Melatonin Regulation of the Oxytocin System in the Pregnant Human Uterus

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MELATONIN REGULATION OF THE OXYTOCIN SYSTEM IN THE
PREGNANT HUMAN UTERUS

By
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To my family and mentors:

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The mechanisms underlying the gestational and circadian timing of parturition in humans are not fully understood. Studies of the timing of initiation of spontaneous labor show a peak between 2400 and 0500. This peak in labor onset coincides with peak serum melatonin levels in humans. Melatonin, N-acetyl-5-methoxytryptamine, is the molecular messenger of circadian night. A monoamine hormone produced by the pineal gland, melatonin, is released into the blood directly in a circadian manner controlled by input from suprachiasmatic nuclei (SCN). Peak levels occur several hours after darkness and its release is inhibited by light via photic input transmitted from the eye via the retino-hypothalamic tract to the SCN. Once in the circulation melatonin can act on numerous tissues via its receptors or via antioxidant mechanisms inferred by its indole ring. Our laboratory recently characterized the expression of the melatonin receptors in the human myometrium and showed that the expression of these receptors is suppressed until late pregnancy. In an effort to understand better the significance of melatonin in the human myometrium, we explored the mechanisms through which this hormone influences the expression of the oxytocin receptor \textit{in vitro}. The stable melatonin analog iodo-melatonin (I-MEL) was presented to cultured telomerase-immortalized smooth muscle myometrial cells of the human telomerase reverse transcriptase line under physiological doses and durations. Pharmacological inhibitors of melatonin binding (4P-PDOT), gene transcription (actinomycin), phospholipase C (U73122), and protein kinase C (C1) signaling were used to define the mechanism of melatonin action. Our results reveal that melatonin significantly reduces oxytocin receptor mRNA levels primarily via the melatonin type 2 receptor, MT2R. We assayed OTR mRNA levels over 24 hours after treatment with the transcriptional inhibitor, Actinomycin, with and without cotreatment melatonin. Our data suggest the melatonin-dependent decrease in oxytocin receptor transcripts involves reduction of the OTR mRNA accumulation rate rather than enhanced rates of transcript degradation. Melatonin effects were abolished by pre-treating the cells with the phospholipase C inhibitor U73122 or the protein kinase C inhibitor C1. Melatonin, like oxytocin, can negatively regulate oxytocin receptor transcription in human myometrial cells via modulation of protein kinase C signaling.
Due to the similarities between the melatonin and oxytocin signaling pathways including reduction of OTR mRNA levels, we next sought to determine the effects of melatonin on contractility and the contractile machinery in telomerase-immortalized human myometrial cells. To ascertain the effect of melatonin on myometrial contractility in cell cultures, we performed gel retraction assays with cells exposed to I-MEL, oxytocin and the pharmacological inhibitors 4P-PDOT, U73122, C1 and combinations of ligands and inhibitors. I-MEL was found to synergistically enhance oxytocin-induced contractility via the MT2R, which is coupled to a protein kinase C-dependent increase in phosphorylation of the myosin light chain protein. The effects of I-MEL on gap junctions were also investigated as gap junction proteins have been shown to be upregulated by melatonin in other tissues and have also been shown to be important for coordination of contractions in the laboring uterus. I-MEL increased expression of the gap junction protein, connexin 43. In vitro dye spread assays showed that I-MEL-treated cells displayed substantially increased intercellular coupling. Increases in connexin 43 mRNA and cell to cell coupling were also found to be mediated via the MT2R in a protein kinase C-dependent manner. Additionally, expression levels of the type 2 melatonin receptor (MT2R) were assessed in myometrial biopsies from term pregnant women with or without labor. MT2R expression was markedly elevated in samples from pregnant women who had entered labor, as compared to matched non-laboring pregnant women.

To ascertain the signaling pathway of melatonin and leading to its effects on myometrial contractility in vitro, we performed gel retraction assays with cells exposed to I-MEL with or without oxytocin and the Rho kinase inhibitor Y27632. I-MEL effects on IP3/DAG/ Protein Kinase C (PKC) signaling were also investigated as these signaling molecules were implicated by our previous pharmacology experiments. I-MEL was found to activate PKCα via the phospholipase C/IP3/DAG signaling pathway which was confirmed by PKC enzyme assay. I-MEL did not affect myosin light chain phosphatase activity and its effects on contractility were insensitive to Rho kinase inhibition. In order to examine another possible method of contractile sensitization, we assayed for caldesmon phosphorylation and upstream Erk1/2 activation. I-MEL did increase phosphorylation of Erk1/2 and caldesmon, which was inhibited by the MEK inhibitor, PD98059 or the PKC inhibitor, C1. These findings lead us to surmise that melatonin sensitizes myometrial cells to subsequent pro-contractile signals in vitro through activation of the phospholipase C/IP3/DAG signaling pathway resulting in specific activation of PKCα and
Erk1/2, thereby phosphorylating caldesmon, which increases actin availability for myosin binding and crossbridging.

This research revealed a new role for melatonin in reproductive physiology, sensitizing myometrial cells to a subsequent pro-contractile oxytocin signal. This function would help explain the increased nocturnal uterine contractility and increased incidence of parturition observed in late term human pregnancy. Synergistic actions of melatonin on oxytocin-induced contractility may be of clinical relevance in that it could provide a means to lower the oxytocin dose used in the induction of labor and thus reducing the contraindications associated with oxytocin induction of labor.
Parturition is a complex, multifactor process. The exact mechanisms controlling the timing and onset of labor are not understood. Prior to labor, the uterus undergoes remodeling and dramatic changes in gene expression to prepare for the process of parturition. These changes result in increases intercellular connectivity and sensitize the myometrium to subsequent pro-contractile signals resulting in the forceful, coordinated contractions required for successful parturition. Understanding these complex processes would allow for the development of more effective treatments to address the medical and financial burdens concurrent with pre-term births which range between 5-15% of all births (Slattery and Morrison 2002; Petrou 2005).

The transition of the myometrium from a quiescent to an active state involves cellular changes that promote myocyte excitability, myosin-actin interaction and intercellular contractility. Changes in ion channel expression and distribution at labor alter the membrane potential allowing for increased response to contractile stimuli and reduced response to anti-contractile signals (Smith 2007). In addition to changes in ion channel expression, expression of receptors for pro-contractile ligands increase from low levels during pregnancy, presumably sustaining uterine quiescence, to high levels which peak at labor (Smith 2007). Concurrently, at the time of labor there is an increase in actin stress fiber formation and alterations in the signaling mechanisms governing the contractile machinery of the cell. Lastly there is an increase in intercellular communication via gap junctions. The increase in gap junctions is due to an increase in connexin 43, the primary component of myometrial gap junctions, which forms multimeric intercellular channels between adjacent myocytes (Smith 2007). These junctions facilitate transmission of ions between cells allowing for a wave of depolarization. The increased intercellular connectivity allows for coordination of contraction within the uterus. Coordination of these events are important to the gestational timing of parturition (Smith 2007).

Oxytocin, a nonapeptide secreted by the neural lobe of the pituitary gland, has long been known for its role in parturition and lactation. Oxytocin is the product of oxytocinergic nerves of the posterior pituitary gland and has been classically linked to parturition and milk ejection. Oxytocin is released to the general circulation in response to uterine dilation, suckling and certain stresses. After its release,
oxytocin circulates until it is bound by its receptor.

The oxytocin receptor, OTR, is a seven transmembrane G-protein coupled receptor in the rhodopsin receptor family. GPCR’s are coupled to heterotrimeric G protein complexes consisting of α, β and γ subunits. Upon ligand binding GTP is bound by the α subunit allowing it to dissociate from the receptor and the β/γ subunits. The α subunit then acts on its target (adenylyl cyclase for Gs and Gi subunits; phospholipase C for Go). The OTR has been shown to couple to both Go/11 and Gi G-protein complexes allowing the receptor to have two different activities based upon which G-protein is coupled to the receptor. The Gq pathway acts on PLCβ, which cleaves inositol triphosphate, IP3, from the cell membrane. Inositol triphosphate then triggers Ca2+ release from internal stores that is bound by calmodulin, activating the calmodulin kinase II, CAMK II. The diacyl glycerol produced as IP3 is cleaved from the cell membrane activates protein kinase C, PKC. CAMK II and PKC phosphorylate multiple target proteins including myosin light chain kinase that triggers smooth muscle contraction, and several membrane channels, facilitating calcium import from the extracellular space.

If OTR is coupled to Gi, the Gi α subunit will inhibit cyclic AMP production by adenylate cyclase thus inhibiting signaling using cAMP as a second messenger. Additionally the β/γ subunits in both instances dissociate from the receptor and can also activate PLC and signal via that path. The Gi pathway is inhibited by pertussis toxin, which prevents the activation of the Gi subunit.

To date, a definite regulatory pathway for OTR expression has yet to be determined. Steroid factors have been shown to influence transcription (Gimpl and Fahrenholz 2001) and the OTR promoter contains many potential transcription factor-binding sites including sites for AP-1, a half estrogen response element and a cAMP response element. Despite the identification of these transcription factor binding sites, the major factors driving transcriptional regulation of the OTR promoter remain unidentified. oxytocin has been reported to inhibit transcription of its own receptor and oxytocin binding in both cells and in human myometrial tissues obtained after induction of labor with oxytocin (Phaneuf, Asboth, Carrasco, Linares, Kimura, Harris and Bernal 1998; Phaneuf, Rodriguez Linares, TambyRaja, MacKenzie and Lopez Bernal 2000) This effect provides a rapid desensitization of the uterus to pro-contractile oxytocin signals after parturition. Prior to labor, OTR is maintained at low levels promoting uterine quiescence. The subsequent upregulation and rapid desensitization point to control of OTR expression being an important factor in the gestational timing of parturition.

Gestational timing is important to ensure the complete development of the offspring. Another important aspect is the circadian timing of parturition. In mammals, the onset of parturition is generally
timed to the rest phase, nocturnal mammals giving birth during the circadian day and diurnal animals during the circadian night. Biologically this is of importance in that it serves to protect the laboring female from predation. In the mouse, oxytocin knockout lines lose the circadian timing of parturition when phase shifted indicating that the circadian rhythm of serum oxytocin levels, which remain unperturbed in wild type mice after phase shifting, determined the timing of birth (Roizen, Luedke, Herzog and Muglia 2007). A study of humans shows a peak in the onset of labor between the hours of 2400 and 0500 regardless of gestational age (Seron-Ferre, Ducsay and Valenzuela 1993). Since humans lack a circadian rhythm in serum oxytocin levels (Gimpl and Fahrenholz 2001) other circadian factors may play a role in the time of day that labor occurs.

Melatonin, N-acetyl-5-methoxytryptamine, is the molecular messenger of circadian night. A monoamine hormone produced by the pineal gland, melatonin, is released into the blood directly in a circadian manner controlled by input from suprachiasmatic nuclei (SCN). Peak levels occur several hours after darkness and its release is inhibited by light via photic input transmitted from the eye via the retino-hypothalamic tract to the SCN. Once in the circulation melatonin can act on numerous tissues via its receptors or via antioxidant mechanisms inferred by its indole ring (Masana and Dubocovich 2001).

Melatonin signals via two G-protein coupled receptors, the type 1 melatonin receptor (MT1R) or the type 2 melatonin receptor (MT2R). Both receptors are seven transmembrane domain, G-protein coupled receptors. MT1R couples with the G\textsubscript{i} alpha subunit and signals through inhibition of adenylate cyclase. MT2R can couple to multiple G\textsubscript{a} subunits including G\textsubscript{i} and G\textsubscript{q/11}. The signaling pathway for MT2R differs in that the G\textsubscript{q/11} subunit complex activates PLC which cleaves membrane phospholipids generating inositol triphosphate (IP\textsubscript{3}) and diacylglycerol (DAG). These second messengers continue their signaling cascade via intracellular calcium release and activation of protein kinase C (PKC) (Masana and Dubocovich 2001). Similar signaling cascades are used by numerous other factors allowing for potential cross-talk between the melatonin signaling pathway and other activated signaling pathways.

