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Melatonin treatment alters glucosensing capacity and mRNA expression levels of peptides related to food intake control in rainbow trout hypothalamus

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ABSTRACT

As demonstrated in previous studies, the functioning of brain glucosensing systems in rainbow trout is altered under stress conditions in a way that they are unable to respond properly to changes in glucose levels. Melatonin has been postulated as necessary for homeostatic control of energy metabolism in several vertebrate groups, and in fish it has been suggested as an anti-stress molecule. To evaluate the possible effects of melatonin on glucosensing, we have incubated hypothalamus and hindbrains of rainbow trout at different glucose concentrations in the presence of increased doses (0.01, 1, and 100 nM) of melatonin assessing whether or not the responses to changes in glucose levels of parameters related to glucosensing (glucose, glycogen and glucose 6-phosphate levels, activities of GK, GSase and PK, and mRNA content of GK, GLUT2, Kir6.x-like, and SUR-like) are modified in the presence of melatonin. While no effects of melatonin were observed in hindbrain, in hypothalamus melatonin treatment up-regulated glucosensing parameters, especially under hypo- and normo-glycaemic conditions. The effects of melatonin in hypothalamus occurred apparently through MT_1 receptors since most effects were counteracted by the presence of luzindole but not by the presence of 4-P-PDOT. Moreover, melatonin treatment induced in hypothalamus increased mRNA expression levels of NPY and decreased mRNA levels of POMC, CART, and CRF. A role of the hormone in daily re-adjustment of hypothalamic glucosensor machinery is discussed.

1. Introduction

Melatonin is involved in the timing of rhythmic events in fish; in addition to a role in the circadian organization, melatonin and the pineal organ can influence a wide range of physiological and behavioral processes such as endocrine function, reproduction, development, growth, sexual maturation, thermoregulation, osmoregulation and smoltification [19]. At present, it is not known how melatonin may exert many of these physiological effects in fish, though at least part of them may be attributed to melatonin actions in the brain where receptors for melatonin have been demonstrated [29,34,54].

In mammals, melatonin is known to be involved in the regulation of glucose metabolism stimulating glucose uptake and utilization in liver and muscle [8,21,35]. Moreover, melatonin is necessary for homeostatic control of energy metabolism since pinealectomy induces insulin resistance [3,26], and melatonin treatment modulates insulin secretion in pancreas [51] through a rapid MT_1/MT_2 membrane receptor-dependent stimulation of the IRS-PI(3)K pathway [4]. Melatonin is therefore involved in the modulation of pancreatic glucosensing systems in a way that an antagonistic relationship exists between the synthesis of insulin and that of melatonin [36,37]. In contrast, in fish, despite melatonin is involved in energy homeostasis [16] and macronutrient selection [52], the action of melatonin in glucose metabolism is still unclear with few evidences obtained to date. These include the contradictory effects of melatonin on glycemia or liver glycogenolysis [15,16,56], and the increased glucose uptake in rainbow trout brain [2].

In previous studies in rainbow trout we demonstrated [41,42,44–46] the presence in central (hypothalamus and hindbrain) and peripheral (Brockmann bodies) locations of a glucosensing system similar to that found in mammalian pancreatic β-cells and glucose-excited (GE) neurons [33,55] based on glucokinase (GK), GLUT2, and inward rectifier potassium channel (K_{ATP}) activity (see review [50]). The glucosensing systems in rainbow trout are disarranged under stress conditions, like those associated with high stocking density, resulting in an inability of the systems to respond to changes in circulating glucose levels [11]. Furthermore, the same stress conditions elicit changes in the mRNA expression levels of several peptides related to the control of food intake including CRF [13] that could be related to the well known anorectic effect of stress in fish [7,57]. Accordingly, we have recently

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demonstrated that the responses to changes in glucose levels of parameters related to glucosensing in hypothalamus and hindbrain of rainbow trout are modified in the presence of CRF [12], in a way comparable to that observed under stress conditions. Melatonin is considered as an anti-stress molecule in humans [31] though such a role has not been clearly established in fish for which a reduction in cortisol levels has been noticed in stressed fish treated with melatonin [6,23]. Moreover, plasma melatonin levels increased in fed gilthead sea bream under stress conditions induced by high stocking density [32], whereas no differences were found in fasted fish. Similar increases in plasma melatonin levels have been described in fish exposed to other stressful conditions [25,30]. However, melatonin is also considered an anorectic factor in diurnal and nocturnal fish species [16,52] though several authors suggested that the effects could be attributed to a sedative action of the molecule [58]. Considering the disruptory effects of stress on glucosensing, an anti-stress molecule like melatonin could also regulate those areas involved in the regulation of food intake in which glucosensing mechanisms are present in rainbow trout like hypothalamus and hindbrain.

