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RESEARCH ARTICLE

Glucosensing capacity in rainbow trout liver displays day–night variations possibly related to melatonin action

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SUMMARY

To assess whether the glucosensing capacity in peripheral (liver and Brockmann bodies) and central (hypothalamus and hindbrain) locations of rainbow trout displays day–night variations in its response to changes in circulating glucose levels, we evaluated the response of parameters related to glucosensing [glucose, glycogen and glucose 6-phosphate levels, activities of glucokinase (GK), glycogen synthetase (GSase) and pyruvate kinase (PK), and mRNA abundance of GK, glucose transporter 2 (GLUT2), and K_{ATP} channel subunits Kir6.x-like and sulfonylurea receptor (SUR)-like] in fish subjected to hyperglycemic treatment under night or day conditions. No day–night significant variations were noticed in the glucosensing capacity of the hypothalamus, hindbrain and Brockmann bodies. In contrast, a clear differential response was noticed in the liver, where glucose levels, GK activity (and mRNA levels) and GSase activity displayed increased values during the day in hyperglycemic fish compared with controls, and lower (GK mRNA levels) or non-existent (glucose, GK and GSase activities, and Kir6.x-like mRNA levels) values during the night. A similar decrease in parameters related to glucosensing in the liver was observed when fish under day conditions were treated with melatonin, suggesting a modulatory role of melatonin in day–night changes of the glucosensing response in the same tissue.

Key words: trout, glucosensing, liver, Brockmann bodies, melatonin, hypothalamus, hindbrain.

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INTRODUCTION

In previous studies in rainbow trout we demonstrated (Soengas et al., 2006; Polakof et al., 2007a; Polakof et al., 2007b; Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c) the presence of a glucosensing system in central (hypothalamus and hindbrain) and peripheral (liver, Brockmann bodies) locations similar to that found in mammalian pancreatic β-cells (Schuit et al., 2001) and glucose-excited (GE) neurons (Marty et al., 2007) based on glucokinase (GK), glucose transporter 2 (GLUT2) and K_{ATP} channels (for a review, see Polakof et al., 2011). We also demonstrated that these glucosensing systems are deregulated under stress conditions, like those associated with high stocking density (HSD), resulting in their inability to respond to changes in circulating glucose levels (Conde-Sieira et al., 2010a). Furthermore, the same stress conditions elicit reduced food intake and changes in the mRNA abundance of several peptides related to the control of food intake, including corticotropin-releasing factor (CRF) (Conde-Sieira et al., 2010b), that could be related to the well-known anorectic effect of stress in fish (Wendelaar Bonga, 1997; Bernier, 2006). Accordingly, we have recently demonstrated that the responses to changes in glucose levels of parameters related to glucosensing in the hypothalamus and hindbrain of rainbow trout are modified in the presence of CRF (Conde-Sieira et al., 2011), in a way comparable to that observed under stress conditions (Conde-Sieira et al., 2010a).

Melatonin is considered an anti-stress molecule (Macchi and Bruce, 2004), and accordingly a reduction in cortisol levels has been observed in stressed fish treated with melatonin (Herrero et al., 2007; Azpeleta et al., 2010). In a recent study (Conde-Sieira et al., 2012), we provided evidence that melatonin treatment induced an up-regulation of glucosensing mechanisms in the hypothalamus (possibly through MT_1 receptors) similar to that observed under CRF treatment. These results do not support the idea that the putative anti-stress role of melatonin in fish is associated with deregulation of central glucosensing mechanisms, allowing us to suggest that the modulatory role of melatonin on glucosensing must be related to other processes, such as synchronization of daily changes in glucose homeostasis. As glucose tolerance in fish is apparently dependent on the time of feeding, with marked differences throughout the day (López-Olmeda et al., 2009), and brain glucose levels increased at night in rainbow trout fed in the morning (Polakof et al., 2007c), we suggest that melatonin could play a role in the modulation of the daily response to glucose, which could be related to the activity of the glucosensor systems.

Therefore, in the present study we aimed to evaluate whether the glucosensing capacity in peripheral (liver and Brockmann bodies) and central (hypothalamus and hindbrain) locations of rainbow trout displays day–night variations in its response to changes in circulating glucose levels – and, if so, how these relate to the effects of melatonin treatment.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 month in 100 l tanks under laboratory conditions and a 12 h:12 h L:D photoperiod in dechlorinated tap water at 15°C. Fish mass was

115±4 g. Fish were fed once daily (09:00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ kg^{-1} of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/EU), and of the Spanish Government (RD 1201/2005) for the use of animals in research.