Melatonin has been shown to have effects on many aspects of the reproductive system. In seasonal breeding species, the day length encoded by melatonin has been shown to regulate the estrus cycle and testicular function (Gunduz and Stetson 1994; Benson and McAsey 1998). In the female human and rat, melatonin modulates sex steroid secretion and also modulates the function of the hypothalamus-pituitary-gonad axis, specifically the release of gonadotropin releasing hormone and prolactin. In the rat and sheep, serum melatonin levels have been shown to maintain a circadian
oscillation during pregnancy (Tamura, Nakamura, Terron, Flores, Manchester, Tan, Sugino and Reiter 2008). Nocturnal serum melatonin levels have also been reported to rise over the course of pregnancy exhibiting significantly higher levels after 32 weeks of gestation followed by a return to non-pregnant levels two days post partum (Tamura, Nakamura, Terron, Flores, Manchester, Tan, Sugino and Reiter 2008). Urine and amniotic fluid melatonin levels also have been shown to peak around the time of delivery. A strong case for a role for melatonin in the circadian timing of parturition is in the rat. Pinealectomized female rats lose the circadian timing of parturition, giving birth across the 24 hr cycle. Diurnal parturition was restored only when melatonin was provided during the normal night time period. Sham and continuous melatonin had no restorative effect (Tamura, Nakamura, Terron, Flores, Manchester, Tan, Sugino and Reiter 2008).

The purpose of my research is to examine the role of melatonin in the uterine physiology of the pregnant human. Previous work by our lab has identified melatonin binding sites in the myometrium (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003). melatonin has also been shown to augment noradrenaline - induced uterine contractility (Martensson, Andersson and Berg 1996). In light of these observations we performed in vitro assays for melatonin effects on uterine physiology using the telomerase immortalized myometrial cell line hTert, as well as primary myometrial cells and frozen myometrial biopsy materials. The hTert cell line is a human myometrial cell line stably transfected with a constitutively expressed telomerase. Characterization of the cell line showed normal expression of numerous key receptors and genes when compared to primary myometrial cells (Condon, Yin, Mayhew, Word, Wright, Shay and Rainey 2002), thus making it a preferred model for our assays.

Melatonin effects on uterine physiology can be assessed using multiple biochemical means. Real time quantitative PCR is a powerful tool to examine changes to gene expression in response to melatonin treatment. Combined with pharmacological treatments it is possible to ascertain certain aspects of the mechanism underlying observed changes in messenger RNA levels of genes of interest. Additionally through the addition of transcriptional inhibitors such as actinomycin, the degradation rate of a specific mRNA can be monitored over time allowing for calculation of the half life of the transcript. These methods proved useful in our examination of the effects of melatonin on oxytocin receptor expression in the myometrium.

Our observations of melatonin’s effect on oxytocin receptor expression led us to investigate the effects of melatonin on contractility. Due to lack of a reliable source of fresh myometrial strips and the necessary equipment to perform contractility measurement of myometrial strips, an alternative method
to assay contractility was required. As an alternative, we used the collagen disc based retraction assay of Devost and Zingg (Devost and Zingg 2007). In this assay for contractility, cells are plated on collagen discs and treated overnight. The discs are fixed and then changes in surface area are measured. Changes in total contractility over the treatment period can then be determined.

Intercellular gap junction activity was assayed using a dye spread assay. Cells were loaded with lucifer yellow dye and the spread to adjacent cells was monitored after 15 minutes. Since lucifer yellow is membrane impermeable the spread of the dye is directly a function of gap junction activity.

The mechanism of melatonin signaling was investigated using a combination of pharmacological treatments, immunoprecipitation and western blotting techniques. These assays for phosphorylated signaling proteins allowed for identification and the elucidation of the activated signaling pathways responsible for melatonin’s effect on contractility. Western blotting for oxytocin and the type 2 melatonin receptors was performed on frozen myometrial biopsies from various gestational ages to investigate the expression of these receptors over the gestational timeline. The western data were confirmed with radioreceptor binding assays using I\(^{125}\)-MEL using the method described by Schlabritz-Loutsevich (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003) to indicate the presence of receptors available for melatonin binding. Inositol phosphate turnover and PKC activity radio assays were performed to confirm PLC and PKC activity suggested by our observations in pharmacological inhibition treatment experiments. Fura-2 based intracellular calcium imaging experiments were also performed to determine if melatonin signaling is occurring through calcium release in the hTert cells.

**Thesis Plan**

The analysis of melatonin’s effects on OTR mRNA levels is described in Chapter 2 and was published as:

The analysis of the synergistic effects of melatonin on oxytocin induced contractility in human myometrial cells is described in Chapter 3 and was published as:


Elucidation of the mechanisms used by melatonin to sensitize human myometrial smooth muscles cells was described in Chapter 4 and has been submitted to the Journal of Clinical Endocrinology and Metabolism for publication.
CHAPTER 2

TRANSCRIPTIONAL INHIBITION OF OXYTOCIN RECEPTOR EXPRESSION IN HUMAN MYOMETRIAL CELLS BY MELATONIN INVOLVES PROTEIN KINASE C SIGNALING

Introduction

Late in pregnancy the human myometrium up-regulates expression of the oxytocin receptor (OTR), a G-protein coupled receptor that transduces the oxytocin signal primarily via the \(G_{\alpha q/11}\) subunit to activate phospholipase C (PLC) and thereby increase intracellular calcium levels and inositol triphosphate production (Sanborn, Qian, Ku, Wen, Anwer, Monga and Singh 1995). A number of protein kinase cascades are also induced by oxytocin, including protein kinase C (PKC) and the p42/44 MAP kinase (Gimpl and Fahrenholz 2001). At parturition the ligand bound OTR contributes to the dramatic increase in uterine contractions. Conversely, the OTR antagonist atosiban is used to delay preterm labor in humans (Akerlund 2006).

Melatonin was reported by Martensson and colleagues (Martensson, Andersson and Berg 1996) to significantly potentiate the contractility of pregnant human myometrial tissues in vitro following application of norepinephrine. This was considered to involve the well-described inhibition of cAMP production by melatonin via its \(G_i\)-mediated suppression of adenylyl cyclase activity and/or the PLC-mediated calcium-elevating effects of melatonin (Masana and Dubocovich 2001).

In a previous study (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003) we determined that the mRNA and protein expression levels of the melatonin receptors (especially the MT2R) were substantially repressed during late pregnancy in the absence of labor. Apart from the hypothesized pro-contractile activity of melatonin its role in the human myometrium remains unclear. To facilitate a better understanding of melatonin’s functions in this tissue we have investigated the influence of melatonin on the expression of the OTR in telomerase immortalized human myometrial cells (hTERT). This model has been demonstrated to accurately reflect the mature myometrial smooth muscle cell (Cohen, Roselle, Chabner, Schmidt and Lippman 1978; Devost and Zingg 2007) and being a nontumor-derived clonal cell line it obviates the high variability of responses seen with primary cells.
and it is amenable to transfection. Our results reveal a significance regulatory impact of melatonin on the transcriptional activity of the OTR gene.

Materials and Methods

Cell Culture Experiments. Human myometrial cells (hTERT) were maintained in Ham’s F-12/DMEM with 4.5 g/liter glucose, 3 mM glutamine, penicillin/streptomycin, and 10% Fetal Plus II serum (Valley Biomedical). For the intracellular signaling studies cells were plated into 6-well culture dishes at a density of 20,000 cells/well. Prior to treatment cells were washed once in complete medium, and then exposed in triplicate to either vehicle or chemical agents. Except for the time-course and dose-response studies, cells were typically treated for 4h with 1 nM iodo-melatonin (I-MEL). In the case of the pharmacological inhibitors pertussis toxin (List), U73122 (Tocris) and C1 (Tocris), cells were preincubated for 1h prior to MEL exposure. For the determination of OTR transcript turnover rates the cells were exposed to 5 μM actinomycin (Sigma) with or without 1 nM I-MEL (Tocris). The cells were then harvested at 0 h, 2h, 4h, 6h, 12h and 24h post-treatment.

Quantitative Polymerase Chain Reaction. Cellular total RNA was extracted with the Qiagen™ RNEasy kit according to the manufacturer’s protocol. The RNA concentration was measured with the Nanodrop™ photometer, then reverse transcribed to cDNA by means of the Biorad® iScript™ reverse transcription system. Quantitative real time PCR was performed on a Biorad iCycler™ using iQ SYBR Green Supermix™ (Biorad), together with one μl of sense and antisense primers (10 pmol/μl) of the transcript of interest and 2 μl of template cDNA. The following thermal cycling parameters were used: Initial heat activation of the DNA-polymerase was performed at 95°C for 5 min. Thereafter, 40 cycles of 95°C (15 sec), 58°C (30 sec) and 72°C (30 sec) were run. After thermocycling the iCycler performs an automatic melting curve, which entails cooling to 55°C for 10 sec, then increasing temperatures in 0.2°C increments up to 90°C. This controls for primer-dimer formation and other nonspecific effects. Quantification of the data was achieved by the Biorad™ iCycler software using a standard curve from a primer-specific dilution series for the PCR product. Data were normalized against expression of the housekeeping gene GAPDH. The primer sequences used for oxytocin receptor (OTR), melatonin receptors (MT1R, MT2R) and GAPDH quantification are as follows:

OTR-forward  5'-TGG CGG AGC ACA GG-3'

8
OTR-reverse  5'-GTG TCA GCA GTC AAG C-3'
MT1R-forward  5'-TCC TGG TCA TCC TGT CCG TGT ATC-3'
MT1R-reverse  5'-CTG CTG TAC AGT TTG TCG TAC TTG-3'
MT2R-forward  5'-TCC TGG TGA TCC TCT CCG TGC TCA-3'
MT2R-reverse  5'-AGC CAG ATG AGG CAG ATG TGC AGA-3'
GAPDH-forward  5'-GTC TTC ACC ACC ATG GAG- 3'
GAPDH-reverse  5'-GTC ATG GAT AAC CTT GGC-3'

Statistical Analyses. Experiments were repeated at least three times. Replicate values for each data point were averaged and differences statistically analyzed using a one-way ANOVA followed by the Bonferroni post-hoc test (GraphPad Prism, CA, USA) with \( P < 0.05 \) as the criterion level for significance. For the determination of OTR mRNA turnover the data were analyzed using a non-linear regression (GraphPad Prism, CA, USA).

Results

Melatonin Treatment Reduces OTR mRNA Levels Via MT2R Activation. I-MEL – much like the OTR ligand oxytocin - was found to substantially reduce OTR expression in immortalized myometrial smooth muscle cells. Concentrations at or above a physiological concentration of 1 nM were equally effective, implying saturation of available receptors (Fig. 2.1A). This is feasible given that the affinity constants for both MT1R and MT2R are < 300 pM. Time course studies showed that the inhibitory effects of I-MEL at 2h and 4h were significant (\( P< 0.05 \)), while a maximal effect (significantly lower than the 2h effect) was seen by 12h of treatment (Fig. 2.1B). No effects of I-MEL on the expression of melatonin receptors (MTR) were noted under these conditions (Fig. 2.2). Similarly, the expression of OTR mRNA did not change with time in the absence of agonist (data not shown).

The inhibitory action of I-MEL (1 nM for 4h) on OTR expression is receptor-mediated in as much as the specific MTR antagonist 4P-PDOT can largely block this effect (Fig. 2.2A). The antagonist alone had no effect on OTR mRNA levels. At the dose of 4P-PDOT that was used (10 nM) only MT2R are likely to be blocked (Masana and Dubocovich 2001), thus we conclude that primarily the MT2R mediates the inhibitory action of I-MEL on OTR mRNA expression in these cells. Both MT1R and MT2R were identified in hTERT cells by qPCR analysis and neither changed its expression level following short-term I-MEL exposure (Fig. 2.2B).
Fig. 2.1. Iodo-melatonin (I-MEL) inhibits OTR mRNA levels in human TERT cells after 4h exposure to various doses (A) and following exposure of cells to 1 nM for 2, 4 or 12h (B). * indicates a statistical difference relative to controls (P<0.05). ** = statistically lower than values at 2h (P<0.05).

Fig. 2.2. A) The inhibition of OTR mRNA expression by I-MEL (1 nM for 4h) was significantly attenuated by the MT2R antagonist 4P-PDOT (10 nM). The antagonist alone was without effect. B) I-MEL (1 nM, 4h) had no effect on the expression levels of the melatonin MT1 or MT2 receptors. * indicates a statistical difference relative to control (P<0.05).
The inhibitory effect of I-MEL largely mimics the effect of oxytocin on OTR expression; hence there may be commonality in their signaling pathways. MTRs are coupled to inhibitory G-protein-coupled mechanisms, hence, we examined whether this pathway is necessary for the inhibitory action of melatonin on OTR mRNA expression. Cells were pre-incubated with pertussis toxin (PTX, 100 ng/ml), which ADP-ribosylates the Ga_i subunit thereby preventing it from signaling. This treatment had no effect on the ability of I-MEL (1 nM for 4h) to suppress OTR mRNA expression (Fig. 2.3).

![Graph showing the effect of PTX and I-MEL on OTR mRNA expression.](image)

**Fig. 2.3.** Pertussis toxin (PTX; 100ng) does not prevent the inhibitory action of I-MEL (1 nM for 4h) on OTR mRNA expression in human myometrial smooth muscle cells. * indicates a statistical difference relative to control (P<0.05).

