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Metabolic effect of fluvoxamine in mouse peripheral tissues

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ABSTRACT

Serotonin leads to reduced food intake and satiety. Disrupted circadian rhythms lead to hyperphagia and obesity. The serotonergic and circadian systems are intertwined, as the central brain clock receives direct serotonergic innervation and, in turn, makes polysynaptic output back to serotonergic nuclei. Our objective was to test the hypothesis that peripherally serotonin alters circadian rhythms leading to a shift towards fat synthesis and weight gain. We studied the effect of serotonin and fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), on the circadian clock and metabolic gene and protein expression in mouse liver, muscle and white adipose tissue (WAT) and cell culture. We found that serotonin and/or the SSRI fluvoxamine led to fat accumulation in mouse liver and hepatocytes by shifting metabolism towards fatty acid synthesis mainly through low average levels of phosphorylated acetyl CoA carboxylase (pACC) and phosphorylated protein phosphatase 2A (pPP2A). This shift towards fat synthesis was also observed in adipose tissue. Muscle cells were only slightly affected metabolically by serotonin or fluvoxamine. In conclusion, although centrally it leads to increased satiety, in peripheral tissues, such as the liver and WAT, serotonin induces fat accumulation.

1. Introduction

Mammals have developed an endogenous circadian clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus that responds to the environmental light–dark cycle. The SCN receives light information from the retina and transmits synchronization cues via neuronal connections or circulating humoral factors to peripheral clocks, such as the liver, heart and lungs, regulating cellular and physiological functions (Panda et al., 2002; Reppert and Weaver, 2002). The clock mechanism in both SCN neurons and peripheral tissues includes CLOCK and BMAL1 (brain-muscle-Arnt-like 1) that heterodimerize and bind to E-box sequences to mediate transcription of a large number of genes, including *Periods* (*Per1*, *Per2*, *Per3*) and *Cryptochromes* (*Cry1*, *Cry2*) (Schibler et al., 2003).

The circadian clock regulates metabolism by mediating the expression and/or activity of certain metabolic enzymes, hormones, and transport systems (Froy, 2010). The rhythmic expression and activity of the metabolic pathways is mainly attributed to the robust and coordinated expression of clock genes in metabolic tissues, such as the liver, adipose tissue and muscle (Lee et al., 2001; Zvonic et al., 2006). Disruption of the coordination between the endogenous clock and the environment leads to a greatly attenuated diurnal feeding rhythm, hyperphagia and obesity, and the metabolic syndrome (Rudic et al., 2004; Sancar and Brunner, 2014; Tsai et al., 2005; Turek et al., 2005).

Fatty acid synthesis starts with the rate-limiting enzyme acetylCoA carboxylase (ACC), whose product malonylCoA is converted to fatty acids through the fatty acid synthase (FAS) enzyme complex. MalonylCoA also inhibits the import of fatty acids into the mitochondria, preventing their oxidation. (Canto et al., 2009). ACC is phosphorylated (pACC), and thus inhibited, by AMP-activated protein kinase (AMPK), the cellular energy gauge, when the AMP/ATP ratio is high. AMPK itself is phosphorylated, and thus activated, by liver kinase 1 (LKB1) and by muscle calcium-calmodulin kinase kinase II (CamKKII) (Witczak et al., 2008). Thus, increased pAMPK levels lead to increased pACC levels. The inhibited phosphorylated pACC is reactivated into ACC by the protein phosphatase 2A (PP2A) (Kowluru et al., 2001).

Serotonin is a monoaminergic neurotransmitter synthesized in the brain raphe nuclei with activities that modulate central functions, such as food intake, sleep, anxiety, sexual behavior and mood (Watanabe et al., 2011). However, serotonin is synthesized by many cells in the periphery to affect vasoconstriction, intestinal motility,

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glucose and lipid metabolism (Hajduch et al., 1999; Watanabe et al., 2010) and adipocyte differentiation *in vitro* (Kinoshita et al., 2010). Serotonin is reuptaken by all secreting cells via the serotonin transporter (SERT), the target of selective serotonin reuptake inhibitors (SSRIs) (Murphy et al., 2004). The serotonergic and circadian systems are intertwined, as the SCN receives direct serotonergic innervation from the median raphe nuclei, and, in turn, makes polysynaptic output back to these mid-brain serotonergic nuclei (Deurveilher and Semba, 2005). SCN neurons express several serotonin receptors. Upon binding, serotonin modulates the response of the circadian system to light as well as the response to non-photic stimuli (Edgar et al., 1993; Glass et al., 2003; Mistlberger et al., 1998). In turn, the biological clock gene network is expressed in serotonergic raphe neurons and the activity of key serotonergic system components varies in a circadian manner (Malek et al., 2007).

Although centrally serotonin leads to reduced food intake and satiety (Halford et al., 2011), one of the side effects of long-term use of SSRIs is weight gain (Masand and Gupta, 2002). Therefore, we hypothesized that peripherally serotonin alters circadian rhythms leading to a shift toward fat synthesis and weight gain. To test this hypothesis, we studied the effect of serotonin and fluvoxamine, an SSRI, on circadian clock and metabolic gene and protein expression in myotube and hepatocyte cell lines and mouse liver, muscle and adipose tissue.

