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Constant light disrupts the circadian rhythm of steroidogenic proteins in the rat adrenal gland

Shin Y. Park [1], Jamie J. Walker [1], Nicholas W. Johnson, Zidong Zhao, Stafford L. Lightman, Francesca Spiga *

Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Sciences, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, UK

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ABSTRACT

The circadian rhythm of corticosterone (CORT) secretion from the adrenal cortex is regulated by the suprachiasmatic nucleus (SCN), which is entrained to the light–dark cycle. Since the circadian CORT rhythm is associated with circadian expression of the steroidogenic acute regulatory (StAR) protein, we investigated the 24 h pattern of hormonal secretion (ACTH and CORT), steroidogenic gene expression (StAR, SF-1, DAX1 and Nurr77) and the expression of genes involved in ACTH signalling (MC2R and MRAP) in rats entrained to a normal light–dark cycle. We found that circadian changes in ACTH and CORT were associated with the circadian expression of all gene targets; with SF-1, Nurr77 and MRAP peaking in the evening, and DAX1 and MC2R peaking in the morning. Since disruption of normal SCN activity by exposure to constant light abolishes the circadian rhythm of CORT in the rat, we also investigated whether the AM–PM variation of our target genes was also disrupted in rats exposed to constant light conditions for 5 weeks. We found that the disruption of the AM–PM variation of ACTH and CORT secretion in rats exposed to constant light was accompanied by a loss of AM–PM variation in StAR, SF-1 and DAX1, and a reversed AM–PM variation in Nurr77, MC2R and MRAP. Our data suggest that circadian expression of StAR is regulated by the circadian expression of nuclear receptors and proteins involved in both ACTH signalling and StAR transcription. We propose that ACTH regulates the secretion of CORT via the circadian control of steroidogenic gene pathways that become dysregulated under the influence of constant light.

1. Introduction

Glucocorticoids are vital hormones that regulate many physiological functions, including metabolic and cardiovascular function, the immune system, and the response to stress (Chrousos, 1995; de Kloet et al., 2005; McEwen and Milner, 2007). In the rat, corticosterone (CORT), the main glucocorticoid, is synthesized in the adrenal gland in response to adrenocorticotropin hormone (ACTH) release from the anterior pituitary, which in turn is under control of corticotropin releasing hormone (CRH) secretion from the paraventricular nucleus (PVN) of the hypothalamus.

In unstressed rats, ACTH and CORT are secreted in an ultradian pulsatile pattern that is modulated by a circadian rhythm; with low pulse amplitude in the morning during the inactive period, and higher pulse amplitude in the evening to anticipate wakening and the active phase (Jasper and Engeland, 1991; Windle et al., 1998). The circadian rhythm is coordinated by outputs from the suprachiasmatic nucleus (SCN) of the hypothalamus, which is entrained to the light–dark cycle (Kalsbeek et al., 2012; Morimoto, 1977). Circadian CORT synthesis is partially mediated by a circadian rhythm of ACTH and CRH release (Carnes et al., 1988; Watts et al., 2004), which in turn is regulated by direct SCN innervation of the PVN (Watts and Swanson, 1987; Watts et al., 1987). In addition to this, circadian CORT synthesis is regulated by the SCN via sympathetic innervation of the adrenal gland, by way of modulation of the adrenal responsiveness to ACTH (Buijs et al., 1999; Dallman et al., 1978; Kaneko et al., 1981; Ulrich-Lai et al., 2006).

Within the adrenal cortex, CORT biosynthesis is induced by ACTH activation of its specific membrane receptor MC2R (melanocortin type 2 receptor), resulting in increased cAMP and activation of PKA, which leads to rapid non-genomic activation of a number of steroidogenic proteins. One of the most important of these is the steroidogenic acute regulatory (StAR) protein (Lin et al., 1995; Stocco and Clark, 1996), which mediates intra-mitochondrial transport of cholesterol, the rate-limiting step in CORT biosynthesis.