Experimental protocol

Experiment 1. Glucosensing capacity at night and during the day

Following acclimation, fish were randomly assigned to 100 l experimental tanks. Fish were fasted for 24 h before experiments began to ensure basal hormone levels were achieved. The day of experiment, at 09:00 h, fish were lightly anesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed, and intraperitoneally injected with 5 ml kg^{-1} body mass of saline alone (control, normoglycemic) or containing D-glucose (500 mg kg^{-1} body mass, hyperglycemic). Immediately after injection, fish were returned to their tanks (two replicates per glycemic treatment), where they remained for 6 h until sampling. The same procedure was carried out with another batch of fish that were injected at 20:00 h and sampled 6 h later (in this case a single low intensity red light was used to aid in sampling at night). Therefore, the four experimental groups (two replicates per treatment) used in this experiment were: (1) normoglycemic fish sampled during the day, (2) hyperglycemic fish sampled during the day, (3) normoglycemic fish sampled at night, and (4) hyperglycemic fish sampled at night. In each group, 8 fish were used to assess enzyme activities and metabolite levels and the remaining 4 fish were used for the assessment of mRNA levels by qRT-PCR. On each sampling, fish were removed from the holding tanks, anesthetized as above and weighed. Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 6% perchloric acid) and neutralized (using 1 mmol l^{-1} potassium bicarbonate) before freezing in liquid nitrogen and storage at –80°C until further assay. The liver was removed, frozen in liquid nitrogen and stored at –80°C until assayed. Brockmann bodies were dissected and cleaned from surrounding vessels, bile ducts and connective tissue, frozen in liquid nitrogen and stored at –80°C until assayed. The brain was removed and placed on a chilled Petri dish; the hypothalamus and hindbrain were obtained as described previously (Polakof et al., 2007b), frozen in liquid nitrogen and stored at –80°C until assayed.

Experiment 2. Effects of melatonin treatment on glucosensing capacity

Following acclimation, fish were randomly assigned to 100 l experimental tanks. Fish were fasted for 24 h before experiments began to ensure basal hormone levels were achieved. The day of experiment, at 09:00 h, fish were lightly anesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed, and intraperitoneally injected with 5 ml kg^{-1} body mass of saline alone (control, normoglycemic) or containing D-glucose (500 mg kg^{-1} body mass, hyperglycaemic) with or without melatonin (0.5 mg kg^{-1}). Immediately after injection, fish were returned to their tanks (two replicates per glycemic treatment) where they remained for 6 h. Therefore, the four experimental groups (two replicates per treatment) used in this experiment were: (1) normoglycemic fish without melatonin, (2) hyperglycemic fish without melatonin, (3) normoglycemic fish treated with melatonin, and (4) hyperglycemic fish treated with melatonin. In each group, 8 fish were used to assess enzyme activities and metabolite levels and the remaining 4 fish were used for the assessment of mRNA levels by qRT-PCR. Fish were sampled as described above for experiment 1.

Assessment of metabolite levels and enzyme activities

Plasma melatonin levels were assayed as described elsewhere (Muñoz et al., 2009), with modifications. Briefly, a 200 µl aliquot of plasma sample was mixed (1:1 v:v) with 0.1 mol l^{-1} acetic acetate buffer (pH 4.6) and 2 ml chloroform was added. The mixture was mixed for 1 min, centrifuged (3800 ***g***, 10 min), and the aqueous phase aspirated. The organic layer was separated from the rest and 500 µl 0.1 mol l^{-1} NaOH was added. After stirring and posterior centrifugation (centrifugal force in the opposite direction to the first case), the aqueous phase was aspirated and the organic layer was dried in a speed-vac system. The residue was dissolved in 100 µl of mobile phase and filtered through 0.5 µm filters. An aliquot (50 µl) of the filtrate was injected into the HPLC system. Data were normalized by the volume (ml) of plasma. The chromatographic system consisted of a Gilson (Middleton, WI, USA) 321 solvent delivery pump equipped with a 50 µl Rheodyne (Index Health, Oak Harbor, WA, USA) injection valve, and a Jasco (Easton, MD, USA) FP-1520 fluorescence detector set at 280 nm/345 nm excitation/emission wavelengths. Melatonin was separated on a Phenomenex (Torrance, CA, USA) Kinetex C-18 column (2.6 µm particles). The mobile phase consisted of a solution of 85 mmol l^{-1} acetic acetate, 0.1 mmol l^{-1} EDTA-Na_2 and 14% (final volume) acetonitrile, pH adjusted to 4.7. All analyses were performed at room temperature at a flow rate of 1.0 ml min^{-1}.

Plasma glucose and lactate levels were determined enzymatically using commercial kits (bioMérieux, Madrid, Spain and Spinreact, Barcelona, Spain, respectively) adapted to a microplate format. The pieces of tissue used for the assessment of metabolite levels were homogenized immediately by ultrasonic disruption with 7.5 volumes of ice-cooled 6% perchloric acid, and neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged, and the resulting supernatant was immediately assayed. Tissue glycogen levels were assessed as described previously (Keppler and Decker, 1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (bioMérieux).

Tissue pieces used to assess enzyme activities were homogenized by ultrasonic disruption with 9 volumes of ice-cold-buffer consisting of 50 mmol l^{-1} Tris (pH 7.6), 5 mmol l^{-1} EDTA, 2 mmol l^{-1} 1,4-dithiothreitol, and a protease inhibitor cocktail (P-2714; Sigma Chemical Co., St Louis, MO, USA). The homogenate was centrifuged and the supernatant used immediately for enzyme assays. Enzyme activities were determined using an INFINITE 200 PRO microplate reader (Tecan, Grödig, Austria) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (10–15 µl), at a pre-established protein concentration, omitting the substrate in control wells (final volume 265–295 µl), and allowing the reactions to proceed at 20°C for pre-established times (3–15 min). Enzyme activities were normalized by protein mass (mg). Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method, with bovine serum albumin (Sigma) as standard. Enzymatic analyses were all carried out at maximum rates, with the reaction mixtures set up in preliminary tests to render optimal activities. GK, glycogen synthetase (GSase) and pyruvate kinase (PK) activities were estimated as described

previously (Polakof et al., 2007a; Polakof et al., 2007b; Polakof et al., 2008a; Polakof et al., 2008b).

Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ1-DNAse (Promega, Madison, WI, USA). Total RNA (2 μg) was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and random hexamers (Invitrogen). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ (BioRad, Hercules, CA, USA). Analyses were performed on 1 μl of the diluted cDNA using the MAXIMA SYBR Green qPCR Mastermix (Fermentas, Vilnius, Lithuania), in a total PCR reaction volume of 15 μl, containing 50–500 nmol l^{-1} of each primer. The mRNA levels of parameters related to glucosensing capacity [GK, GLUT2, and K_{ATP} channel subunits Kir6.x-like and sulfonylurea receptor (SUR)-like] were assessed by qPCR as previously described for rainbow trout (Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c).

Relative quantification of the target gene transcripts was done using β-actin gene expression as a reference, which was stably expressed in these experiments. Thermal cycling was initiated by incubation at 95°C for 15 min using hot-start iTaq DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15 s for denaturation, and at the specific annealing temperature for 30 s, followed by extension at 72°C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C s^{-1} from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analyzed in triplicate. All the replicates of each sample were located in the same plate for each gene to allow comparisons. For all plates, we included the standard curve (in triplicate), and blanks for DNA, PCR and retrotranscription (in duplicate). Only efficiency values between 85 and 100% were accepted (the R^2 for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl method (Pfaffl, 2001).

Statistics

In experiment 1, comparisons among groups were carried out using two-way ANOVA with time of day (day–night) and glycemic levels (normoglycemic and hyperglycemic) as main factors. In experiment 2 comparisons among groups were carried out using two-way ANOVA with glycemic levels (normoglycemic and hyperglycemic) and melatonin treatment as main factors. In both experiments *post hoc* comparisons were made using Student–Newman–Keuls tests, and differences were considered statistically significant at $P<0.05$.

RESULTS

Parameters assessed for the plasma samples are shown in Fig. 1. In experiment 1, melatonin levels were higher at night than during the day but this difference was not significant in the hyperglycemic group. In experiment 2, melatonin treatment induced a 10-fold increase in melatonin levels compared with the non-treated group, and the hyperglycemic group showed lower levels than controls with melatonin treatment. Plasma glucose levels increased after hyperglycemic treatment in a similar way under day and night conditions, and melatonin treatment did not modify this response. Plasma lactate levels increased after hyperglycemic treatment only under night conditions (experiment 1) or after melatonin treatment (experiment 2).

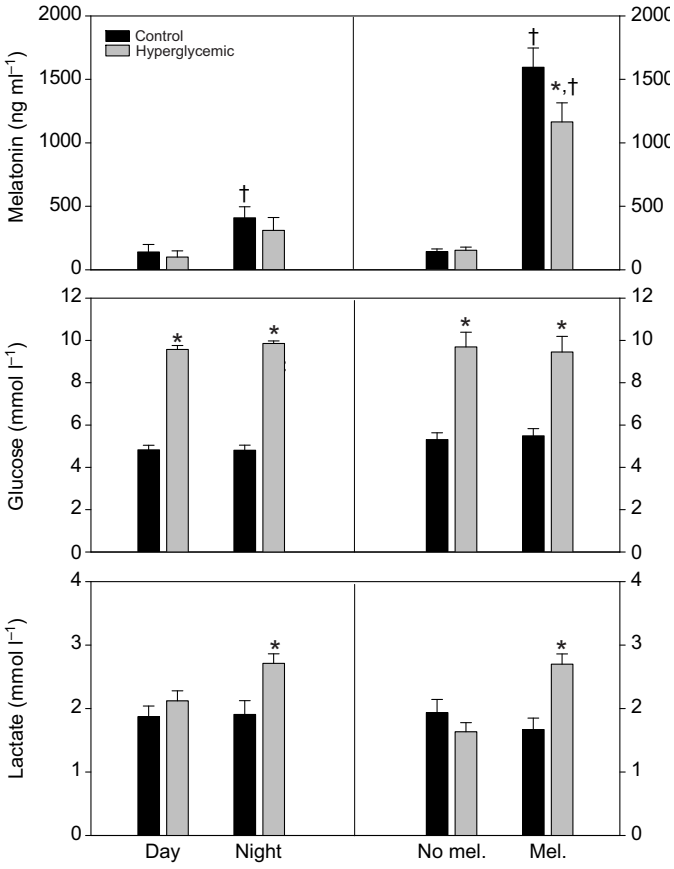

Fig. 1. Melatonin, glucose and lactate levels in plasma of rainbow trout under different glycemic conditions elicited by intraperitoneal administration of saline alone (control, normoglycemic) or containing 500 mg D-glucose kg^{-1} (hyperglycemic). Left panels (experiment 1): fish injected during the day (09:00 h) or night (20:00 h) and sampled 6 h later. Right panels (experiment 2): fish injected at 09:00 h with Cortland saline alone (control, no melatonin) or containing 0.5 mg kg^{-1} melatonin and sampled 6 h later. Data represent means + s.e.m. of 12 measurements. *Significantly different ($P<0.05$) from control (normoglycemic) fish under the same treatment. †Significantly different ($P<0.05$) from day (left panels) or no melatonin (right panels) treatment under the same glycemic conditions.

Changes in the levels of liver metabolites are shown in Fig. 2. Glucose levels increased after hyperglycemic treatment during the day but not during the night; melatonin treatment abolished the increase in glucose observed under hyperglycemic conditions. Glycogen levels increased under hyperglycemic conditions during both day and night (experiment 1), whereas the presence of melatonin abolished this increase (experiment 2).

