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Regulation of the ovine MT_1 melatonin receptor promoter: Interaction between multiple pituitary transcription factors at different phases of development☆

Jonathan D. Johnston[a,*,1], Carole Schuster[b,1,2], Perry Barrett[b], David G. Hazlerigg[c]

[a] *School of Biomedical and Molecular Sciences, University of Surrey, Guildford GU2 7XH, UK*
[b] *Molecular Neuroendocrinology Group, Rowett Research Institute, Aberdeen AB21 9SB, UK*
[c] *School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK*

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Abstract

Pineal secretion of melatonin provides a neuroendocrine representation of the light–dark cycle, which is used to synchronise daily and annual rhythms of physiology and behaviour. In mammals, melatonin primarily acts through MT_1 melatonin receptors that exhibit a highly restricted tissue distribution. Expression of MT_1 receptors is subject to developmental and circadian control, which likely modulates the physiological actions of melatonin. To investigate the mechanisms controlling MT_1 expression we cloned the proximal 1.5 kb region of the ovine MT_1 promoter.

Sequence analysis revealed putative *cis*-elements for transcription factors involved in pituitary development, namely Pitx-1 and Egr-1, and multiple putative E-boxes, which are involved in both circadian and developmental gene regulation. Nuclear protein from ovine pars tuberalis (PT) cells, a site of high endogenous MT_1 expression, stimulated gene expression from a MT_1 expression construct, indicating the presence of a functional promoter. Pitx-1 was strongly expressed in the ovine PT and stimulated MT_1 promoter activity in transfection assays. Co-transfection with Egr-1 induced promoter-specific effects: Pitx-1-stimulated MT_1 activity was inhibited, whereas βLH promoter activity was enhanced.

In addition to *Pitx-1* the circadian clock genes *Clock* and *Bmal1* were also expressed in the PT. However, despite multiple putative E-boxes in the MT_1 promoter, transfected *Clock* and *Bmal1* were unable to regulate either basal or Pitx-1-stimulated MT_1 promoter activity.

The current data, in conjunction with our previous study of the rat MT_1 promoter, suggests a general model in which melatonin receptor expression in the mammalian pituitary is determined by the developmentally changing balance between stimulatory and inhibitory transcription factors. Furthermore, our data suggest that circadian variation in MT_1 gene expression does not depend upon the direct action of circadian clock genes on E-box *cis*-elements.

Keywords: Pitx-1; Egr-1; Pars distalis; Gonadotroph; Pars tuberalis; E-box

1. Introduction

The nocturnal secretion of melatonin from the pineal gland provides an endocrine representation of both circadian and seasonal time (Simonneaux and Ribelayga, 2003). In mammals, melatonin acts via two major receptor subtypes, of which MT_1 (previously termed Mel_{1a}) is the more prevalent (Reppert et al., 1996). Throughout life, melatonin receptor populations exhibit considerable plasticity. During early stages of development, melatonin receptors are transiently expressed in multiple neural and endocrine tissues (Davis, 1997). In adult animals, many melatonin receptor populations exhibit 24 h variation in receptor density (Piketty and Pelletier, 1993; Gauer et al., 1994; Recio et al., 1996; Schuster et al., 2001; Poirel et al., 2002). Currently, the significance of this plasticity and the mechanisms that drive changes in melatonin receptor expression are poorly understood.

The best characterised region of foetal melatonin receptor expression is the pituitary pars distalis (PD). In rodents, melatonin binding sites are present in the pituitary after approximately 12–15 days of gestation (Rivkees and Reppert, 1991;

☆ The sequence of the ovine MT_1 gene described in this study has been submitted to the Genbank database under accession number AY524665.

* Corresponding author. Tel.: +44 1483 686470; fax: +44 1483 686401.
E-mail address: j.johnston@surrey.ac.uk (J.D. Johnston).

[1] These two authors contributed equally to this work.

[2] Current address: SERVIER MONDE, 22 rue Garnier, 92578 Neuilly Sur Seine Cedex, France.

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Williams et al., 1991; Duncan and Davis, 1993) and decline over the first 2–3 weeks of neonatal life (Vanecek, 1988; Williams et al., 1991; Vanecek and Kosar, 1994). In the ovine PD, melatonin binding is present from 31 days of gestation through to birth, although labelling becomes more diffuse with gestational age (Helliwell and Williams, 1994). Current evidence suggests that these binding sites correspond to MT_1 receptors expressed in gonadotroph cells (Martin and Klein, 1976; Martin et al., 1982; Johnston et al., 2003b, 2006). The development and regulation of this cell type is dependent on many transcription factors, including pituitary homeobox-1 (Pitx-1; Szeto et al., 1999) and the lineage specific factor early growth response factor-1 (Egr-1; Lee et al., 1996; Topilko et al., 1998). Pitx-1 is of particular interest as it stimulates promoter activity of many pituitary-specific genes (Tremblay et al., 1998) and is also highly expressed in the pituitary pars tuberalis (PT) (Lanctot et al., 1999), an important site of melatonin action in seasonal mammals (Hazlerigg et al., 2001; Johnston, 2004).

Putative *cis*-elements for these gonadotroph-associated transcription factors have recently been identified in the rat MT_1 promoter (Johnston et al., 2003a). Pitx-1 and Egr-1 were subsequently shown to be potent regulators of MT_1 promoter activity (Johnston et al., 2003b). The MT_1 receptors in rat gonadotroph cells appear to be down-regulated by the onset of hypothalamic gonadotrophin-releasing hormone (GnRH) secretion, suggesting that the establishment of a mature hypothalamic input pathway shuts down melatonin sensitivity in these cells in this species (Johnston et al., 2003b). Whether the same pathway of developmental control also applies in larger non-rodent species, in which developmental organisation is far more advanced at the time of birth (precocial), or in other endocrine tissues showing transient melatonin receptor expression, remains to be established.