ACTH also regulates StAR at the level of transcription, via several cAMP/PKA-activated nuclear receptors. StAR transcription is increased by the binding of SF-1 (steroidogenic factor 1) and Nurr77 to the StAR promoter (Caron et al., 1997; Song et al., 2001; Sugawara et al., 1996). In turn, the activity of SF-1 and Nurr77 at the StAR promoter is repressed by DAX1 (the nuclear receptor dose-sensitive sex reversal, adrenal hypoplasia congenita

* Corresponding author. Tel.: +44 (0)117 3313120; fax: +44 (0)117 3313169.
E-mail address: F.Spiga@bristol.ac.uk (F. Spiga).
[1] These authors contributed equally to this work.

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determining region on the X-chromosome-1) (Song et al., 2004; Zazopoulos et al., 1997). While SF-1 and Nurr77 expression is induced by ACTH (Ragazzon et al., 2006; Song et al., 2004; Winnay and Hammer, 2006), DAX1 expression is repressed by ACTH (Gummow et al., 2006).

A further regulatory role of ACTH on CORT biosynthesis and StAR expression is the induction of MC2R (Mountjoy et al., 1994) and the MC2R accessory protein MRAP (Metherell et al., 2005), which controls MC2R transport to the cell membrane as well as receptor functionality (Cooray et al., 2008; Webb et al., 2009).

In rodents, the circadian CORT rhythm is associated with a circadian rhythm in the expression of StAR (Girotti et al., 2009; Son et al., 2008) and other genes involved in the regulation of StAR transcription, including MC2R, SF-1 and Nurr77 (Ishida et al., 2005; Kalsbeek et al., 2012; Oster et al., 2006a). In contrast, DAX1 demonstrates a lack of circadian variation in the mouse (Oster et al., 2006b; Son et al., 2008).

Disruption of SCN signalling to the HPA axis, by either electrolytic lesion or exposure to constant light, results in a loss of circadian CORT rhythm (Abe et al., 1979; Buijs et al., 1993; Claustrat et al., 2008; Sage et al., 2001; Scheving and Pauly, 1966; Szafarczyk et al., 1979). Furthermore, we have recently shown that the loss of circadian CORT is characterized by increased ultradian CORT pulsatility during the nadir phase (Waite et al., 2012). In agreement with this, maternal exposure to constant light during the last period of gestation has been shown to result in lowered foetal corticosterone levels and a suppressed StAR circadian rhythm (Mendez et al., 2012).

Given the pivotal role of StAR in CORT biosynthesis, we investigated whether a loss of the circadian CORT rhythm in adults rats exposed to constant light is also associated with changes in the circadian rhythm of StAR expression and other genes involved in regulating StAR expression in the rat adrenal.

2. Materials and methods

2.1. Animals

All experiments were conducted on adult male Sprague–Dawley rats (Harlan Laboratories, Inc., Blackthorn, UK). Animals were given a 1-week acclimatization period prior to the start of the experiments. During this period, animals were maintained under a 14 h light, 10 h dark schedule (lights on at 0500 h) and they were housed four per cage with ad libitum access to food and water. All animal procedures were conducted in accordance with Home Office guidelines and the UK Animals (Scientific Procedures) Act, 1986.

2.2. Experimental groups and procedures

Experiment 1: Circadian rhythm of hormones, gene expression and protein levels. Rats (250–300 g, 7–9 weeks old) were maintained under a normal 14 h light, 10 h dark schedule and were euthanized every 4 h across the 24 h cycle at the clock times described in the results (n = 5–6/time point).

Experiment 2: Effect of constant light on hormones, gene expression and protein levels. After a 1-week acclimatization period, rats weighing 175–200 g (5–6 weeks old) were group housed in 24 h light conditions (~200 lux, LL) for 5 weeks in an environmentally controlled chamber (Waite et al., 2012). Age-matching control rats weighting 300–350 g at the time of kill (9–11 weeks old) were group housed under a normal 14 h light, 10 h dark schedule (LD). Rats from both LL and LD groups were euthanized either at 0900 h (AM group) or at 1700 h (PM group) (n = 6/group).