Activities of enzymes in the liver are shown in Fig. 3. GK activity increased after hyperglycemic treatment during the day but during the night control levels were similar to those of hyperglycemic fish, whereas the increase noticed after hyperglycemic treatment in experiment 2 was not observed after melatonin treatment. GSase activity increased sharply in hyperglycemic fish during the day, whereas the increase was lower when hyperglycemic treatment occurred at night; the increase due to hyperglycemic treatment disappeared when melatonin was administered, as observed in experiment 2. PK activity increased under hyperglycemic conditions only at night, whereas melatonin treatment did not modify the response from that observed in the non-treated group.

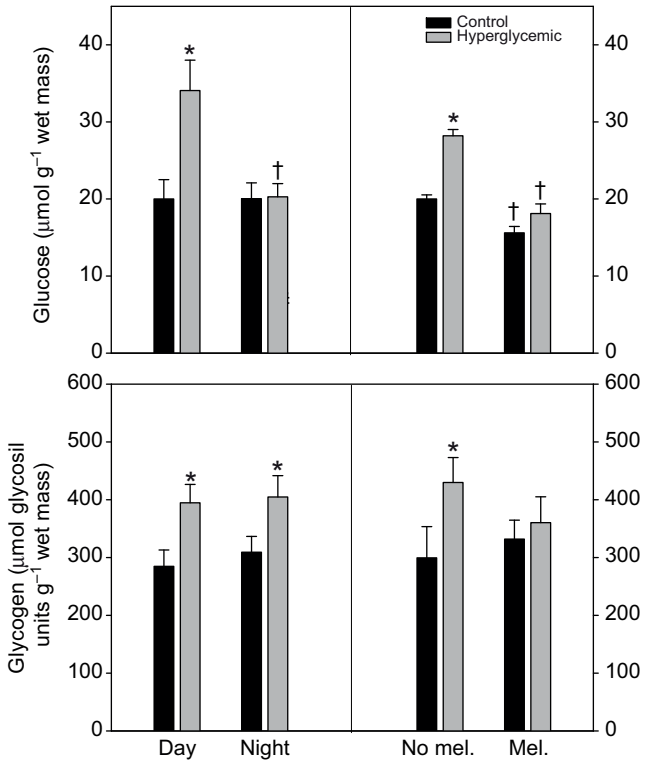

Fig. 2. Glucose and glycogen levels in liver of rainbow trout under different glycemic conditions elicited by intraperitoneal administration of saline alone (control, normoglycemic) or containing 500 mg D-glucose kg^{-1} (hyperglycemic). Left panels (experiment 1): fish injected during the day (09:00 h) or night (20:00 h) and sampled 6 h later. Right panels (experiment 2): fish injected at 09:00 h with Cortland saline alone (control, no melatonin) or containing 0.5 mg kg^{-1} melatonin and sampled 6 h later. Data represent means + s.e.m. of 8 measurements. For further details, see legend to Fig. 1.

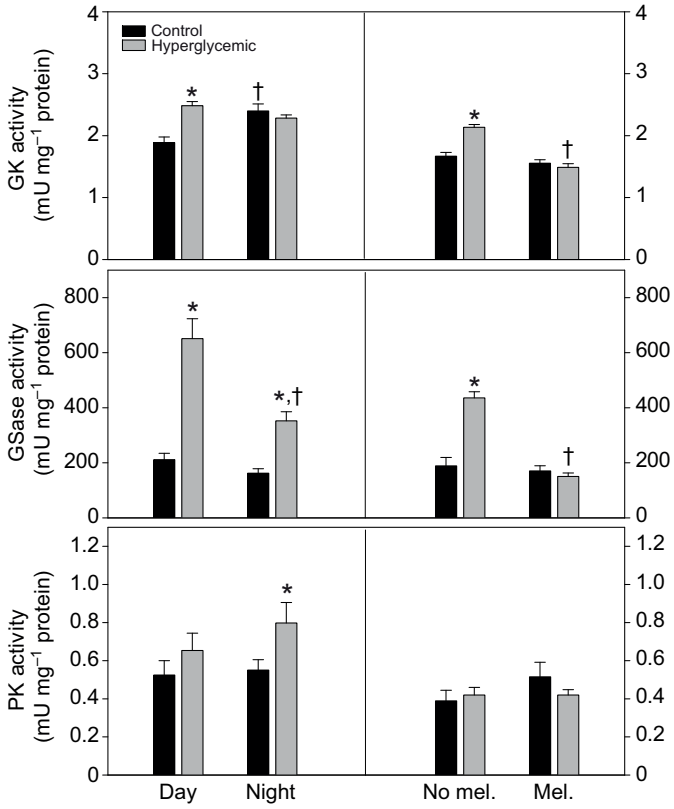

Fig. 3. Activities of glucokinase (GK), glycogen synthetase (GSase) and pyruvate kinase (PK) in liver of rainbow trout under different glycemic conditions elicited by intraperitoneal administration of saline alone (control, normoglycemic) or containing 500 mg D-glucose kg^{-1} (hyperglycemic). Left panels (experiment 1): fish injected during the day (09:00 h) or night (20:00 h) and sampled 6 h later. Right panels (experiment 2): fish injected at 09:00 h with Cortland saline alone (control, no melatonin) or containing 0.5 mg kg^{-1} melatonin and sampled 6 h later. Data represent means + s.e.m. of 8 measurements. For further details, see legend to Fig. 1.

