver homogenates were measured by HPLC. Rats were subjected to a regime of light: dark = 12:12 h. The shaded zone represents the dark phase. Food availability for the RFS group is indicated by dark boxes (from 12:00 to 14:00 hours). Controls of food condition are shown (Fa and Fa–Re). Mesor values are represented as a dashed line for the AL group and a dotted line for the RFS group. The graphs represent the mean ± SEM of four rats per time point. *$P < 0.05$ vs. 02:00 hours by one-way ANOVA and post hoc Bonferroni test, a = $P < 0.05$ vs. Fa, by Student's *t*-test.

glutamine concentrations in the corresponding time points of hepatic glutamine acrophases (highest value) and valleys (lowest value) were similar in both groups (data not shown), which support the fact that the rhythmic behaviour is intrinsic of the liver and not for blood contamination. These results suggest that daytime RFS modifies the daily rhythmicity of liver GS at multiple levels.

Discussion

Glutamine synthetase is a key enzyme in nitrogen metabolism. GS is formed by eight identical subunits of ~44 kDa molecular weight that contains four active sites (34), and it is transcriptionally and allosterically regulated (35). In the present study, we have shown that a 3-week protocol of daytime restricted food availability modified liver GS and the amount of its catalytic product, glutamine, at different levels as measured by different rhythmic parameters.

In the mouse liver, circadian regulation has been demonstrated for many genes implicated in the nutrient and intermediate metabolism (23), such as enzymes and transporters for glucose metabolism, as well as cholesterol biosynthetic and catabolic enzymes (36). In mammals, transcriptional regulation of GS is the key mechanism responsible for the liver-specific zonal expression of GS (37). Our study revealed that mRNA expression of GS did not show a rhythmic pattern over a 24-h period; however, under restricted feeding a rhythm appeared whose acrophase coincided with the end of food access (~14:00 hours). Although there is no evidence for the presence of consensus sites of regulation by circadian control genes in either the GS promoter or its enhancers, circadian regulation could be the indirect result of other factors that are influenced by or drive circadian output rhythms in target tissues, such as corticosteroids, and by the presence of glucocorticoid-response elements in the GS promoter (38, 39). In fact, the acrophase of mRNA expression in RFS rats occurred after the peak of serum corticosterone in rats under the same restricted feeding protocol (18), supporting this possibility.

It is known that the circadian transcriptome comprises 5–10% of the genes studied by microarray analysis in different tissues (40, 41). Regarding the proteosome, up to 20% of soluble proteins, predominantly enzymes, of the mouse liver are subject to circadian control (13), and interestingly, almost half of them lack a correspondingly cycling transcript. By inference, these observations strongly suggest that several proteins are subjected to post-transcriptional mechanisms of circadian control. In this context, we analysed if there was a correlation between gene expression, protein levels and activity of liver GS, as well as with the level of glutamine. Overall, there was no a clear relation among these parameters (Table 1): some of them were rhythmic, such as GS activity and glutamine, whereas others did not show 24-h variations at all, such as levels of GS protein. Interestingly, RFS differentially modulated several of them: it induced rhythmicity in GS mRNA, promoted enhanced GS activity in the mitochondrial fraction, preserved the diurnal rhythm of the GS activity measured in the liver homogenate and promoted the phase shift in the levels of hepatic glutamine. These findings indicate that the GS properties and glutamine metabolism can be influenced by the circadian molecular clock at different steps, and by different mechanisms that remain to be elucidated.

Metabolic zonation in the liver is based on the efficient adaptation of functions that occur in the hepatocytes in response to changes in the environment (42), including nutritional supply and levels of oxygen, metabolites and hormones (42, 43). This functional hepatocyte heterogeneity implies modification of gene expression and regulation of enzymatic activities by mechanisms that include allosteric modulation. Ammonia metabolism displays a dynamic and static zonation (7): whereas ureogenesis is performed in periportal hepatocytes, synthesis of glutamine is carried out in pericentral hepatocytes (7, 44). According to our study, the RFS protocol did not modify the presence of GS around the pericentral vein, as measured by two approaches: fluorescence intensity and percent positive cells. In a panoramic analysis of the fluorescent signal, a similar pattern of distribution of GS along the acinus was observed in both the AL and RFS groups. Thus, the

presence of protein analysed by Western-blot and by immunohistochemistry was not influenced by daytime RFS. Many clock proteins are post-transcriptionally regulated to control their rates of accumulation and degradation, allowing cell functions to oscillate even when genes are expressed constitutively or without a rhythmic pattern. Hence, in spite of the changes in its expression, protein content in cellular fractions, and activity, GS kept its characteristic liver zonation during the RFS protocol.