**Melatonin Inhibits OTR mRNA Transcription.** In order to establish whether the decline in OTR mRNA levels following application of I-MEL is due to inhibitory transcriptional mechanisms as opposed to a shortening of half-life of the OTR transcript, we exposed cells to the transcriptional blocker actinomycin D (5 μg/ml). As would be expected, the levels of the OTR transcript decay exponentially following this treatment alone with a half-life of 1.48h. The presence of 1 nM I-MEL had no additional effect on the rate of OTR mRNA degradation (Fig. 2.4) indicating that I-MEL’s actions are likely to involve transcriptional repression.
Fig. 2.4. The rate of decline of the myometrial OTR transcript after exposure to I-MEL (1nM) for 2, 4, 6, 12 or 24h is identical to that after no I-MEL when transcription is blocked by pre-incubation of cells with actinomycin D (5 μg/ml). No additional effect was noted when actinomycin and I-MEL (1 nM) were given together.

**Melatonin Inhibition Of OTR Is Mediated By PLC/PKC Signaling.** Both MTR and OTR use the phospholipase C signaling pathway upon activation by their cognate ligands. We therefore assessed the effect of PLC blockade on I-MEL’s action to suppress OTR transcription. The PLC inhibitor U73122 at a dose of 1 μM prevented I-MEL’s inhibitory effect (Fig. 2.5) as well as preventing oxytocin’s inhibitory effect (data not shown). The inhibition of OTR transcription could be reinstated by addition of phorbol 12, 13- dibutyrate (10 nM), a phorbol ester that directly activates PKC.

To confirm that PKC signaling is required for I-MEL’s inhibitory effect on OTR transcription, cells were pretreated in the presence or absence of 10 μM of the PKC inhibitor C1 for 1h. Inhibition of PKC prevented I-MEL (1 nM for 4 h) from lowering OTR transcription in immortalized myometrial smooth muscle cells (Fig. 2.6).
Fig. 2.5. Pharmacological inhibition of phospholipase C activity with U73122 (1 μM) prevents I-MEL inhibition (1 nM for 4h) of OTR transcription in cultured human myometrial cells. The PKC activator PDBu (10 nM) also inhibits OTR transcription, and can circumvent the effect of U73122. * indicates a statistical difference relative to control (P<0.05).

**Discussion**

An influence of melatonin on parturition in rodents was suggested recently (Takayama, Nakamura, Tamura, Yamagata, Harada, Nakata, Sugino and Kato 2003) by findings that female rats lacking endogenous melatonin synthesis failed to deliver their young exclusively during the daytime, which is the normal birthing phase for these nocturnal animals. Instead, they gave birth randomly across the 24-hour light-dark cycle. Melatonin was effective in restoring
Fig. 2.6. The inhibitory effect of 1 nM I-MEL (4h) on OTR transcription in hTERT cells is prevented by pre-incubation with the protein kinase C inhibitor C1 (10 μM). * indicates a statistical difference relative to control (P<0.05).

the daytime birth pattern when it was administered in the evening (the time at which endogenous melatonin secretion would normally rise), but was ineffectual when given in the morning or continuously. These interesting results demonstrate that the timing of birth in the rat is under circadian control, and that melatonin may serve as a key circadian signal for this event.

The precise mode of action of melatonin in the uterine myometrium is still unclear. Earlier reports (Hertz-Eshel and Rahamimoff 1965; Burns 1972) described direct inhibitory effects of pharmacological doses of melatonin on uterine contractility in rodents as well as the presence of melatonin-specific binding sites in the uterus (Cohen, Roselle, Chabner, Schmidt and Lippman 1978). Later studies confirmed inhibitory effects of melatonin on uterine contractility in vitro following stimulation by oxytocin (Gimeno, Landa, Sterin-Speziale, Cardinani and Gimeno 1980; Abd-Allah, El-Sayed el, Abdel-Wahab and Hamada 2003). The large-conductance, calcium-dependent potassium channel (BK_{Ca}) has also been identified as a target for melatonin action in rat myometrial cells (Steffens, Zhou, Sausbier, Sailer, Motejlek, Ruth, Olcese, Korth and Wieland 2003). In this study, melatonin modulated the BK_{Ca} channel via the G_{i}-mediated inhibition of cAMP signaling (thereby inhibiting mechanisms that promote myometrial quiescence) in nonpregnant but not in pregnant myometrium. In
contrast, melatonin consistently activated the G_q/phospholipase C pathway in myometrial cells, which promotes contractility. The BK_{Ca} potassium channel is the predominant potassium channel in the nonpregnant and pregnant myometrium of the human (Khan, Matharoo-Ball, Arulkumaran and Ashford 2001) and has been implicated in maintaining uterine quiescence during pregnancy (Matharoo-Ball, Ashford, Arulkumaran and Khan 2003).

Given that human labor and delivery are – in contrast to the nocturnal rodent -statistically more common during the nocturnal phase (Glattre and Bjerkedal 1983; Cooperstock, England and Wolfe 1987; Panduro-Baron, Gonzalez-Moreno and Hernandez-Figueroa 1994), it would seem reasonable to propose that melatonin may contribute to human parturition. Previously, we demonstrated MTR mRNA and protein expression in the human myometrium during pregnancy without labor and in non-pregnant women (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003). A substantial decline in melatonin receptor expression (as compared to nonpregnant tissues) was seen in the myometria of women in late pregnancy prior to the onset of labor. Additionally, the inhibitory effects of melatonin on intracellular cyclic AMP signaling that were typical to cultured myometrial cells of nonpregnant women were absent in the cells of pregnant women, possibly because of the low MTR expression in the latter case. Our finding of low myometrial MTR expression in late pregnancy may represent a physiological mechanism to balance contractile against quiescent mechanisms until the proper phase of late gestation has been reached. Changes in myometrial receptor expression and cAMP signaling mechanisms during pregnancy are well known. For example, the expression of the stimulatory G-protein G_s is upregulated and then subsequently down-regulated before the onset of labor (Europe-Finner, Phaneuf, Tolkovsky, Watson and Lopez Bernal 1994). Conversely, the myometrial expression of the FP prostaglandin receptor is dramatically down-regulated in pregnancy when compared to non-pregnant tissue, but it then increases dramatically with labor (Brodt-Eppley and Myatt 1999).

Substantial evidence points to a role for the pituitary hormone oxytocin in stimulating myometrial contractility. Many, but not all studies have found that the expression of the oxytocin receptor in the human myometrium is significantly upregulated in late pregnancy (Akerlund 2006), when nocturnal plasma oxytocin levels are also increasing (Fuchs, Husslein and Fuchs 1981). Continuous infusion of oxytocin agonists is commonly used to induce labor. However, prolonged labor induction by application of continuous oxytocin is only effective when high amounts of the hormone are given, due to oxytocin receptor down-regulation at both the transcriptional and post-translational levels (Phaneuf, Rodriguez Linares, TambyRaja, MacKenzie and Lopez Bernal 2000). Despite numerous
studies demonstrating significant modulation by steroids, cytokines, etc. there remains a paucity of data on the specific regulatory mechanisms controlling the oxytocin receptor (OTR) gene in the human myometrium (Kimura, Saji, Nishimori, Ogita, Nakamura, Koyama and Murata 2003).

The results of our present studies provide novel insights into the regulation of OTR expression in human myometrial smooth muscle cells. The effects of I-MEL on OTR mRNA levels are remarkably similar to those of the OTR ligand itself in as much as oxytocin suppression of OTR in myometrial cells also involves PKC signaling (Gimpl and Fahrenholz 2001; Kimura, Saji, Nishimori, Ogita, Nakamura, Koyama and Murata 2003). At 1 nM - a dose approximating nocturnal levels in late pregnancy (Wierrani, Grin, Hlawka, Kroiss and Grunberger 1997) - and at exposure times of >2h I-MEL suppressed OTR expression by 30-60 % (Fig. 2.1). No significant additive effects of oxytocin + I-MEL were noted (data not shown), suggesting near maximal recruitment of PLC/PKC pathways by I-MEL. This notion is further supported by the non-additive effects of I-MEL and the PKC activator PDBu on OTR mRNA levels (Fig. 2.5).

In our in vitro experiments neither I-MEL nor oxytocin was tested for possible effects on the contractile activity of human myometrial cells. Thus, it is not possible at this time to draw any definitive conclusions regarding the influence of melatonin on contractility of the pregnant human uterus. Earlier studies in pregnant non-human primates (Matsumoto, Hess, Kaushal, Valenzuela, Yellon and Ducsay 1991) reported no alteration of rhythmic uterine contractility after blockade of melatonin secretion by constant light exposure, nor following subsequent melatonin infusion. Whether this reflects species-specific differences in myometrial MTR expression or underscores endocrine redundancy in the mechanisms controlling uterine contractility remains to be investigated.

The I-MEL-dependent inhibition of OTR transcription that we have identified in human myometrial cells can be blocked by the antagonist 4P-PDOT (Fig. 2.2) at a dose that is selective for the MT2R (Masana and Dubocovich 2001). The inhibition of OTR transcription by I-MEL can also be relieved by pharmacological blockade of phospholipase C (Fig. 2.5) and protein kinase C (Fig. 2.6), pointing to the involvement of a PLC/PKC signaling pathway in this effect. Melatonin receptors have been shown to couple to PKC in other cell systems (Masana and Dubocovich 2001; Ramirez-Rodriguez, Ortiz-Lopez and Benitez-King 2007). Although kinase cascades are known to modulate numerous transcription factors, the precise molecular mechanism for PKC-mediated suppression of myometrial OTR transcription, either by oxytocin or by I-MEL, remains to be elucidated.
The findings in the present report illuminate new avenues for a better understanding of human myometrial physiology. The interplay between a circadian signal, such as melatonin, and a contractile stimulus, such as oxytocin, during the course of late pregnancy may contribute to the maintenance of normal term pregnancy as well as the circadian timing of labor. Further characterization of such regulatory networks in the human uterus can be expected to be of value in the search for novel therapies to prevent pre-term labor - the etiology of which still not clear.
CHAPTER 3
MELATONIN SYNERGIZES WITH OXYTOCIN TO INCREASE CONTRACTILITY OF HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS

Introduction

Continuous monitoring of normal uterine contractile activity during late term pregnancy in humans has shown increased frequency between the hours of 8:30 PM and 2:00 AM (Zahn and Hattensperger 1993). Studies on the timing of human labor onset and deliveries show that the initiation of labor peaks between the hours of 24:00 and 05:00, regardless of gestational age (Seron-Ferre, Ducsay and Valenzuela 1993). Current models describe parturition as a multi-step process beginning with myometrial activation in late pregnancy followed by stimulation leading to uterine contraction and subsequent delivery of the infant. Myometrial activation encompasses cellular remodeling with appropriate changes in gene expression. The increased expression of these “contraction-associated proteins” marks the transition of the myometrium from a quiescent to activated state. These proteins facilitate the powerful uterine contractions necessary to deliver the infant by increasing the excitability of the myometrial cells, enhancing smooth muscle myosin-actin interactions, and increasing intercellular connectivity, thereby facilitating synchronous myometrial contractions (Smith 2007).

After its activation the myometrium can be stimulated by multiple factors including oxytocin, prostaglandins and noradrenaline (Berg, Andersson and Ryden 1986; Blanks, Shmygol and Thornton 2007). Oxytocin, a nonapeptide hormone secreted by the pituitary gland is one of the most potent uterine contractants. Oxytocin, upon binding to its G\textsubscript{q} -protein coupled receptor (OTR), activates the membrane-bound phospholipase C (PLC) and subsequently protein kinase C (PKC). Inositol triphosphate (IP\textsubscript{3}), cleaved from membrane phospholipids, binds receptors on the sarcoplasmic reticulum triggering the release of Ca\textsuperscript{2+} from intracellular stores as well as the influx of extracellular Ca\textsuperscript{2+} via membrane calcium channels. Increases in intracellular calcium concentrations result in activation of the Ca\textsuperscript{2+}/calmodulin-independent enzyme, myosin light chain kinase (MLCK), thereby leading to the phosphorylation of the myosin light chain and myometrial contraction (Word, Tang and Kamm 1994; Arthur, Taggart and Mitchell 2007).
Melatonin, a monoamine hormone secreted by the epithalamic pineal gland, is a major molecular messenger of the nocturnal phase of the light-dark cycle. Melatonin signals via two G-protein coupled receptors, melatonin receptor 1 (MT1R) and melatonin receptor 2 (MT2R) (Masana and Dubocovich 2001). We have previously shown that human myometrium is a target for melatonin and expresses both melatonin receptors (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003; Sharkey and Olcese 2007). These studies pointed to a potential point of interaction between oxytocin and melatonin signaling pathways. An earlier report demonstrated that melatonin potentiates norepinephrine-induced contractility in a dose dependent manner in human myometrial strips (Martensson, Andersson and Berg 1996).