The mRNA abundance of genes related to glucosensing potential in the liver are shown in Fig. 4. GK and Kir6.x-like mRNA levels were increased by hyperglycemic treatment during the day but not during the night, and the increase observed in the non-treated group was abolished when fish were additionally treated with melatonin, as observed in experiment 2. GLUT2 mRNA abundance was not affected by hyperglycemic, day–night or melatonin treatments. SUR-like mRNA abundance increased after hyperglycemic treatment during the night but not during the day, whereas melatonin treatment did not affect mRNA levels.

Parameters assessed in the hypothalamus, hindbrain and Brockmann bodies are shown in Table 1. In the hypothalamus, the increase observed after hyperglycemic treatment during the day in glucose and glycogen levels as well as in GK, GSase and PK activities and GK mRNA levels remained basically the same when fish were treated at night; melatonin treatment abolished the increase in GSase activity and GLUT2 mRNA levels. In the hindbrain, the hyperglycemic treatment induced similar increases during the day and night in glucose and glycogen levels, GSase activity and GK mRNA levels; melatonin treatment did not modify the responses to hyperglycemic treatment. In Brockmann bodies, hyperglycemic treatment during the day induced increased levels of glucose and glycogen, and increased activity and mRNA levels of GK that were not modified when the treatment occurred at night; melatonin treatment did not alter in general the responses to hyperglycemic conditions.

DISCUSSION

Several parameters assessed in controls (for instance, GK activity in liver or PK activity in hypothalamus and hindbrain) displayed clear day–night differences, which in some cases agree with those described for the same species in the literature (Boujard and Leatherland, 1992; Reddy and Leatherland, 1994; Polakof et al., 2007c; Polakof et al., 2007d), allowing us to postulate the existence of daily rhythms. Treatment with glucose during the day induced hyperglycemia, which is in agreement with similar studies carried out previously in the same fish species (Polakof et al., 2007a; Conde-Sieira et al., 2010a). Moreover, the increase in circulating glucose levels during the day elicited in all tissues changes in parameters related to glucosensing capacity, indicative of an activation of glucosensor systems, such as increased glucose and glycogen levels, increased GK, GSase and PK activities, and increased mRNA abundance of GK, GLUT2, Kir6.x-like and SUR-like, in agreement with previous studies in the same species (for a review, see Polakof et al., 2011). All these results validate the experimental design.

The capacity of glucosensing systems in central and peripheral locations of rainbow trout to inform about changes in glucose levels had previously been assessed only under daytime conditions

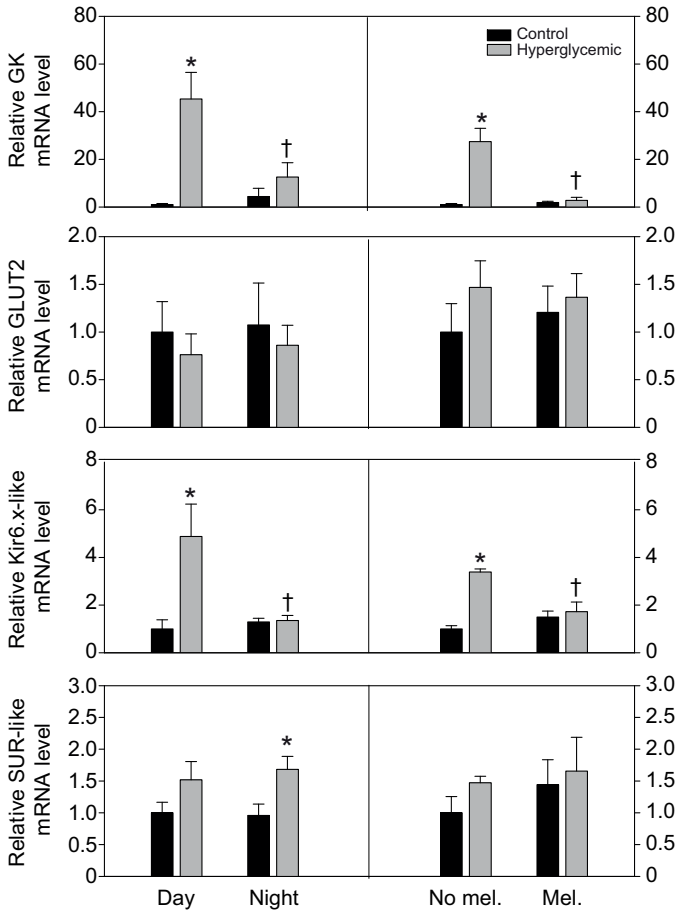

Fig. 4. mRNA levels of GK, glucose transporter 2 (GLUT2), and K_{ATP} channel Kir6.x-like and sulfonylurea receptor (SUR)-like in liver of rainbow trout under different glycemic conditions elicited by intraperitoneal administration of saline alone (control, normoglycemic) or containing 500 mg D-glucose kg^{-1} (hyperglycemic). Left panels (experiment 1): fish injected during the day (09:00 h) or night (20:00 h) and sampled 6 h later. Right panels (experiment 2): fish injected at 09:00 h with Cortland saline alone (control, no melatonin) or containing 0.5 mg kg^{-1} melatonin and sampled 6 h later. Each value is the mean + s.e.m. of 4 measurements. Differences in mRNA levels are presented as an *x*-fold induction with respect to control. Expression results were normalized to β-actin mRNA levels (no changes in β-actin mRNA levels were observed when comparing all groups used in all experiments). For further details, see legend to Fig. 1.