2.3. Sample collection

Rats were euthanized with isoflurane and decapitated using a guillotine. Trunk blood was collected on ice into tubes containing 50 μl of EDTA (0.5 m; pH 7.4) and 50 μl of Aprotinin (5000 KIU/ml, Trasylol; Bayer, EDTA, Newbury, UK). Plasma was separated by centrifugation and then stored at −80 °C until processed for CORT and ACTH measurement. Adrenal glands were collected and quickly dissected free of fat and decapsulated to separate the outer capsule containing the zona glomerulosa and the inner zones comprising the zona fasciculata and zona reticularis of the cortex and the medulla. Individual inner zones were immediately frozen in dry ice until processing for isolation of RNA for a reverse transcription quantitative polymerase chain reaction (RT-qPCR) (left inner adrenal) and protein extraction for Western blotting and CORT measurement (right inner adrenal).

2.4. RNA isolation and RT-qPCR

Total RNA was extracted from the inner zone of individual adrenals using TRIzol reagent (Invitrogen, Hopkinton, MA, USA), followed by purification using RNeasy mini kit reagents and column DNase digestion (Qiagen, Valencia, CA, USA) to remove genomic DNA contamination. Complementary DNA was reverse transcribed from 1 μg of total RNA using Cloned AMV First-Strand cDNA synthesis kit (Invitrogen) and mRNA accumulation was evaluated using primer sequences designed to amplify StAR mRNA (Spiga et al., 2011a), SF-1 and DAX1 mRNA (Burger et al., 2011), MC2R and MRAP mRNA (Spiga et al., 2011b). The following primer set was used for Nurr77: forward GCGGAACCGCTGCCAGTTCT; reverse GCATCTGGGGGCTGCTTGGG. Power SYBR green PCR mix (Applied Biosystems, Foster City, CA, USA) was used for the amplification mixture with each primer at a final concentration of 200 nm and 2 μl of cDNA for a total reaction volume of 25 μl. PCR reactions were performed on spectrofluorometric thermal cycler (7500 Real-Time PCR System; Applied Biosystems). Samples were amplified by an initial denaturation at 50 °C for 2 min, 95 °C for 10 min and then cycled (45 times) using 95 °C for 15 s and 60 °C for 1 min. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as determined in a separate real-time PCR reaction. The absence of RNA detection when the reverse transcription step was omitted indicated the lack of genomic DNA contamination in the RNA samples.

2.5. Western blotting

Whole cell lysate from the inner zone of individual adrenals were prepared using RIPA buffer (Sigma) supplemented with 0.2 mM Na orthovanadate, 2 mM NaF, and Complete Protease Inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK). Protein concentration was quantified by spectrophotometry using the Pierce BCA protein assays, (Thermo Fisher Scientific Inc. Rockford, IL, USA). Aliquots of each sample (15–20 μg of protein) were loaded and separated in a 5–15% Tris–glycine gel (BioRad, Hercules, CA, USA), transferred to a PVD membrane (GE Amersham Biosciences, Piscataway, NJ, USA), blocked with 5% non-fat milk in 1 × Tris-buffered saline plus 0.05% Tween 20 (TBST) and incubated with rabbit anti-StAR (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-SF-1 (1:1000; Upstate Biotechnology, EMD Millipore Corporation, Billerica, MA, USA), rabbit anti-Dax1 (1:1000, Santa Cruz) or mouse anti-Nurr77 (1:500; BD Bioscience, Franklin Lakes, NJ, USA). After washing with TBST, the membranes were incubated with a horseradish peroxidase-conjugated donkey anti-rabbit IgG at a dilution of 1:10,000. Immunoreactive bands were visualized using ECL Plus TM reagents (GE Amersham Biosciences)

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