Our previous work showed striking similarities between melatonin regulation of OTR mRNA expression and regulation of OTR mRNA expression by oxytocin (Phaneuf, Asboth, Carrasco, Linares, Kimura, Harris and Bernal 1998; Sharkey and Olcese 2007) leading us to further explore the similarities between the melatonin and oxytocin signaling pathways in the myometrium. Herein, we investigated the effects of melatonin on myometrial contractility in vitro by conducting experiments with the well characterized hTERT telomerase-immortalized human myometrial smooth muscle cell line which have been shown to express oxytocin receptor (Condon, Yin, Mayhew, Word, Wright, Shay and Rainey 2002). The results of the present studies show that melatonin acts synergistically via the MT2R/PLC/PKC signaling pathway to significantly increase sensitivity of myocytes to oxytocin and increase oxytocin-induced contractility in a dose dependent manner. In addition, we also investigated the potential effects of melatonin on expression of the gap junction protein, connexin43 (Cx43). Expression of Cx43 is known to increase late in human pregnancy thereby facilitating myometrial cell coupling and synchronization of uterine contraction (Smith 2007). Our data reveal that melatonin treatment of cultured myometrial cells increased both mRNA and protein levels of Cx43 via the MT2R signaling cascade. Taken together these studies point to a novel regulatory function of the circadian hormone melatonin in “gating” human myometrial activity. More specifically, our data provide a model system to investigate the mechanism through which melatonin interacts with the oxytocin pathway to promote uterine contractility and parturition.
Materials and Methods

**Cell Culture.** Telomerase immortalized myometrial cells (hTert) were maintained in DMEM/F12 (Mediatech) medium with 10% FBS (HyClone) with penicillin/streptomycin and gentamycin at 37°C and 5% CO₂. Cells were trypsinized at 90% confluence and plated in T175 cell culture flasks at a 1:5 dilution or 6 well plates at 20,000 cells per well. For pharmacological experiments cells were treated with iodo-melatonin (Tocris; Ellisville, MO, USA), oxytocin (Sigma-Aldrich; USA) or co-treated as described in the Results. Pharmacological inhibitors, 4-phenyl-2-propionamidotetralin (4P-PDOT), a MT2R specific antagonist, the general PKC inhibitor C1, and the phospholipase C inhibitor, U73122 (all from Tocris) were applied as a pretreatment 1 hr prior to application of I-MEL or oxytocin. After treatment the cells were trypsinized, pelleted, washed in phosphate-buffered saline, and frozen at -20°C until further analysis.

**Immunoblotting.** Frozen myometrial samples from pregnant women before and after the onset of labor and nonpregnant women were obtained from an NIH-funded tissue repository as described previously (Word, Tang and Kamm 1994). Term pregnancy without labor was defined as gestational week 38-40 with no sign of uterine contractions or cervical changes, while term pregnancy with true labor was defined as undergoing at least 3 spontaneous (not induced), regular uterine contractions in a 10 min interval in association with progressive increases in cervical effacement and dilation, or dilation of >4 cm. Uterine contractions in the absence of cervical change were considered “false labor”. Myometrial tissues from women with clinical or histological chorioamnionitis, rupture of membranes >12h, placenta previa, APLA syndrome, abnormal vaginal discharge, or positive myometrial cultures for β-strep, gonorrhea, trichomonas or syphilis were also excluded.

For in vitro investigations cultured hTERT cells were collected by trypsinization and gentle scraping. Cells were suspended in PBS and pelleted by centrifugation. Protein extraction was performed according to the method of Shearman and colleagues (Shearman, Sriram et al. 2000). Following electrophoretic separation on a 10% SDS-polyacrylamide gel, proteins were semi-dry blotted in buffer onto a Whatman PROTRAN Nitrocellulose membrane (Whatman; Dassel, Germany). Molecular size markers (Amersham) were included. The membrane was incubated for 60 min at 20°C in blocking buffer containing 5% milk powder before overnight incubation at 4°C with anti-OTR, anti-MT2R (Santa Cruz Biotechnology), anti-Cx43 antiserum (Millipore) or Anti-actin (Sigma) at a dilution of 1:1000 in
blocking buffer. Following washing in buffer (20 mM Tris, pH 7.6+137 M NaCl+0.05% Tween-20), the membrane was incubated at 20°C for 1h with a peroxidase-conjugated affinity purified goat anti-rabbit immunoglobulin (Sigma) in a 1:2000 dilution. Chemiluminescent signals were then detected with the Pierce ECL Western Blotting Substrate (Pierce; Rockford, IL, USA) using CL-X Posure film (Pierce).

Western blotting for phospho-myosin light chain kinase (Ser19) (Millipore) was performed with the following exceptions. Membranes were blocked with a 5% BSA/TBS solution for one hour. The primary antibody was diluted in TBS at a 1:500 dilution at 4°C overnight with shaking. The goat anti-rabbit immunoglobulin was diluted 1:2000 in a 5% milk/TBST solution. Densitometric analysis was performed using AIS Image Analysis software (Ontario, Canada) of images acquired with a digital camera. Criteria for assessment of samples as immunonegative was the absence of a band after 1 hr of exposure. Western blots were repeated a minimum of three times to insure reproducibility.

**Myometrial Cell Contractility Assay.** Myometrial cell contractility was assayed using a collagen disk retraction assay as described by Devost and Zingg (Devost and Zingg 2007) plating 10,000 hTERT cells per well. Samples were treated as described in the results and each treatment was performed in triplicate. Myometrial cell contractility was quantified by capturing images of the fixed collagen disks with a digital camera and analyzing for total area using AIS Image Analysis Software (Ontario, Canada). The results were normalized to the cell-free control sample areas and expressed as a percentage of untreated control area.

**Quantitative PCR.** Cellular total RNA was extracted with the RNEasy kit (QIAGEN; Valencia, CA, USA) according to the manufacturer’s protocol. The RNA concentration was measured with the Nanodrop photometer and then reverse transcribed to cDNA by means of the iScript reverse transcription system (Bio-Rad Laboratories; Foster City, CA, USA). Quantitative real-time PCR was performed on a Bio-Rad iCycler using iQ SYBR Green Supermix (Bio-Rad), together with 1 µl of sense and antisense primers (10 pmol/ µl) of the transcript of interest and 2 µl of template cDNA. The following thermal cycling parameters were used: initial heat activation of the DNA-polymerase was performed at 95° C for 5 min. Thereafter 40 cycles at 95° C (15 sec), 58° C (30 sec), and 72° C (30 sec) were run. After thermocycling the iCycler performs an automatic melting curve, which entails cooling to 55° C for 10 sec and then increasing temperatures in 0.2° C increments up to 90° C. This controls for primer-dimer formation and other nonspecific effects. Quantification of the data was achieved by the Bio-Rad iCycler software using a standard curve from a primer-specific dilution series for the PCR
product. Data were normalized against expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used for Cx43, and GAPDH quantification are as follows:

Cx43 forward 5’-ATG AGC AGT CTG CCT TTC GT-3’
Cx43 reverse 5’-TCT GCT TCA AGT GCA TGT CC-3’
GAPDH forward 5’-GTC TTC ACC ACC ATG GAG-3’
GAPDH reverse 5’-GTC ATG GAT AAC CTT GGC-3’.

**Lucifer Yellow Dye Migration Assay.** Lucifer yellow scrape loading assays were performed in accordance with the procedures of El-Fouly et al (El-Fouly, Trosko and Chang 1987). Cells were grown to confluency in 30 mM cell culture dishes, washed with warm phosphate buffered saline (PBS) and treated with 2mL of pre-warmed 0.05% lucifer yellow in PBS solution. The cells were then scraped by drawing a scalpel across the plate. After two minutes at room temperature the plates were washed three times with prewarmed PBS and returned to normal medium. The plates were then photographed at 10 minutes after scraping using a digital camera through a Zeiss Axiovert 40CFL Microscope at a 100x and 200x magnification. Each treatment condition was repeated three times to ensure reproducibility.

**Statistical Analyses.** Experiments were repeated a minimum of three times. Replicate values for each data point were averaged and differences statistically analyzed using a one-way ANOVA followed by the Bonferroni post hoc test (Prism; GraphPad, San Diego, CA, USA) with $P<0.05$ as the criterion level for significance. For testing the observed vs. expected percentages of MT2R-immunopositive myometrial samples, a Chi square test was employed with $P<0.01$ as criterion level.

**Results**

**Human Myometrium Is a Target For Melatonin.** Myometrial tissues express both isoforms of melatonin receptors at the transcript and ligand binding levels (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003; Sharkey and Olcese 2007). Using frozen myometrial samples, we conducted immunoblotting experiments and in some cases radioreceptor assays. Western blot analysis was inconclusive using commercial antibodies for MT1R but clearly confirmed the presence of MT2R in both hTERT cells and term pregnant myometrium (Fig. 3.1A). Remarkably, MT2R immunoreactive signals were detected in 89% of myometrial samples from women in labor compared with only 38% of
tissues from pregnant women before the onset of labor (Fig. 3.1B, C, D). Myometrial tissues from nonpregnant women were completely devoid of MT2R mRNA and immunoreactivity (data not shown). Additional immunoblotting experiments were also conducted to ascertain a potential correlation between OTR and MT2R expression. As shown in Fig. 3.1B, the MT2R-immunopositive myometria were all likewise OTR-immunopositive, whereas MT2R-negative tissues were typically OTR-negative or in a single case only very weakly OTR-positive. We also found that MT2R mRNA levels were elevated in tissues from laboring patients (Fig. 3.1E). These results point to a certain degree of regulated co-expression of MT2R and OTR in human myometrial tissue at the time of labor. Additionally, $^{125}$I-MEL binding was increased significantly in MT2R-immunopositive tissues (from laboring women) while melatonin binding was barely detectable in samples (from nonlaboring women) in which the MT2R was not detected by Western blot. The observed 247-fold difference in specific $^{125}$I-melatonin binding implicates the MT2R receptor as the primary site of melatonin binding in myometrial smooth muscle (data not shown).

**Melatonin Increases Oxytocin Sensitivity and Myometrial Contractility In Vitro Via the MT2R/Phospholipase C/Protein Kinase C Signaling Pathway.** Since melatonin has been reported to act via the MT2R to activate Gq mechanisms involving PLC and PKC (Masana and Dubocovich 2001), and these same signaling pathways are utilized by the OTR, we hypothesized that cross talk between the MT2R and OTR might modulate myometrial contractions induced by oxytocin. To investigate this possibility, we performed collagen gel retraction assays in accordance with the published protocols of Devost and Zingg (Devost and Zingg 2007). Co-treatment of cultured hTERT myometrial smooth muscle cells with 1nM I-MEL and 1 nM oxytocin resulted in a two-fold statistically significant increase in contractility compared to treatment with oxytocin alone. I-MEL acted to increase oxytocin-induced contractility in a dose dependent manner (Fig. 3.2A) as well as increasing the sensitivity to oxytocin (Fig. 3.2B). Pharmacological experiments were then conducted with the MT2R specific antagonist 4P-PDOT (10 nM) and the general PKC inhibitor, C1 (10 nM). Treatment with 4P-PDOT reduced the synergistic effect of I-MEL to levels corresponding to treatment with oxytocin alone. Treatment with C1 reduced myometrial cell contractility when cells were treated alone or in combination with oxytocin, I-MEL, or both (Fig. 3.2C). Treatment with the PLC inhibitor, U73122, completely abolished contractility in response to all treatments (data not shown). Western blot analysis for phosphorylated myosin light chain at Ser19 indicated that although I-MEL treatment alone resulted in modest increases in myosin
Fig. 3.1. Functional melatonin receptors in the human myometrium.  A, Western blot for the MT2R in hTERT cells, uteroleioma cells stably transfected with MT1R, MT2R, or neither (neg).  B and C, MT2R immunoreactivity in myometrial punches from pregnant nonlaboring patients (B) and patients in active labor (C).  Numbers in B and C represent individual donor samples.  D, Results from panels B and C in histogram form.  *, $P < 0.05$ by $\chi^2$ statistic.  E, MT2R mRNA expression in tissues from the same laboring (IL) and nonlaboring (NIL) patients as in B and C.  *, $P < 0.05$ relative to NIL.
light chain phosphorylation, oxytocin-induced increases in myosin light chain phosphorylation were increased dramatically by I-MEL (Fig. 3.2D). I-MEL-induced increases in myosin light chain phosphorylation were abolished by 4P-PDOT or C1 pretreatment (Fig. 3.2D) suggesting that MT2R and PKC are essential for I-MEL-induced increases in myosin light chain phosphorylation.