(Polakof et al., 2011) and no studies had been carried out to assess whether changes in glucosensing capacity could be dependent upon day–night variations. In this respect, several studies in diurnal fish species such as goldfish (López-Olmeda et al., 2009) have revealed that glucose tolerance is higher in the morning than in the afternoon or evening, whereas in other diurnal species like rainbow trout, brain glucose levels are known to be increased at night when fish are fed in the morning (Polakof et al., 2007c). If there are daily changes in the response of fish to glucose, these changes could be related to the activity of the glucosensor systems characterized in rainbow trout. Therefore, in our first experiment, we aimed to assess whether the glucosensing response to a similar increase in glucose was different during the day and night. The results obtained displayed two different patterns. In the first pattern, illustrated by the results obtained in the hypothalamus, hindbrain and Brockmann bodies, the glucosensing response was similar when comparing day and night conditions for most of the assessed parameters involved in glucosensing. The second pattern was basically observed in the liver, where a similar increase in plasma glucose under day and night conditions did not result in similar changes in several parameters related to glucosensing. Thus, glucose levels, GK activity (and mRNA levels) and GSase activity displayed increased values during the day in hyperglycemic fish compared with controls but not during the night (lower for GK mRNA levels; no change for glucose, GK activity, GSase activity and Kir6.x-like mRNA levels). Therefore, it seems that under night conditions the glucosensing response is reduced in liver compared with day conditions whereas the response is unaltered in other glucosensor systems (either central or peripheral). The differential response between day and night conditions could be associated with any factor(s) displaying day–night variations in rainbow trout, such as levels of cortisol (Boujard and Leatherland, 1992) or melatonin (Ceinos et al., 2008). In a recent study (Conde-Sieira et al., 2012) we suggested that the modulatory role of melatonin in central and peripheral glucosensors in rainbow trout could be related to daily readjustment of hypothalamic glucosensing.

To assess whether day–night changes in glucosensing capacity could be related to the modulatory action of melatonin, we carried out a second experiment in which we treated fish with melatonin during the day to assess whether under conditions of melatonin levels comparable to those observed during the night the response of glucosensing systems would be similar. In preliminary experiments, we evaluated the increase in circulating melatonin levels after treatment with different doses of melatonin and chose the dose that induced a 10-fold increase, thus simulating nocturnal melatonin peak levels. However, the actual increase was higher than that observed when comparing day and night melatonin levels in the first experiment. The experimental design was further validated by the changes displayed by hyperglycemic treatment in fish not treated with melatonin, which were in general (with the exception of PK activity, and Kir6.x-like and GLUT2 mRNA levels in hypothalamus and glucose levels in hindbrain) similar to those of hyperglycemic fish treated during the day in the first experiment. Circulating melatonin in rainbow trout is mainly produced in the pineal organ, where melatonin synthesis is basically regulated by direct light action on pineal photoreceptive cells, as this species lacks a circadian system like that demonstrated in other teleosts (Bolliet et al., 1996). It has also been shown that melatonin production is related to changes in circulating metabolite levels, as melatonin levels change in food-deprived and refed rainbow trout (Ceinos et al., 2008). Therefore, it is likely that any of the endocrine systems related to changes in circulating glucose levels, like insulin (Peschke et al., 2007), could be related to the regulation of melatonin production in rainbow trout.

In plasma, glucose levels were not affected by the sampling time, in the same way that the increase in glucose levels elicited by glucose treatment was similar when comparing melatonin-treated with non-melatonin-treated fish. In contrast, plasma lactate levels, which only increased at night in hyperglycemic fish (first experiment), clearly increased in hyperglycemic fish treated with melatonin (second experiment), suggesting that melatonin could be involved in the differential day–night response.

In the glucosensor systems again two different types of response were noticed when evaluating the effects of melatonin. The first type of response was characterized by the results obtained in the hypothalamus, hindbrain and Brockmann bodies, where the presence of melatonin did not modify in general the response of glucosensing parameters to hyperglycemic treatment, with a few exceptions