Despite the developmental loss of melatonin receptors in PD gonadotroph cells, MT_1 mRNA remains strongly expressed in the "PT-specific" thyrotrophs of the adult PT (Klosen et al., 2002; Dardente et al., 2003). Daily rhythms of both MT_1 expression and melatonin binding sites have been reported in the PT and suprachiasmatic nuclei (SCN) of the hypothalamus (Piketty and Pelletier, 1993; Gauer et al., 1994; Recio et al., 1996; Schuster et al., 2001; Poirel et al., 2002). Furthermore, both of these tissues rhythmically express multiple circadian clock genes (Reppert and Weaver, 2001; Lincoln et al., 2002; Hazlerigg et al., 2004; Johnston et al., 2005; von Gall et al., 2005), whose possible role in melatonin receptor expression has not yet been investigated. These clock genes could interact directly with the MT_1 promoter, or modulate the activity of other factor(s), such as Pitx-1 expressed in the PT, to further regulate receptor plasticity.

In order to further explore the mechanisms through which changes in MT_1 expression occur we have cloned a fragment of the ovine MT_1 promoter. We identified putative *cis*-elements for a range transcription factors, including those involved in circadian gene expression and in pituitary differentiation. We then assessed the functional relevance of these elements using a combination of *in vitro* transcription and reporter assays, together with analysis of gene expression *in vivo*.

2. Materials and methods

2.1. Cloning and sequence analysis

An ovine genomic library (Barrett et al., 1994) was screened with a ^{32}P-labelled oligonucleotide for exon 1 of the ovine MT_1 receptor sequence. Hybridising plaques were plaque purified with several rounds of replating and screening. One clone was analysed by restriction digestion followed by Southern analysis for a fragment likely to contain ~2 kb of sequence upstream of the coding region start site. Based on the known restriction digestion pattern for the ovine MT_1 sequence, a NotI/SphI fragment was deemed to fulfil these requirements. This fragment was cloned into the NotI/SphI site of the vector pGEM-T (Promega, UK). The sequence of the cloned fragment was determined in both directions using ABI dye terminator sequencing reagents and analysed on an ABI 377 DNA sequencer. The fragment was 1.68 kb in length containing 111 bp of coding sequence.

Restriction fragments were prepared with lengths as indicated in the manuscript, and subcloned into the luciferase reporter vector pGL3-Basic (Promega UK), according to the manufacturer's protocol. These fragments covered regions −1534 to −26, −716 to −26, −440 to −26 and −90 to −26, relative to the translation start site.

Putative transcription factor *cis*-elements were selected using MatInspector software (Quandt et al., 1995; Cartharius et al., 2005).

2.2. In vitro transcription run off assay

Nuclear extracts from a primary cell culture of ovine PT cells were prepared using the protocol of Andrews and Faller (1991) using up to 10^7 cells. Briefly cells were resuspended in 400 μl of ice cold buffer A (10 mM HEPES-KOH pH 7.9 at 4 °C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and allowed to swell on ice for 10 min. Cells were then spun at full speed in a microfuge for 10 s at room temperature. The nuclear pellet was then resuspended in 200 μl of buffer C (20 mM HEPES-KOH pH 7.9 at 4 °C, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). The nuclear extract was then spun at full speed in a microfuge for 2 min. The resultant supernatant was dialysed overnight in buffer D (24 mM HEPES pH 7.9 at 25 °C, 24% (v/v) glycerol, 120 mM KCl, 0.24 mM EDTA, 0.6 mM DTT, 0.5 mM PMSF. Dialysis was carried out in 0.5–3 ml cassettes (3500 MW, Pierlabo Scientific) according to the manufacturer's protocol.

The MT_1 promoter-luciferase constructs were linearised with SalI, facilitating a run off transcript of 2 kb. The *in vitro* transcription assay was carried out in a volume of 100 μl containing 12 mM HEPES (pH 7.9), 12% glycerol, 0.3 mM DTT, 0.12 mM EDTA, 60 mM KCl, 12 mM $MgCl_2$, 600 μM each rNTP, 10–20 μg/ml linearised DNA template. The transcription reaction was incubated at 30 °C for 90 min at which point RQ1 DNAse (Promega UK, 2 U/μg DNA) was added to the reaction and incubated for a further 30–60 min at 37 °C. The synthesised RNA was purified with the RNeasy kit (RNA clean-up protocol, Qiagen, UK). The RNA was eluted in 30 μl DEPC water and concentrated by overnight ethanol precipitation at −20 °C following the addition of a one-tenth volume of 3 M NaAc pH 5. The RNA was then resuspended in 5 μl of DEPC treated water and prepared for electrophoresis on a denaturing formaldehyde 1% agarose gel. Following electrophoresis, the RNA was transferred to a nylon membrane (Dupont, UK). The RNA was UV cross-linked. The membrane was then probed with a ^{32}P-labelled DNA fragment containing the luciferase coding sequence prepared from the plasmid pGL3 basic (Promega, UK).

2.3. Transient transfections

Assays were performed as described previously (Johnston et al., 2003b). Briefly, COS-7 cells at 50–80% confluence were transfected with appropriate plasmid combinations, using FuGene6 (Roche), according to the manufacturer's protocol. Forty-eight hours after transfection, cells were washed, enzymatically detached and pelleted. Cells were then resuspended in DMEM and aliquots were either assayed for β-galactosidase activity or lysed in Steady-Glo reagent (Promega) prior to measurement of luciferase activity. Each treatment was performed in triplicate per experiment.