**Melatonin Increases Expression of the Gap Junction Protein Cx43 In Vitro Through a PKC-Dependent Pathway.** Cx43 expression has shown to be up-regulated in human myometrium at term and by PKC activation in cultured cells (Geimonen, Boylston, Royek and Andersen 1998). Initially we investigated the acute effects of I-MEL on Cx43 mRNA levels in cultured hTERT myocytes by quantitative PCR. Treatment with I-MEL (1 nM) resulted in significant, but transient increases in Cx43 mRNA after 4 hrs (Fig. 3.3A). Western blot analysis confirmed upregulation of Cx43 protein levels in I-MEL-treated cells (Fig. 3.3B). Co-treatment with 4P-PDOT abolished the effect of I-MEL on Cx43 expression as did treatment with U73122 or C1. These results indicate that induction of myometrial cell Cx43 by I-MEL involves MT2R receptor signaling via PLC and PKC (Fig. 3.3C).

To ascertain the effects of I-MEL in a physiological context, we investigated the effects of I-MEL on Cx43 mRNA expression in an 8 hr time course experiment. Cells were treated with I-MEL (1 nM) for 8 hrs to mimic a natural nocturnal phase of the light-dark cycle. Cx43 mRNA levels were assayed at times 0, 4, and 8 hrs. Cx43 mRNA expression was elevated at 4 hrs post I-MEL treatment but had returned to untreated levels 8 hrs after treatment. These data indicate that Cx43 mRNA expression may be nocturnally stimulated by physiological exposures to melatonin (Fig. 3.3A). The return of Cx43 mRNA expression to control levels after 8 hrs indicates other methods of regulation of Cx43 mRNA expression.

**Melatonin Increases Intercellular Connectivity In Vitro.** I-MEL-induced increases in Cx43 mRNA and protein led us to predict that intercellular communication would be increased due to the formation of additional gap junctions. To test this hypothesis we performed lucifer yellow dye migration assays on I-MEL-treated hTERT cells. Results of these experiments show that the cells I-MEL-pretreated plates show an increased amount of cell coupling, allowing for a greater spread of the lucifer yellow dye from the site of uptake and subsequent blocking of this effect by pretreatment with 4P-PDOT or C1 (Fig. 3.4). Treatment with 4P-PDOT or C1 resulted in dye migration comparable to control levels (data not shown).
Fig. 3.2. Effects of I-MEL on oxytocin-induced contractility. A, Contractility of hTERT myometrial cells treated with oxytocin (dark bars) or cotreated with 1 nM I-MEL (light bars). *, $P < 0.05$ relative to control values; **, $P < 0.05$ relative to all columns marked with single asterisk. B, Effects of treatment with 10 µM of the PKC inhibitor C1, or 10 nM of the MT2R-specific antagonist 4P-PDOT, on I-MEL induced contractility. *, $P < 0.05$ relative to control. C, Effect of 4P-PDOT pretreatment on the contractility of samples cotreated with I-MEL and oxytocin. **, $P < 0.05$ relative to oxytocin-treated and I-MEL/oxytocin/4P-PDOT-treated samples. *, $P < 0.05$ significantly elevated over controls. D, Effects of treatment with 1 nM of I-MEL (M) and/or oxytocin on the phosphorylation of the myosin light chain regulatory subunit and the effect of cotreatment with 10 nM 4P-PDOT (PD) or 10 µM C1. Un, Untreated control cells; S, size markers.
Fig. 3.3. Effects of I-MEL on the expression of the gap junction protein, Cx43. A, Effects of treatment with 1 nM I-MEL (filled bars) or control vehicle (open bars) on Cx43 mRNA levels in hTERT cells collected at 0, 4, and 8 h (mean ± SEM; n = 9). B, Effects of treatment with I-MEL (6 hrs) on Cx43 protein levels. Samples were loaded in duplicate. C, Effect of cotreatment with 4P-PDOT on Cx43 mRNA levels. * indicates a statistical difference relative to control (P<0.05).

Discussion

The molecular mechanisms leading to forceful uterine contractions of labor involve many interacting factors and regulatory pathways. Our data present a novel mechanism of interaction of the nocturnal brain hormone melatonin with the oxytocin-signaling pathway in human myometrial cells. These interactions likely occur at multiple points in the signaling cascade. Herein, we demonstrate that co-treatment with physiological concentrations of melatonin increases both basal- and oxytocin-induced contractility of myometrial cells and that this stimulatory effect can be blocked by the application of the
MT2R specific antagonist 4P-PDOT, the general PKC inhibitor C1, and the phospholipase C inhibitor U73122. From these data it can be concluded that melatonin acts synergistically via MT2R to activate PLC, which likely triggers an increase in intracellular calcium. This increase in intracellular Ca\(^{++}\), while presumably sufficient to only modestly augment basal contractility alone (see Fig. 3.2A), appears to

Fig. 3.4. Effects of I-MEL on gap junction communication in hTERT cells. The top row shows the cells under bright field, whereas the remaining figures were photographed under fluorescent light to demonstrate lucifer yellow dye spread within 10 min after scrape loading. Treatments include 1 nM I-MEL, or I-MEL after pretreatment of the cells with 10 nM 4P-PDOT or 10 μM C1. The bottom two images at higher magnification reveal lucifer yellow dye spread in greater detail.
greatly facilitate oxytocin-induced contractility by sensitizing myometrial cells to oxytocin. Kitazawa previously described a calcium sensitization phenomenon in which PKC resulted in increased force generation in permeabilized uterine strips clamped at a constant Ca\(^{++}\) environment (Kitazawa, Kobayashi, Horiuti, Somlyo and Somlyo 1989). Since melatonin has also been shown to activate PKC in multiple tissues (Sampson, Lupowitz, Braiman and Zisapel 2006), and we have previously reported that melatonin regulates OTR expression in myometrial cells through PKC (Sharkey and Olcese 2007), we suggest that melatonin not only increases sensitivity to oxytocin through Ca\(^{++}\)-sensitization, but also through modulated OTR expression. This agrees with our data and our model that melatonin acts synergistically to promote contractility in uterine smooth muscle cells.

Oxytocin-induced contractility is initiated by increased phosphorylation of myosin light chain (MLC) at Ser19, which facilitates conformational changes in myosin and interactions with actin. Investigation of melatonin treatment on MLC phosphorylation (Fig. 3.2D) showed changes in MLC which agree with our observations from the contractility studies. These data support the conclusion that the responsible receptor is a G\(_q\) coupled MT2R rather than a G\(_i\) coupled MT2R or MT1R (Masana and Dubocovich 2001) which could theoretically augment contractility by suppressing cAMP levels.

Although high levels of cAMP have been shown to relax myometrium (Smith 2007), the myometrium is somewhat refractory to cAMP-induced relaxation relative to other smooth muscles. Our previous work has shown that in this system, melatonin signaling is pertussis toxin-insensitive and PKC-dependent (Sharkey and Olcese 2007) and that melatonin treatment inhibits forskolin-induced cAMP accumulation only in myometrial samples from non-pregnant women (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003). Taken together, these findings further strengthen the notion that the synergistic effect of melatonin on oxytocin induced contractility is via MT2R.

MTR and OTR expression levels were similar in myometrial samples from non-laboring and laboring women. MT2R immunoreactive signals were detected in 89% of myometrial samples from women in labor but only 38% of samples from women not in labor (P <0.05). All samples that were immunopositive for MT2R were also OTR positive. This suggests that low MT2R levels during pregnancy may preclude the synergistic input of melatonin on contractility and thus serve to maintain myometrial quiescence. Activation of gene expression at labor also suggests the MT2R, like the OTR, is necessary to provide maximum uterine contractility during parturition, and that the nocturnal surges in melatonin temporally gate these contractions to occur primarily at night. To date the mechanism of regulation of gene expression for both the OTR and MTR are poorly understood, indeed these results
appear to be the first report of positive regulation of MT2R in humans. The similar regulation of both OTR and MT2R and the fact that both serum oxytocin (Gimpl and Fahrenholz 2001) and melatonin levels (Kivela, Kauppila, Leppaluoto and Vakkuri 1989; Kivela 1991) increase over the course of pregnancy support our hypothesis that melatonin acts synergistically to promote oxytocin-induced contractility in pregnant women at term.

Activation of myometrium late in gestation is associated with marked increases in gap junction proteins. The increase in gap junctions is thought to facilitate greater communication between myometrial cells to allow for synchronized contractions. Cx43 protein is the primary component of myometrial gap junctions in term myometrium (Geimonen, Boylston, Royek and Andersen 1998; Smith 2007). We show here that unlike oxytocin which to date has been shown to have little effect on Cx43 protein levels and gap junction formation in humans (Ciray, Backstrom and Ulmsten 1998), melatonin treatment also results in significant increases in Cx43 mRNA and protein levels, and that it leads to increased intercellular coupling between uterine myocytes. These data indicate that melatonin acts to promote contractility not only through direct action on the contractile machinery, but also acts to facilitate synchronized contractions via increased gap junction-mediated intercellular communication. Both actions would promote enhanced nocturnal uterine contractility at term.

In conclusion we propose the model shown in Fig. 3.5 to explain the action of melatonin in promoting nocturnal contractility during labor. Release of melatonin from the pineal gland into the circulation at night leads to binding of melatonin to myometrial MT2Rs. Bound melatonin activates PLC which generates IP3 and increases intracellular calcium thus activating MLCK. The phosphorylation of MLC results in increased contractility and enhanced sensitivity to OTR-mediated signals. Additionally, the DAG released by melatonin binding to the MT2R activates PKC, which has been shown to act via c-fos and c-jun to increase Cx43 expression (Mitchell and Lye 2001). It is important to note that modest increases in contractility by melatonin alone were always detected. However, the greatest increases in contractility were achieved when both melatonin and oxytocin were provided concomitantly. We propose that this neuroendocrine synergy plays a key role in the increase in births observed in the late evening and early morning in humans.

The results of this study point to melatonin playing a pro-contractile role in human myometrial physiology during pregnancy. Combined with our previous observations regarding the action of melatonin on OTR mRNA expression it appears that melatonin and oxytocin signaling are very similar in the myometrium. These data provide new insights into the mechanisms underlying the timing of birth
and regulation of the contractile machinery in the myometrium and reveal a novel physiological mechanism whose further characterization may serve in the development of new pharmacological strategies for the management of preterm and/or delayed parturition.

Fig. 3.5. Proposed model for the synergy between melatonin and oxytocin on nocturnal myometrial contractility in the laboring pregnant uterus. Melatonin acts synergistically with oxytocin to increase PLC activity and associated signaling mechanisms, thereby enhancing myometrial contractility and gap junction-associated intercellular communication. DAG, Diacylglycerol.
CHAPTER 4

MELATONIN SENSITIZES HUMAN MYOMETRIAL CELLS IN A PKCα/ERK-DEPENDENT MANNER

Introduction

The initiation of parturition is a complex multifactorial process. Our previous work investigating the role of melatonin in human myometrial physiology (Sharkey and Olcese 2007; Sharkey, Puttaramu, Word and Olcese 2009) indicated that melatonin signaling plays a significant role in the inhibition of oxytocin receptor (OTR) mRNA transcription, increases intercellular communication via gap junctions and synergistically enhances oxytocin- induced contractility. In all of these processes melatonin signaling occurred via the melatonin type 2 receptor (MT2R) and protein kinase C (PKC).

MT2R and OTR are both G-protein-coupled receptors that have been shown to couple with the G_{q/11} alpha subunit (Gimpl and Fahrenholz 2001; Masana and Dubocovich 2001). In the myometrium oxytocin has been shown to activate phospholipase C (PLC) leading to the generation of inositol triphosphate (IP_3) which mediates intracellular calcium release. This results in calmodulin-dependent phosphorylation of the myosin light chain (MLC) by myosin light chain kinase (MLCK) and activation of PKC (Gimpl and Fahrenholz 2001). Oxytocin has also been shown to activate the Rho kinase signaling cascade, leading to inhibition of myosin light chain phosphatase (MLCP) (Smith 2007). The similarities between the melatonin and oxytocin signaling pathways provide multiple points for potential cross-talk and potentiation which could lead to increased MLC phosphorylation and increased contractility.