To assess such hypothesis, we have incubated hypothalamus and hindbrains of rainbow trout at different glucose concentrations (indicative of hypo-, normo-, and hyper-glycaemic conditions) in the presence of increased doses of melatonin (0.01, 1, and 100 nM) to assess whether or not the presence of melatonin modifies the response to changes in glucose levels of several parameters involved in the glucosensing response, such as levels of metabolites (glycogen, glucose, and glucose 6-phosphate), glucose transport capacity (GLUT2 mRNA content), glucose phosphorylation capacity (GK activity and mRNA content), glycolytic potential (pyruvate kinase, PK, activity), glycogen metabolism (glycogen synthetase, GSase, activity), and potential of K^+_{ATP} (inward rectifier K^+ channel pore type 6.-like, Kir6.x-like; sulfonylurea receptor-like, SUR-like). Moreover, we have also assessed the response of mRNA expression levels of different peptides related to the control of food intake in fish (NPY, POMC, CRF, and CART). Since melatonin effects were reported in a first experiment, we carried out a second experiment to assess the specificity of the response to melatonin by using antagonists of MT_1 and MT_2 melatonin receptors.

2. Materials and methods

2.1. 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 12:12 L:D photoperiod in dechlorinated tap water at 15 °C. Fish mass was 65 ± 4 g. Fish were fed once daily (10:00 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 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.

2.2. Experimental protocol

2.2.1. Dose–response effects of melatonin treatment

Every morning of an experiment, fish that were fasted for 24 h were dipnetted, anaesthesized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed and sacrificed by decapitation. Hypothalamus and hindbrain were dissected as described previously [42]. Tissues were rinsed by immersion in modified Hanks' medium (92.56 mM NaCl; 3.63 mM KCl, 2.81 mM $NaHCO_3$, 0.85 mM $CaCl_2$, 0.55 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.23 mM Na_2HPO_4, 7.5 mM HEPES, 50 U ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin sulphate, pH 7.0; referred to a basal medium), sliced in chilled Petri dishes, placed in a chilled Petri dish containing 100 ml of modified Hanks' medium g^{-1} tissue, and gassed with a 0.5% CO_2/99.5% O_2 mixture. In order to have enough mass, tissues were pooled from different fish resulting in pools of 3–4 tissue samples (hypothalamus or hindbrain). On each pool, tissue was finely sliced and mixed and then placed in 48-well culture plates (25 mg of tissue in 250 µl of modified Hanks' medium per well).

In each experiment, freshly obtained tissues were incubated as previously described [42] in 48-well culture plates at 15 °C for 1 h with 250 µl of modified Hanks' medium per well containing 25 mg of tissue that was gassed with a 0.5% CO_2/99.5% O_2 mixture. In control wells, medium contained three different concentrations of D-glucose: 2, 4 and 8 mM (indicative of hypo-, normo- and hyperglycaemic conditions in rainbow trout). In treated wells medium contained the same glucose concentration and different concentrations (0.01, 1, and 100 nM) of melatonin. Melatonin was previously dissolved in ethanol (5% final concentration, same amount used in control wells, no vehicle effects were seen). The concentrations of melatonin were selected according to previous *in vitro* studies carried out in rainbow trout [18]. After 1 h incubation, tissues were quickly removed, freeze-clamped in liquid nitrogen, and stored at −80 °C until assayed.

In each experiment, three different sets of tissue pools ($n = 12$; 4 treatments × 3 glucose concentrations) were used. The first set was used to assess enzyme activities (GK, GSase, and PK). The second set was assayed for tissue metabolites measurement (glucose, glycogen and glucose 6-phoshate levels). Finally, the third set was used for quantification of gene expression of parameters related to glucosensing capacity (GK, GLUT2, Kir6.x-like, and SUR-like), and peptides related to the control of food intake (NPY, POMC, CRF, and CART). The same procedure was performed in four independent replicates per set ($N = 4$ experiments).

2.2.2. Effects of melatonin receptor antagonists on melatonin action

In each experiment, freshly obtained tissues were incubated as described above in 48-well culture plates at 15 °C for 1 h with 250 µl of modified Hanks' medium per well containing 25 mg of tissue that was gassed with a 0.5% CO_2/99.5% O_2 mixture. In control wells, medium contained 4 mM D-glucose whereas in treated wells medium contained the same glucose concentration and 1 nM melatonin alone, or 1 nM melatonin plus a melatonin MT_1/MT_2 receptor antagonist (100 nM luzindole), or 1 nM melatonin plus a melatonin MT_2 receptor antagonist (100 nM 4-P-PDOT). The inhibitors were added 15 min prior to the addition of melatonin. The concentration of melatonin was selected according to results obtained in the experiments described above whereas those of the different receptor antagonists used were selected on the basis of previous studies *in vitro* carried out in fish [18,29]. After 1 h incubation, tissues were quickly removed, freeze-clamped in liquid nitrogen, and stored at −80 °C until assayed.

Three different sets of tissue pools were used in each experiment. The first set was used to assess enzyme activities (GK, GSase, and PK). The second set was assayed for tissue metabolites measurement (glucose, glycogen and glucose 6-phoshate levels). Finally, the third set was used for quantification of mRNA abundance of parameters related to glucosensing capacity (GK, GLUT2, Kir6.x-like, and SUR-like), and peptides related to the control of food intake (NPY, POMC, and CRF). The same procedure was performed in four independent replicates per set ($N = 4$ experiments).