Table 1. Parameters assessed in the hypothalamus, hindbrain and Brockmann bodies

	Experiment 1				Experiment 2			
	Day		Night		No melatonin		Melatonin	
Parameters	Control	Hyperglycemic	Control	Hyperglycemic	Control	Hyperglycemic	Control	Hyperglycemic
Hypothalamus								
Glucose levels	2.14±0.06	6.32±0.35*	1.58±0.08	5.92±0.43*	2.16±0.24	5.52±0.78*	1.40±0.30	4.98±0.78*
Gycogen levels	0.32±0.05	0.51±0.05*	0.34±0.04	0.54±0.06*	0.30±0.04	0.44±0.04*	0.37±0.06	0.48±0.05
GK activity	0.15±0.03	0.28±0.01*	0.12±0.03	0.23±0.01*	0.20±0.02	0.22±0.01	0.25±0.02	0.23±0.02
GSase activity	6.77±5.00	41.96±3.52*	5.16±5.25	60.15±6.98*	6.44±1.19	45.70±3.16*	3.83±0.90	8.65±1.52†
PK activity	0.93±0.43	6.94±0.76*	5.15±0.84†	8.44±1.05*	1.31±0.16	1.13±0.13	1.29±0.15	0.93±0.18*
GK mRNA levels	1.00±0.15	1.87±0.15*	0.91±0.15	1.84±0.13*	1.00±0.25	1.51±0.12*	0.96±0.22	1.91±0.21*
Kir6.x-like mRNA levels	1.00±0.13	0.81±0.25	0.80±0.43	0.73±0.18	1.00±0.21	2.05±0.23*	1.11±0.32	0.99±0.08†
SUR-like mRNA levels	1.00±0.09	1.30±0.41	1.29±0.35	0.99±0.34	1.00±0.40	1.51±0.20	0.80±0.21	1.11±0.10
GLUT2 mRNA levels	1.00±0.24	0.60±0.19	1.31±0.41	1.55±0.47	1.00±0.28	2.45±0.48*	0.82±0.27	3.27±1.29*
Hindbrain								
Glucose levels	1.40±0.07	5.49±0.27*	1.31±0.15	4.62±0.33*	1.32±0.14	3.06±0.49*	1.20±0.19	2.66±0.23*
Gycogen levels	0.27±0.03	0.55±0.05*	0.36±0.03	0.65±0.03*	0.24±0.05	0.59±0.02*	0.31±0.02	0.72±0.03*
GK activity	1.63±0.22	1.73±0.08	2.06±0.09	2.30±0.15	1.29±0.11	1.68±0.18	1.49±0.21	1.78±0.27
GSase activity	0.96±0.03	67.64±4.65*	1.16±0.25	84.06±5.54*	0.56±2.62	61.63±3.78*	8.93±4.93	68.09±1.82*
PK activity	8.67±0.43	10.21±0.26	9.75±0.41	10.04±0.60	8.42±0.70	12.30±0.42*	7.53±0.68	11.56±0.96*
GK mRNA levels	1.00±0.13	1.60±0.12*	1.13±0.08	1.80±0.11*	1.00±0.38	1.58±0.05*	0.87±0.07	1.52±0.07*
Kir6.x-like mRNA levels	1.00±0.17	0.86±0.26	1.16±0.27	1.12±0.31	1.00±0.35	1.07±0.42	0.89±0.19	0.95±0.28
SUR-like mRNA levels	1.00±0.34	0.86±0.24	1.02±0.25	1.07±0.38	1.00±0.28	0.82±0.14	0.81±0.31	0.80±0.17
GLUT2 mRNA levels	1.00±0.39	0.35±0.09	1.21±0.34	0.75±0.30	1.00±0.43	0.89±0.27	0.69±0.28	1.01±0.29
Brockmann bodies								
Glucose levels	5.75±0.48	8.64±0.41*	4.71±0.33	7.65±0.75*	5.89±0.45	7.52±0.15*	4.57±0.37	7.21±1.38*
Gycogen levels	1.02±0.03	1.57±0.08*	1.06±0.08	1.78±0.05*	1.11±0.43	1.83±0.07*	1.15±0.61	1.58±0.09*
GK activity	0.20±0.06	0.36±0.04*	0.21±0.03	0.34±0.03*	0.19±0.06	0.33±0.03*	0.15±0.06	0.40±0.03*
PK activity	2.69±0.82	1.85±0.28	1.25±0.25†	1.90±0.43	2.31±0.94	1.93±0.75	1.31±0.38	2.38±0.69
GK mRNA levels	1.00±0.44	5.25±0.94*	1.05±0.56	6.13±0.62*	1.00±0.46	4.22±0.13*	0.78±0.26	4.73±0.33*
Kir6.x-like mRNA levels	1.00±0.31	0.75±0.25	0.67±0.16	0.97±0.27	1.00±0.14	1.22±0.29	1.21±0.30	2.05±0.47
SUR-like mRNA levels	1.00±0.33	0.62±0.30	0.76±0.21	1.06±0.29	1.00±0.29	1.01±0.24	1.14±0.35	2.71±0.96†
GLUT2 mRNA levels	1.00±0.19	3.44±1.40	1.80±0.14	8.26±5.93	1.00±0.47	0.42±0.33	0.69±0.23	3.59±0.96*,†

Glucose and glycogen levels, activities of glucokinase (GK), glycogen synthetase (GSase) and pyruvate kinase (PK), and mRNA levels of GK, glucose transporter 2 (GLUT2), and K_{ATP} channel subunits Kir6.x-like and SUR-like in the hypothalamus, hindbrain and Brockmann bodies of rainbow trout under different glycemic conditions elicited by intraperitoneal administration of saline alone (control, normoglycemic) or containing 500 mg D-glucose kg^{-1} (hyperglycemic).

Experiment 1: fish injected during the day (09:00 h) or night (20:00 h) and sampled 6 h later. Experiment 2: fish injected at 09:00 h with saline alone (control, no melatonin) or containing 0.5 mg kg^{-1} melatonin and sampled 6 h later.

Data represent means ± s.e.m. of 8 measurements for glucose (mmol g^{-1} wet mass) and glycogen (mmol glycosyl units g^{-1} wet mass) levels and enzyme activities (mU mg^{-1} protein) and 4 measurements for mRNA levels (*x*-fold induction with respect to control; expression results were normalized by β-actin mRNA levels; no changes in β-actin mRNA levels were observed when comparing all groups used in all experiments).

*Significantly different (P<0.05) from control (normoglycemic) fish under the same treatment.

†Significantly different (P<0.05) from day (Experiment 1) or no melatonin (Experiment 2) treatments under the same glycemic conditions.