In addition to modulation of the phosphorylation of MLC, the availability of actin for myosin binding is regulated by caldesmon and calponin (Morgan and Gangopadhyay 2001). The phosphorylation of caldesmon by the extracellular-regulated kinases Erk 1/2 reduces the affinity of caldesmon for actin, which makes actin more readily available for myosin binding during the crossbridge cycle. This effect is modulated by PKC activity and presents a mechanism for sensitization of myometrial cells to pro-contractile signals.
In the present studies we sought to obtain a better understanding of the specific details of the signaling pathway that facilitates melatonin effects on human myometrial smooth muscle cells. We focused on similarities between the MT2R and OTR signaling pathways as these have been proposed to underlie the synergy of melatonin and oxytocin action on myometrial contractions (Sharkey, Puttaramu, Word and Olcese 2009). Our experiments identify the specific PKC isoform responsible for melatonin effects on contractility in the human myometrium and show that melatonin increases the availability of myometrial actin for the myosin crossbridge cycle through modulation of caldesmon phosphorylation.

Materials and Methods

Cell Culture. Telomerase immortalized myometrial cells (hTert) were maintained in DMEM/F12 (Mediatech) medium with 10% Fetal II Plus serum (Valley Biomedical) with penicillin/streptomycin and gentamycin at 37°C and 5% CO₂. Cells were trypsinized at 90% confluency and plated in T175 cell culture flasks at a 1:5 dilution or 6 well plates at 20,000 cells per well. For pharmacological experiments cells were treated with iodo-melatonin (1 nM; Tocris; USA), oxytocin (1 nM; Sigma-Aldrich; USA) or co-treated as described in the Results section. The pharmacological inhibitors, 4-phenyl-2-propionamidotetralin (4P-PDOT; 10 nM - an MT2R specific antagonist), PD 98059 (10 μM - a specific inhibitor of MAP kinase kinase), Y27632 (10 μM - a Rho kinase inhibitor), microcystin-LW (1 μM - an inhibitor of the myosin light chain phosphatase), C1 (10 μM - an inhibitor of PKC), or the phospholipase C inhibitor, U73122 (1 μM) (all obtained from Tocris) were applied as a pretreatment 1 hr prior to application of iodo-melatonin (I-MEL) or oxytocin. I-MEL was used due to its increased stability in cell culture. After treatment the cells were trypsinized, pelleted, washed in phosphate-buffered saline, and frozen at -20°C until further analysis.

Immunoblotting and Immunoprecipitation. For in vitro investigations cultured hTERT cells were collected by trypsinization and gentle scraping. Cells were suspended in PBS and pelleted by centrifugation. Protein extraction was performed according to the method of Shearman and colleagues (Shearman, Sriram et al. 2000). Following electrophoretic separation on a 10% SDS-polyacrylamide gel, proteins were semi-dry blotted in buffer onto a Whatman PROTRAN Nitrocellulose membrane (Whatman; Germany). Molecular size markers (BioRad) were included. The membrane was incubated for 60 min at 20°C in blocking buffer containing 5% milk powder before overnight incubation at 4°C.
with phospho-PKCα/β (Cell Signaling Technologies) or anti-actin (Sigma) at a dilution of 1:1000 in blocking buffer. Following washing in buffer (20 mM Tris, pH 7.6 + 137 M NaCl + 0.05% Tween-20), the membrane was incubated at 20°C for 1h with a peroxidase-conjugated affinity purified goat anti-rabbit immunoglobulin (Sigma) in a 1:2000 dilution. Chemiluminescent signals were then detected with the Pierce ECL Western Blotting Substrate (Pierce; USA) using CL-X Posure film (Pierce).

Western blotting for phospho-caldesmon (AbCam), p42/44 MAPK (phospho-Erk 1/2), phospho-PKC α/β II (Ser638/641), delta (Thr505), δ/θ (Ser643/676), θ (Thr538), ζ/λ (Thr410/403), phospho-PKD/PKC μ (Ser916), phospho-myosin light chain 2 (Ser19) and myosin light chain 2 (Cell Signaling) was performed in accordance with manufacturer’s protocol at a dilution of 1:500. The goat anti-rabbit immunoglobulin was diluted 1:2000 in a 5% milk/TBST solution. Densitometric analysis was performed using AIS Image Analysis software (Ontario, Canada) of images acquired with a digital camera. Western blots were repeated a minimum of three times to insure reproducibility.

Immunoprecipitation was performed as follows. Protein G Sepharose beads were equilibrated in protein extraction buffer for 1 hr at room temperature. Beads were then washed three times for 20 min in 500 μL protein extraction buffer. Rabbit anti-myosin light chain 2 antibody (Cell Signaling) was linked to Protein G Sepharose beads (Amersham Biosciences) at a 1:50 dilution for 1 hr at room temperature. Sample protein extracts were pre-cleared with 10 μL of equilibrated Protein G Sepharose beads for 20 minutes. Samples were then spun down for 10s at 3,000 rpm. Samples were transferred to antibody linked beads and incubated overnight at 4°C. Samples were spun at 3000 rpm for 10s and supernatant transferred to tubes as immunodepleted samples. Beads were then washed six times in 1 mL protein extraction buffer (10s spin at 3000 rpm). Protein loading buffer was then added to samples for western blotting and the samples were denatured at 95°C for 5 minutes, then rocked at room temperature for 5 minutes. Samples were spun for 1 minute at 12000 rpm and supernatant was used in western blot analysis for MLC or pMLC.
**Protein Kinase C Activity Assay.** hTert cells were cultured and treated in T75 plates, harvested, extracted for protein, and immunoprecipitated for PKCα as described for Western blotting with the following exception. Samples for PKC activity were assayed after the final 1mL wash and were not denatured. PKC activity assay was performed in accordance with manufacturer’s protocol (Millipore) but the PKC activator cocktail was not added. ³²P-ATP gamma (3000Ci/µmol) was purchased from Perkin-Elmer.

**Myometrial Cell Contractility Assay.** Myometrial cell contractility was assayed using a collagen disk retraction assay as described by Devost and Zingg (Devost and Zingg 2007) plating 10,000 hTERT cells per well. Samples were treated as described in the Results and each treatment was performed in triplicate. Myometrial cell contractility was quantified by capturing images of the fixed collagen disks with a digital camera and analyzing for total area using AIS Image Analysis Software (Ontario, Canada). The results were normalized to the cell-free control sample areas and expressed as a percentage of untreated control area.

**Inositol Triphosphate Turnover Assay.** Cells were cultured for at least 24h. Following a brief wash in fresh medium, the cells were then incubated overnight with myo- [2-³H]–inositol (10 µCi/ml) (Perkin-Elmer) to label cell inositol phospholipids (IP). The cells were rinsed three times with physiological saline to remove the unincorporated radioactivity and equilibrated in medium for 60 min. The cells were treated for 15 min with 10 mM lithium chloride (final conc.) prior to stimulation to inhibit inositol-1-monophosphatase activity. The cells were then treated for 30 min with I-MEL, oxytocin or both in LiCl at 37°C. The cells were lysed and cellular proteins precipitated by addition of 1 ml of 95°C water, followed by freezing and thawing and scraping. The extracts were then centrifuged 2 min at high speed. The supernatant was used for IP determination and pellet for ³H incorporation into membrane lipids. The ³H–IPs in the supernatant were purified by anion exchange column chromatography on Dowex-1 columns (AG 1-X8, formate form; BIORAD).The cell extracts were applied to the top of columns without disturbing resin and flow-through was discarded. Sample tubes were rinsed three times with 0.5 ml distilled water and the rinses were added to the columns. The columns were washed with 8 ml of room temperature 60 mM ammonium formate/ 5 mM Na₂B₄O₇. The sample fractions were collected into scintillation vials as follows. The columns were eluted with two successive aliquots of each elution buffer and then washed with 8mL of the previous elution buffer prior to application of successive elution buffers. The buffer compositions were IP₁: 200 mM ammonium
formate/100 mM formic acid, IP₂: 400 mM ammonium formate/100 mM formic acid, IP₃: 1M ammonium formate/100 mM formic acid.

The cell pellets were processed by adding 0.5 mL of lipid clearing solution (500 mM KCl / 5 mM myoinositol) and 0.5 ml chloroform. The pellets were then vortexed heavily, then shaken vigorously for 10 min at RT and centrifuged for 20 sec at 5000xg. Two-hundred µl of the organic phase were transferred to scintillation vials and evaporated to dryness at RT. The eluted radioactivity in each sample was quantified by adding 5 mL scintillation fluid (Fisher Scientific) and then counting in a liquid scintillation counter. The data are reported as cpm/well. Results are expressed as IP₃ turnover (Huckle and Conn 1987) for IP₃ and total IP (IP₁ + IP₂ + IP₃) since IP₁ and IP₂ are dependent on, and highly correlated with, IP₃ formation (Flint, Leat, Sheldrick and Stewart 1986; Huckle and Conn 1987).

Statistical Analyses. Experiments were repeated a minimum of three times. Replicate values for each data point were averaged, and differences were statistically analyzed using a one-way ANOVA followed by the Bonferroni post hoc test (Prism; GraphPad, San Diego, CA) with P < 0.05 as the criterion level for significance.

Results

Melatonin Effects on Contractility and MLC Phosphorylation Do Not Involve Rho Kinase Signaling or Inhibition of MLCP. Rho kinase activation has been shown to increase myometrial contractility through inhibition of myosin light chain phosphatase (Kim, Appel, Vetterkind, Gangopadhyay and Morgan 2008). We hypothesized that melatonin could act to inhibit MLCP activity via the Rho kinase signaling pathway promoting contractility. To determine melatonin’s effect on the Rho kinase pathway we performed collagen retraction assays (Sharkey, Puttaramu, Word and Olcese 2009). Cells were treated with I-MEL, oxytocin, and/or the Rho kinase inhibitor Y27632. Treatment with Y27632 reduced basal and oxytocin-induced contractility but had no significant effect on I-MEL-induced contractility. Y27632 treatment of samples co-treated with oxytocin and I-MEL showed a modest inhibition of contractility down to levels seen in samples treated with I-MEL alone, I-MEL and Y27632, and oxytocin and Y27632 (Fig. 4.1A). This effect suggests that contractility due to I-MEL does not involve Rho kinase signaling.
Fig. 4.1. Dependence of melatonin actions on the Rho kinase signaling pathway and myosin light chain phosphatase (MLCP) activity. A) Gel contraction assay using human telomerase-immortalized hTERT myometrial smooth muscle cells. Cells were treated as noted at the following concentrations I-MEL (1 nM), oxytocin (1 nM), and the Rho kinase inhibitor Y27632 (10 μM). ‘a’ denotes P < 0.05 versus control, ‘b’ P < 0.05 versus control and I-MEL-treated cells, ‘c’ P < 0.05 versus control, I-MEL and oxytocin-treated cells, ‘d’ P < 0.05 versus control, I-MEL, oxytocin and I-MEL/oxytocin-cotreated cells. B) Western blot for phospho-myosin light chain 20. hTert cells were treated with 1 nM I-MEL, 1 μM microcystin-LW (an MLCP inhibitor) or in combination. Microcystin was applied 30 minutes prior to treatment with I-MEL.
We next performed Western blots for phospho-MLC20 (pMLC). MLC is phosphorylated by myosin light chain kinase at two sites, Thr$^{18}$ and Ser$^{19}$, both of which are detected by our pMLC antibody. To confirm that I-MEL-induced contractility is not due to inhibition of MLCP, cells were pre-treated with the MLCP inhibitor microcystin-LW and then treated with I-MEL. Only the extracts from cells treated with both I-MEL and microcystin showed an increase in MLC phosphorylation levels, specifically the appearance of a second band corresponding to Thr$^{18}$/Ser$^{19}$ phosphorylated MLC, which is absent in all other treatment groups. Neither inhibition of the phosphatase with microcystin alone nor treatment with I-MEL alone resulted in the multiple MLC phosphorylation pattern. The absence of the second band in samples treated with I-MEL alone indicates that MLCP activity was unaffected by I-MEL (Fig. 4.1B), thus suggesting that I-MEL’s action is via increased kinase activity rather than MLCP inhibition.