2. Methods and materials

2.1. Animal and experimental design

Eight-week-old C57BL/6 male mice (Harlan Laboratories, Jerusalem, Israel) were housed in a temperature- and humidity-controlled facility (23–24 °C, 60% humidity). Mice were entrained to 12 h light and 12 h darkness (LD) for one week with food available *ad libitum* and then were randomly assigned to either the control (n = 24) or fluvoxamine treatment (9 mg/kg/day; n = 24) groups for 3 weeks. Fluvoxamine (Cayman chemical company, Ann Arbor, MI, USA) was provided in the drinking water designed to achieve the maximal recommended dose of fluvoxamine for humans per basal water intake. Body weight was recorded weekly and at the end of the experiment. Food intake was monitored every day on the last week of the experiment. Liquid consumption was monitored every day throughout the experiment. Mice were placed on a 12 h fast before they were anesthetized with isoflurane and blood, liver, muscle and epididymal fat samples were removed every 4 h around the circadian cycle in total darkness (DD) under a dim red light to avoid the masking effects of light after which mice were humanely killed. Fasting blood glucose levels were determined using a glucometer (Optium Xceed, Abbott Laboratories, Maidenhead, UK). Tissues were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. The joint ethics committee of the Hebrew University and Hadassah Medical Center approved this study.

2.2. Cell culture, differentiation and treatment

C2C12 myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Biological Industries, Beit HaEmek, Israel) supplemented with 10% bovine calf serum and 5% CO_2 at 37 °C. Differentiation of cells to myotubes was achieved by allowing the cells to reach confluence in 0.1% gelatin-coated flasks. When cells were confluent, the medium was replaced with DMEM supplemented with 2% horse serum. Every day thereafter, fresh medium (DMEM plus 2% horse serum) was added to the cells and differentiation was achieved after 72–96 h. Mouse AML-12 hepatocytes were maintained in DMEM supplemented with 10% bovine calf serum, 100 mg/l L-glutamine and 100 mg/l penicillin at 37 °C in 5% CO_2. For circadian gene and protein expression, differentiated C2C12 myotubes and AML-12 hepatocytes were synchronized with a 2-h pulse of 1 μM dexamethasone (Sigma, Rehovot, Israel). After 2 h (t = 0), the medium was replaced with fresh medium (control) or medium-supplemented with 10 μM serotonin (MP Biomedicals, Strasbourg, France), 0.001 μM ketanserin (Sigma, Rehovot, Israel) or their combination. Following 3 h of incubation (t = 3), cells were harvested in triplicates per treatment per time-point every 6 h for 48 h. Three independent experiments were performed.

2.3. Serum separation and ELISA

Blood was kept at room temperature for 30 min for clotting and subsequently centrifuged at 2000 *g* for 15 min. Serum was collected and stored at −80 °C for further analysis. Corticosterone levels were determined using ELISA (Assaypro, St. Charles, MO, USA) according to the manufacturer's instructions.

2.4. Enzymatic colorimetric tests

Serum triglycerides, total cholesterol and HDL cholesterol levels were determined by Cobas kits (Roche Diagnostics, Burgess Hill, UK) and analyzed in a Roche/Hitachi analyzer (Roche Diagnostics, Indianapolis, IN, USA). Assays were performed according to the manufacturers' instructions.

2.5. Lipid content quantification

Liver tissue samples weighing 100 mg were homogenized in a mixture of chloroform:methanol (2:1) and left shaking for 30 min. The samples were then centrifuged at 2500 g for 30 min, and water was added. The samples were left open for evaporation and the test tubes were then weighed. Lipid quantification in C2C12 and AML-12 cells was performed using Oil Red O staining. Cells were fixed in 10% formaldehyde in aqueous phosphate buffer overnight, washed with 60% isopropanol, and stained with Oil Red O solution (in 60% isopropanol) for 10 min. Cells were then repeatedly washed with water and destained in 100% isopropanol for 15 min. The optical density of the isopropanol solution was measured at 500 nm.

2.6. RNA extraction and quantitative real-time PCR

RNA was extracted from liver, muscle, epididymal fat and cell lines using TRI Reagent (Sigma, Rehovot, Israel). Total RNA was DNase I-treated using RQ1 DNase (Promega, Madison, WI, USA) and reverse-transcribed using qScript cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA) and random hexamers (Promega). The reaction was subjected to quantitative real-time PCR using primers spanning exon–exon boundaries and the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers for all genes (Barnea et al., 2012) were tested alongside the normalizing gene *Actin*. The fold change in target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ relative quantification method (Applied Biosystems).

2.7. Western blot analysis

Liver, muscle and epididymal fat tissue samples were homogenized in lysis buffer, as was described (Sherman et al., 2011). Samples were run on a 10% SDS polyacrylamide gel and transferred onto nitrocellulose membranes as was described (Sherman et al., 2011). Blots were incubated with AMPK/pAMPK, ACC/pACC, FAS (Cell Signaling Technology, Beverly, MA, USA), PP2A/pPP2A (Santa

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