(GSase activity and Kir6.x-like mRNA abundance in hypothalamus and GLUT2 mRNA abundance in Brockmann bodies). In a recent study (Conde-Sieira et al., 2012), we observed that melatonin treatment in the hypothalamus and hindbrain during the day under *in vitro* conditions induced a higher response in glucosensing parameters, which was not observed in the present *in vivo* studies, suggesting that the effect of melatonin on central glucosensing *in vivo* is masked by other regulatory factors or that melatonin is acting at different targets preventing us from seeing any specific changes. In contrast, in a second type of response, characterized by the results obtained in the liver, melatonin treatment induced a response to hyperglycemic treatment in several parameters, similar to that observed under night conditions, e.g. glucose levels, GK activity and mRNA abundance, and Kir6.x-like mRNA abundance. However, treatment with melatonin did not induce similar responses to hyperglycemia to those observed at night in parameters like glycogen levels, PK and GSase activities, and SUR-like mRNA levels. Therefore, the glucosensing capacity of the liver is apparently diminished during the night and this decrease could be related to the modulatory action of melatonin as treatment with this hormone induced similar changes to those observed at night in several (not all) parameters assessed in liver. The fact that several parameters were not affected by melatonin treatment suggests that the effect of melatonin is mediated by specific metabolic targets that do not affect all parameters, or that melatonin is interacting with other factors regulating hepatic glucosensing function.

It is interesting to note that the effect of melatonin in liver was in general a down-regulation of the glucosensing response, which is just the converse of that previously observed in the hypothalamus and hindbrain *in vitro*, where melatonin treatment induced an up-regulation of the glucosensing response (Conde-Sieira et al., 2012). The differences in the performance of both types of tissue could be related to the different functions in which glucosensing systems might be involved in these tissues, such as the control of food intake and counter-regulation of hypoglycemia in central areas, and homeostatic control of glucose in liver (Polakof et al., 2011). There are almost no studies regarding the circadian regulation of liver metabolism in fish, but in mammals the metabolism of the liver is in part under the control of circadian regulation, and several metabolic genes follow a circadian expression in this tissue, such

as GK, phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase) or GSase (Desvergne et al., 2006; Cailotto et al., 2008). The daily variations in liver glucosensing capacity, possibly modulated by melatonin, could be associated with the circadian regulation of metabolism within this tissue. Correspondingly, it is known in mammals that several clock genes regulate liver energy metabolism through the expression of different genes including sirtuins (Grimaldi et al., 2009) for which there are currently no references available in fish.

The different actions of melatonin inducing converse changes in the same parameter in different tissues may be related to the presence of different receptor types in those tissues and/or to the interaction with other endocrine system(s) involved in metabolic regulation in the liver. It is important to emphasize that in mammals melatonin is known to interact with insulin such that melatonin inhibits insulin release (Peschke and Mühlbauer, 2010). In humans (diurnal species), the circadian variation in glucose tolerance (better in the morning) could be due to a reduction in insulin secretion and/or a decrease in insulin sensitivity in the morning whereas in rat (nocturnal species) the situation is reversed: greater insulin efficiency and an increase in insulin secretion in response to a glucose load have been repeatedly observed at night (Bizot-Espiard et al., 1998). If a similar situation is present in rainbow trout (diurnal species), this would concur with at least some of the results obtained here, as insulin treatment in fish is known to stimulate glycogen accumulation in liver as well as inhibiting glycolysis (for a review, see Caruso and Sheridan, 2011). However, as no measurements of plasma insulin are available, this suggestion has to be taken with caution.

As liver and pancreas have to function in concert to ensure maintenance of the correct blood glucose levels, one would expect rhythm synchronization between these organs, and therefore similar day–night changes in metabolic parameters in the two tissues (Mühlbauer et al., 2009). In mammals, melatonin has been hypothesized to be the synchronizing factor linking the two tissues, though glucose can also act as a synchronizing agent itself (Hirota et al., 2002). It is interesting to note that changes caused by hyperglycemic treatment in Brockmann bodies did not show any differences between day and night, suggesting that the difference in day–night responses induced by melatonin in the liver is linked to particular mechanisms present in the liver and possibly not to other tissues including endocrine pancreas. In mammals, a circadian rhythm was retained in mice pancreas devoid of melatonin receptor signaling, and therefore other factors may be able to complement melatonin in a zeitgeber function in the pancreas (Mühlbauer et al., 2009). A similar situation could be present in rainbow trout in this study, helping us to explain the different metabolic behavior displayed by the liver and Brockmann bodies.

CONCLUSIONS

In summary, we have demonstrated in rainbow trout the existence of day–night variations in the glucosensing response of liver to increased circulating glucose levels, whereas the other glucosensing regions assessed such as the hypothalamus, hindbrain and Brockmann bodies displayed basically the same response under day and night conditions. The response to changes in circulating glucose levels in the liver was lower under night than under day conditions. A comparable decrease in several parameters related to glucosensing in the liver was observed when fish were treated with melatonin, suggesting a modulatory role of melatonin in day–night changes of parameters related to glucose metabolism in the same tissue. The physiological meaning of these findings could be related to the mechanisms involved in daily rhythmic control of food intake and utilization in this species (Sánchez-Vázquez and Tabata, 1998). Further studies are also necessary in liver to characterize the presence of circadian system-related changes in parameters of energy metabolism in general, and to glucosensing in particular, and on the potential role of melatonin in these functions.

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