Melatonin Activates PKCα in Human Myometrial Smooth Muscle Cells. Next we explored the IP$_3$/DAG signaling mechanisms that are utilized by melatonin. Our previous work has shown that melatonin signaling in the human myometrium is mediated by PLC and PKC (Sharkey and Olcese 2007; Sharkey, Puttaramu, Word and Olcese 2009). To specifically dissect this signaling mechanism, we used IP$_3$ turnover assays, immunoprecipitation and western blotting to determine the activation levels of the pathway and identify the specific PKC isoform. Cells were treated with I-MEL, oxytocin, and the inhibitors 4P-PDOT and U73162 (see Methods). IP$_3$ turnover was significantly increased in samples treated with I-MEL (179% of control levels), oxytocin (238% of control levels), and after I-MEL/oxytocin co-treatment (360% of control levels). Pretreatment with 4P-PDOT reduced IP$_3$ turnover in I-MEL/oxytocin-cotreated cells to oxytocin levels, and pretreatment with U73122 reduced IP$_3$ levels to those of control (Fig. 4.2A). Total inositol phosphate turnover displayed the same trend confirming that I-MEL treatment augments PLC activity (Fig. 4.2B). Subsequent standard fura-2 calcium imaging experiments, however, showed no detectable increase in intracellular calcium due to I-MEL treatment, whereas treatment with oxytocin showed a dramatic and rapid increase in intracellular calcium (data not shown), suggesting that I-MEL acts to sensitize myometrial cells to subsequent pro-contractile signals rather than significantly elevating calcium itself.
Fig. 4.2. Melatonin actions on IP₃ and total IP turnover. A) IP₃ turnover after treatment with I-MEL (1 nM), oxytocin (1 nM), the MT2R antagonist 4P-PDOT (10 nM), and the PLC inhibitor U73122 (1 μM). ‘a’ denotes P < 0.05 versus control, ‘b’ P < 0.05 versus control and I-MEL-treated cells, ‘c’ P < 0.05 versus control, I-MEL and oxytocin-treated cells, ‘d’ P < 0.05 versus all treatments, except ‘b’. B) Total IP turnover after treatments. ‘a’ denotes P < 0.05 versus control, ‘b’ P < 0.05 versus control, oxytocin and I-MEL-treated cells, ‘c’ P < 0.05 versus I-MEL/oxytocin co-treatment.

Western blots for a panel of phospho-PKC isoforms (see Methods) were performed to define those activated by treatment with I-MEL. I-MEL treatment resulted in an increase only in phospho-PKCα/β. To specifically identify which of these two isoforms was activated, samples were immunoprecipitated using an antibody against PKCα and western blotted with the phospho-PKCα/β antibody. This produced a band in the immunoprecipitated samples (Fig. 4.3A). Western blots on the immunodepleted supernatant (i.e. containing the PKCβ isoform) showed no detectable phospho-PKCα/β
Fig. 4.3. Melatonin activates PKCα. A) The hTert cells were treated with 1 nM I-MEL +/- 10 nM 4P-PDOT. Treated samples were immunoprecipitated with anti-PKCα (right panel) and then western blots were performed for phospho-PKCα/β. B) PKC activity assays were also performed on immunoprecipitated (black bars) as well as on immunodepleted (grey bars) samples for confirmation of the western blot results. ‘a’ denotes P < 0.05 versus control.

signal (data not shown). Treatment with the MT2R antagonist 4P-PDOT reduced phospho-PKC levels to control levels (Fig. 4.3A). To further confirm this result we performed PKC enzyme assays. $^{32}$P incorporation into the PKC enzyme substrate was significantly increased in I-MEL-treated samples (Fig.
4.3B) and in samples immunoprecipitated for PKCα. Activity was absent in immunodepleted samples (i.e. containing the PKCβ isoform only), confirming PKCα activation by I-MEL.

**Melatonin Treatment Increases ERK1/2 and Caldesmon Phosphorylation.** Another regulator of smooth muscle contractility is caldesmon. Phosphorylation of caldesmon results in a decrease in its affinity for actin, allowing for increased actin/myosin binding (Morgan and Gangopadhyay 2001). We hypothesized that PKCα activation by I-MEL would result in caldesmon phosphorylation in an ERK1/2-dependent manner. Western blots for phospho-caldesmon and p42/44 MAPK (pERK1/2) confirmed that after 15 minutes I-MEL increases both ERK1/2 and caldesmon phosphorylation. This action could be abolished by pretreatment of cells with the MEK inhibitor PD98059, and by the general PKC inhibitor C1 (Fig. 4.4).

![Western blots for phospho-Erk 1/2 and phospho-caldesmon](image)

**Fig. 4.4.** Melatonin activates ERK1/2-dependent phosphorylation of caldesmon. The figure shows representative western blots for phospho-Erk 1/2 and phospho-caldesmon in cell extracts collected after 15 minute treatments with I-MEL (1 nM), oxytocin (1 nM). The middle two lanes indicate samples pretreated with the MEK inhibitor PD98059 (10 μM) or the PKC inhibitor C1 (10 μM) before I-MEL addition.
Discussion

Continuous monitoring of normal uterine contractile activity during late term pregnancy in humans has shown increased frequency between the hours of 8:30 PM and 2:00AM (Zahn and Hattensperger 1993). Studies on the timing of human labor onset and deliveries show that the initiation of labor peaks between the hours of 24:00 and 05:00, regardless of gestational age (Seron-Ferre, Ducsay and Valenzuela 1993). Little is known regarding the physiological mechanisms underlying the timing of birth to the night phase. Melatonin, the molecular messenger of circadian night, peaks during the night. Our previous work has identified PKC-mediated melatonin signaling as playing a role in regulation of myometrial contractility and gap junction-mediated intercellular communication (Sharkey and Olcese 2007; Sharkey, Puttaramu, Word and Olcese 2009).

The purpose of the present investigations was to more fully elucidate the melatonin signaling pathway leading to its effects on contractility in human myometrial cells. Our previous work implicated involvement of the PLC/IP\(_3\) signaling pathway and involvement of PKC as the basis for the synergistic effect of melatonin on oxytocin-induced contractility. This synergistic effect could be accomplished theoretically by multiple mechanisms which regulate the activities of the MLCK, MLCP, and actin availability for myosin crossbridge cycling (Morgan and Gangopadhyay 2001; Somlyo and Somlyo 2003; Sward, Mita, Wilson, Deng, Susnjar and Walsh 2003). Herein we followed the melatonin signaling cascade from IP\(_3\)/DAG generation through the signaling cascade to phosphorylation of caldesmon. I-MEL treatment resulted in an increase in IP\(_3\) and total IP turnover in myometrial cells treated with I-MEL alone and in cells co-treated with I-MEL + oxytocin (Fig. 4.2A, B). As expected the increase in I-MEL+oxytocin co-treated samples was abolished by treatment with the MT2R specific antagonist 4P-PDOT. This agrees fully with our previous data indicating that I-MEL effects on contractility are mediated through MT2R receptor. Unexpectedly, we were not able to detect an increase in intracellular Ca\(^{2+}\) in cells treated with I-MEL alone. Treatment with oxytocin did, however produce a strong increase in intracellular calcium (data not shown). Melatonin has been shown to synergistically augment intracellular calcium signaling in MCF-7 cells without a detectable effect on calcium levels (Dai, Inscho, Yuan and Hill 2002). Also, melatonin has been shown to augment norepinephrine-induced vasoconstriction in a calcium-independent manner in the rat (Vandeputte, Giummelly, Atkinson, Delagrange, Scalbert and Capdeville-Atkinson 2001).
PKC has long been implicated as an important factor in the uterine contractile machinery in pregnancy. PKCα has been reported to be expressed in the human myometrium at term (Hurd, Fomin, Natarajan, Brown, Bigsby and Singh 2000) and to have a direct role in myometrial contractility (Fomin, Kronbergs, Gunst, Tang, Simirskii, Hoffman and Duncan 2009). Our data show that I-MEL increases PKCα phosphorylation as well as PKC enzyme activity. The lack of PKC activity in the immunodepleted fraction of the samples (Fig. 4.3B) points to a very specific activation of only PKCα (as opposed to PKCα and β activation) by I-MEL. PKCα has been shown to phosphorylate Raf (Kolch, Heidecker, Kochs, Hummel, Vahidi, Mischak, Finkenzeller, Marme and Rapp 1993), which initiates the MAPK signaling cascade (MEK and subsequently ERK1/2 phosphorylation). This can then subsequently phosphorylate targets such as caldesmon and MLCK (Kim, Appel, Vetterkind, Gangopadhyay and Morgan 2008). We followed the PKC cascade to ERK1/2 and caldesmon. I-MEL increased phosphorylation of both caldesmon and ERK1/2 and, importantly, this effect was abolished by inhibition of MEK and PKC (Fig. 4.4). Samples treated with oxytocin alone showed an increase in caldesmon phosphorylation in the absence of a detected elevation in phosphorylated Erk1/2. Devost and colleagues have reported rapid activation and inactivation of Erk1/2 as well as activation of p38MAPK by oxytocin in the hTert cell line (Devost, Carrier and Zingg 2008). Both Erk1/2 and p38MAPK have been reported to phosphorylate caldesmon (Li, Je, Malek and Morgan 2003; Kim, Kim, Bae, Cho, Kwon, Jung, Park and Ahn 2004).

In conclusion the present studies expand on our previous model for pro-contractile melatonin signaling in human myocytes (Fig. 4.5). We confirmed melatonin activation of PLC via the MT2R by showing increased IP₃ turnover. Additionally, we identified PKCα as the mediator of melatonin’s downstream effects. PKCα is necessary for activation of the ERK1/2 signaling cascade. ERK1/2 activities include phosphorylation of caldesmon, which has been shown to be a negative regulator of smooth muscle contractility by binding actin and thus preventing myosin crossbridge formation. Phosphorylation of caldesmon greatly reduces its affinity for actin thus making actin more readily available for myosin binding. This mechanism has been proposed as a mechanism of sensitization in smooth muscle (Kim, Appel, Vetterkind, Gangopadhyay and Morgan 2008). Combined with our previous observations showing melatonin enhancement of oxytocin-induced contractility (Sharkey, Puttaramu, Word and Olcese 2009), the present data provide a mechanism by which melatonin can sensitize the human myometrium to pro-contractile oxytocin signaling. This mechanism explains our previous observation that melatonin sensitzes human myometrial cells to the effects of oxytocin,
thereby serving to increase myometrial contractility. Sensitization of the myometrium to subsequent pro-contractile signals in vivo would provide a mechanism underlying the reported increase in nocturnal uterine contractility and likelihood of nocturnal parturition in late term pregnancy. These data provide new insights into the mechanisms underlying the timing of birth and regulation of the contractile machinery in the human myometrium and reveal a novel physiological mechanism for melatonin actions whose further characterization may serve in the development of new pharmacological strategies for the management of preterm and/or delayed parturition.

Fig. 4.5. Proposed model for myometrial sensitization by melatonin. Melatonin activates PLC, which generates DAG and subsequent activation of PKCα. This initiates the Raf/MEK/Erk1/2 signaling cascade leading to phosphorylation of caldesmon (hCaD) which is bound to actin. In this state, hCaD has less affinity for actin causing it to release the actin strand making actin more available for binding to myosin and crossbridge cycling, thereby enhancing myometrial contractions.
CHAPTER 5
CONCLUSIONS

Much of the research on human parturition has focused on the gestational timing of birth. This is due in large part to the health and economic burdens associated with pre-term birth (Smith 2007). To date very little research has been performed investigating the mechanisms underlying the nocturnal prevalence of labor onset in the human. Our research proposes a role for the circadian factor, melatonin, in the circadian timing of human parturition.

Melatonin’s role in the reproductive system has been studied since its discovery in 1958. Since then melatonin has been shown to affect the reproductive system on multiple levels. In seasonal breeding species, the day length encoded by melatonin has been shown to regulate the estrus cycle and testicular function (Gunduz and Stetson 1994; Benson and McAsey 1998). In the female human and rat, melatonin modulates sex steroid secretion and also modulates the function of the hypothalamus-pituitary-gonad axis, specifically the release of gonadotropin releasing hormone and prolactin. In the rat and sheep, serum melatonin levels have been shown to maintain a circadian oscillation during pregnancy (Tamura, Nakamura, Terron, Flores, Manchester, Tan, Sugino and Reiter 2008). Nocturnal serum melatonin levels have also been reported to rise over the course of pregnancy exhibiting significantly higher levels after 32 weeks of gestation followed by a return to non-pregnant levels two days post partum (Tamura, Nakamura, Terron, Flores, Manchester, Tan, Sugino and Reiter 2008). Urine and amniotic fluid melatonin levels also have been shown to peak around the time of delivery. A strong case for a role for melatonin in the circadian timing of parturition is in the rat. Pinealectomized female rats lose the circadian timing of parturition, giving birth across the 24 hr cycle. Diurnal parturition was restored only when melatonin was provided during the normal night time period. Sham and continuous melatonin had no restorative effect (Tamura, Nakamura, Terron, Flores, Manchester, Tan, Sugino and Reiter 2008).

Regarding parturition in humans, the role of melatonin outside of its protective anti-oxidant capacity in the event of fetal hypoxia and preeclampsia (Reiter, Tan, Manchester, Paredes, Mayo and Sainz 2009) in the physiology of the pregnant human myometrium is largely unclear. The serum profile of melatonin, the importance for maintaining time of birth in the rat, synergistic effects on
norepinephrine-induced contractility (Martensson, Andersson and Berg 1996), and identification of both melatonin receptor subtypes in the human myometrium (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003) provide strong impetus to investigate melatonin’s effects on human myometrial physiology. Also of interest was that comparison of melatonin receptor mRNA expression in pregnant versus non-pregnant myometrial tissues showed a down regulation in RNA levels in pregnant tissues concurrent with an increase in oxytocin receptor mRNA levels (Schlabritz-Loutsevitch, Hellner, Middendorf, Muller and Olcese 2003). These observations lead us to hypothesize that melatonin signaling inhibited oxytocin receptor mRNA levels.