The enzymatic activity of several proteins has been demonstrated to play a key role in rhythmic behaviour of their target proteins. It is the case for enzymes that are associated with food intake and energy status, such as the NAD^+-dependent deacetylase SIRT1, which functions in a circadian manner, and CLOCK, a key transcription factor in the molecular circadian machinery that has intrinsic acetyltranferase activity (45, 46).

Most enzymes with quaternary structure are susceptible to allosteric regulation. In mammals, GS is an octamer, and substrate binding can stabilize the active form to facilitate the binding of a second substrate. Liver GS contains in its structure various kinds of allosteric sites for regulation that can recognize substrates, products, other metabolites and divalent cations (3, 47). Thus, GS actions ultimately depend upon regulation of its activity and probably, according to the present results, on temporal regulation as well. Our study showed differential patterns of GS activity when the liver homogenate and mitochondrial fraction were compared. In homogenate, although there was neither a rhythmic pattern nor changes in protein abundance, GS activity showed a robust diurnal rhythm that was not modified by the daytime RFS in its rhythmic parameters: mesor, amplitude and acrophase. It seems that the light/dark cycle drove the rhythm of cytoplasm GS activity, whereas the mealtime schedule influenced the mitochondrial activity.

Since GS is mostly bound to the outer membranes of diverse organelles, it is considered a cytosolic enzyme in mammalian liver (1). In this study, the GS activity detected in the mitochondrial fraction is accounted for the attachment of GS to the external mitochondrial membrane. The GS activity in the mitochondrial fraction did not show a rhythmic pattern; however, it was significantly higher in RFS than AL groups at all the times measured. The FEO, promoted by the RFS, appears to influence the GS activity differentially, in a compartment-specific manner. We suggest that GS in the mitochondrial fraction adopts a conformation with higher activity when the FEO is expressed. However, so far we have not identified the biochemical mechanism (s) underlying this phenomenon.

Glutamine is not only a substrate for the biosynthesis of proteins and purine rings, but it is also fundamental in the handling of nitrogen, both as a source and as a carrier. The turnover of liver glutamine is rapid and depends on breakdown by glutaminase and re-synthesis by GS to ensure a very efficient regulation of body nitrogen balance and acid-base homeostasis. (35). Our study demonstrated that concentrations of intrahepatic glutamine showed a robust daily pattern, both in AL and RFS rats, with similar rhythmic characteristics, but in an antiphasic manner. Taking into consideration the fact that the glutamine concentration in liver depends on anabolism by GS and catabolism by glutaminase, as well as excretion via the SNAT3 transporter, it is possible that the rhythmicity of this parameter is influenced by several metabolic factors and environmental conditions. The antiphasic oscillation suggests an influence of the circadian clock on liver GS under AL conditions, but this influence is modified by the establishment of the FEO under RFS.

It is clear that daytime RFS has an influence on several steps in the handling of nitrogen by the liver, for example, the 24-h rhythmicity of serum urea and the activities of ornithine transcarbamylase and carbamoyl phosphate synthetase-1 (18), the rate-limiting enzyme in the urea cycle, and in this work, we showed evidence that GS characteristics are also affected.

In conclusion, daytime RFS is a strong entraining stimulus that promotes the expression of the FEO, influences liver GS at different levels (transcription, post-transcriptional modifications and enzymatic activity), and regulates rhythmic parameters (the appearance or disappearance of a rhythm, shift of phase and changes in mesor). All these changes seem to be part of the metabolic, physiological and chronobiological adaptations promoted by the RFS during the rheostatic adaptation associated with FEO expression, and they differ from those responses provoked by acute Fa or Fa–Re conditions, maybe to optimize the handling of nutrients and their related compounds, including ammonia. These changes show the plasticity of hepatic GS system when the organism faces a metabolic challenge such as RFS.

Acknowledgements

We thank Fernando López-Barrera for his expert technical assistance and Dr. Dorothy Pless for critically editing the manuscript.

Financial support: This project was supported by grants IA200713 and IB200311 from PAPIIT, UNAM, México.

Conflict of interest: The authors do not have any disclosures to report.

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