The purpose of our first study was to examine the effects of melatonin on expression of the oxytocin receptor in human myometrial cells. We found that I-MEL treatment inhibits oxytocin receptor transcription through Phospholipase C and Protein Kinase C. This would be the first of several similarities we observed between melatonin and oxytocin signaling. Treatment with the transcriptional inhibitor actinomycin and I-MEL resulted in no change in the half life of the mRNA message (Fig. 2.4). Treatment with the MT2R antagonist, 4P-PDOT, abolished I-MEL induced OTR inhibition. I-MEL inhibition of OTR mRNA expression was insensitive to pertussis toxin. These observations implicate the MT2R as the activated receptor responsible for transcriptional repression of OTR.

MT2R and OTR both can bind the G<sub>q</sub> α subunit and thus activate similar downstream signaling pathways providing a potential for cross talk between the two signaling pathways. Oxytocin, a powerful uterotonic agent, has been shown to inhibit the transcription of its own receptor (Phaneuf, Asboth, Carrasco, Linares, Kimura, Harris and Bernal 1998; Phaneuf, Rodriguez Linares, TambyRaja, MacKenzie and Lopez Bernal 2000). The interplay between these two signaling pathways leads us to next explore the effect of melatonin on myometrial contractility. OTR has been shown to be desensitized in both homologous and heterologous manners. For example, IL-1α (Soloff, Izban, Cook, Jeng and Mifflin 2006) and IL-1β (Rauk and Friebe-Hoffman 2000), have both been shown to inhibit OTR mRNA levels. Both these receptors are Toll-like receptors that activate the IP<sub>3</sub> signaling cascade.

Our second study focused on melatonin’s effects on oxytocin-induced contractility and the expression of the contractile associated protein, connexin 43. Our results showed melatonin increased basal myometrial contractility and acted synergistically to increase oxytocin induced contractility. We observed increase in phosphorylated myosin light chain levels were observed in samples treated with I-MEL and a synergistic increase in phospho-myosin light chain levels in I-MEL/oxytocin co-treated samples which is in agreement with our contractility data. We also observed that melatonin treatment
acted to sensitize the hTert cells to oxytocin (Fig. 3.2). Melatonin’s contractile effects were mediated by MT2R and abolished by treatment with PKC inhibitors.

The observation of a pro-contractile role for melatonin lead us to investigate the regulation of MT2R over the gestational timeline. OTR is maintained at low levels over the course of gestation and is dramatically up-regulated prior to labor (Gimpl and Fahrenholz 2001). MT2R was found to follow a similar expression profile in myometrial biopsy samples from the lower segment of the uterus. Radioreceptor binding assays showed $I^{125}$-MEL binding is 247 times higher in MT2R immunopositive myometrial samples as compared to MT2R-immunonegative samples. In these tissues mRNA transcripts for both MT1R and MT2R were present. The lack of a reliable commercial antibody against MT1R prevented us from analyzing for MT1R protein. The negligible specific binding of $I^{125}$-MEL in MT2R immunonegative samples would indicate that both receptors were kept at low levels simultaneously in these tissues. This finding is significant in that if melatonin is playing a pro-contractile role in the uterus, it would be critical to maintain melatonin receptor levels low to reduce pro-contractile stimuli to maintain uterine quiescence during gestation. MT1R signaling has been shown to inhibit adenylate cyclase activity thus reducing intracellular cyclic AMP levels (Masana and Dubocovich 2001) which would serve as a pro-contractile stimulus as well (Smith 2007). It is important to note that all myometrial tissue samples were obtained from the lower uterine segment. There is evidence of differential gene expression between the upper and lower uterine segments. For example, Sparey et al show differential expression of cyclooxygenase 1 and 2 and connexin 43 between uterine segments (Sparey, Robson, Bailey, Lyall and Nicholas Europe-Finner 1999). These data point to the importance of examining melatonin receptor and oxytocin receptor expression in the upper uterine segment in the future.

Another important factor in successful parturition is the coordination of uterine contractions. The gap junction protein, connexin 43, has been shown to be dramatically up-regulated in both uterine segments in labor (Sparey, Robson, Bailey, Lyall and Nicholas Europe-Finner 1999), thus facilitating increased intercellular connectivity and contractile coordination in the myometrium (Smith 2007). We found that I-MEL treatment increases connexin 43 mRNA and protein levels and increased intercellular connectivity in hTert cells (Fig. 3.4). Increased intercellular connectivity would allow for the passage of ions and other second messengers between the cells facilitating coordinated contractions. This effect was also found to be mediated by PKC.
Melatonin’s reliance on PKC mediated signaling to facilitate its effects on contractility and OTR and connexin 43 expression led us to attempt to identify the specific PKC isoform that is activated by the melatonin signaling cascade. Western blot screening for activated PKC isoforms followed by immunoprecipitation for PKCα identified PKCα as the isoform activated in the melatonin signaling cascade. PKC activity assays on the immunoprecipitated and immunodepleted fraction confirmed that PKC activity was confined to the PKCα-immunoprecipitated fraction.

Sensitization of the myometrium to pro-contractile signals can be accomplished through multiple pathways, including inositol triphosphate mediated calcium release, Rho kinase mediated inhibition of myosin light chain phosphatase, increased myosin light chain kinase activity or mechanisms regulating actin availability for myosin binding and subsequent cross bridge cycling. Melatonin’s effects on contractility were not affected by inhibition of Rho kinase. Additionally, inhibition of myosin light chain phosphatase and subsequent treatment with melatonin yielded additional phosphorylated myosin light chain. These data indicate that melatonin’s synergistic effects are not mediated by inhibition of myosin light chain phosphatase, and instead are mediated by modulation of myosin light chain kinase. Our data indicate that the melatonin signaling cascade also sensitizes the myometrium to pro-contractile signaling through an Erk1/2 mediated phosphorylation of caldesmon which triggers its release from actin. Erk1/2 mediated caldesmon phosphorylation has been previously characterized as a sensitization mechanism in smooth muscle (Kim, Appel, Vetterkind, Gangopadhyay and Morgan 2008).

The data presented in this study provide a new aspect of melatonin physiology in the pregnant human myometrium. We show that melatonin acts to prepare the myometrium for subsequent pro-contractile signals, in this study oxytocin, by augmenting intercellular communication via gap junctions and through increasing actin availability for myosin binding. We also observed an increase in phospho-myosin light chain protein levels in hTert extracts treated with melatonin alone and a synergistic effect in melatonin/oxytocin co-treated samples apparently through an increase in myosin light chain kinase activity. These effects promote the strong, coordinated contractions required for successful parturition and would also provide a model for the observed increase in uterine contractile activity and the increased incidence of labor onset during the night (Seron-Ferre, Ducsay and Valenzuela 1993).

We show that melatonin, like oxytocin, inhibits the transcription of OTR, presumably through cross-talk between the two signaling pathways. OTR desensitization has been described at both the receptor level through binding of G-protein coupled receptor kinase 6 (Willetts, Brighton, Mistry, Morris, Konje and Challiss 2009) and at the transcriptional level by NF-κb, p50/p65 subunits, binding of
the OTR promoter after IL-1β treatment (Rauk and Friebe-Hoffman 2000). Melatonin has also been reported to activate NF-κb, p50/p65 subunits, in a human promonocytic cell line. This provides a potential mechanism for melatonin repression of OTR transcription.

Of particular clinical relevance is the increase in oxytocin-induced contractility and oxytocin sensitivity in cells co-treated with I-MEL. Clinically, there is a current trend amongst clinicians toward use of a minimal oxytocin dose for the induction of labor. This trend is due to the variability of contractile response among patients, the vague dosage recommendations, and the prevalence of contraindications which are a result of uterine hyperactivity (Clark, Simpson, Knox and Garite 2009). Interestingly, a randomized, comparative study of oxytocin-induced deliveries performed in the evening (induction at 2100 hours) versus the morning (0700 hours) showed a modest but statistically insignificant reduction in duration of labor. The study did, however, find a significant improvement in neonatal outcome in oxytocin-induced deliveries performed in the evening versus those performed in the morning (Bakker, De Vos, Pel, Wisman, Van Lith, Mol and Van Der Post 2009). At 2100 hrs serum melatonin are elevated in the human (Arendt 1998). Melatonin treatment could provide a means of reducing the necessary oxytocin dose for labor induction by sensitizing the myometrium to oxytocin. Another benefit from melatonin treatment would be incurred by its free-radical scavenging properties as both the mother and fetus are exposed to the high oxidative stress incurred during parturition (Reiter, Tan, Manchester, Paredes, Mayo and Sainz 2009).

In conclusion our data provide new insight into the physiology of pregnancy and parturition. We propose that melatonin acts synergistically in the pregnant myometrium by increasing the sensitivity of the myometrium to oxytocin as well as increasing the intercellular gap junction communication to promote increased contractility at night. The subsequent repression of OTR mRNA transcription appears to be a desensitization mechanism which defines a contextual window for an increased effect of a subsequent oxytocin stimulus. By defining a nocturnal period of enhanced contractility due to oxytocin and/or other pro-contractile stimuli, melatonin contractile synergy would provide a mechanism for the circadian rhythm of uterine contractility and labor onset in the human.
Future Directions

Our research provides a basis for future examination of melatonin physiology in the pregnant human myometrium. A logical direction for that research would include additional examination in the areas of contractility and intracellular calcium signaling, examination of the regulation of the melatonin and oxytocin receptors, receptor localization, and the effects of hormonal status. Investigation in these areas would build upon this study and provide valuable insight into the areas of melatonin and reproductive physiology.

The collagen retraction assays used in this study to measure contractility provided valuable data regarding myocyte contractility due to oxytocin and melatonin. A weakness of this assay is a lack of temporal resolution. This could be achieved by measuring the contractility of myometrial strips using an isometric tension recording system which would provide a means to quantify the acute effect of melatonin treatment on contractile force and frequency of myometrial contractions. Another advantage of this system is that it is ex vivo and would provide more insight into melatonin’s effect on myometrial contractility in vivo.

Another experiment that would provide valuable insight would involve suppressing nocturnal melatonin secretion in near term pregnant women and externally monitoring uterine contractility. This is easily accomplished by light exposure (McIntyre, Norman, Burrows and Armstrong 1989) as photic input inhibits melatonin production by the pineal gland. Data gathered from these experiments would provide insight into melatonin’s effects in vivo on uterine contractility specifically contractile frequency.

Preliminary fura-2 intracellular calcium measurements showed no detectable effects of melatonin on intracellular calcium in hTert cells. Our data did show an increase in inositol triphosphate turnover in samples co-treated with melatonin and oxytocin. One would expect to see an increase in calcium release from intracellular stores due to the increase in inositol triphosphate. The lack of a detectable intracellular calcium increase seen in treatments with melatonin alone could be due to insufficient IP<sub>3</sub> production to produce a strong calcium release. A low level calcium release would be rapidly buffered by cytosolic proteins, PKC or calmodulin, and/or rapidly transported from the cytosol by calcium efflux pumps (sodium/calcium exchanger or calcium ATPase pump) or sarcoplasmic calcium reuptake pumps (Marín, Encabo, Briones, García-Cohen and Alonso 1998). These mechanisms would keep free calcium below the detectable limits of fura-2. Further investigation into this observation is absolutely critical to fully understanding melatonin signal transduction in the myometrium. Pharmacological inhibitors
against internal and external calcium channels could also be used to determine the nature of any observed synergistic effects on intracellular calcium release. Electrophysiology experiments could also be performed to further characterize the effects of melatonin on membrane potential and external ion channel function. Melatonin has been reported to modulate the activity of the large conductance calcium-activated potassium channel in the rat myometrium (Steffens, Zhou, Sausbier, Sailer, Motejlek, Ruth, Olcese, Korth and Wieland 2003) and thus modulating membrane potential; however no information is available on melatonin’s effects on human myometrial smooth muscle excitability.

Another area of interest would be characterization of the expression profile of the MT2R in all areas of the uterus. The myometrial biopsy samples we were provided were exclusively from the lower segment of the uterus. Differential expression of the prostaglandin E2 receptor between the lower and upper uterine segments has been reported in humans (Astle, Thornton and Slater 2005). It would not be surprising if melatonin receptors showed differential regulation between the segments as well. These data would provide additional understanding of melatonin’s actions in the uterus and could provide insights into the coordination of contractions between the upper and lower segments.

Additional experiments looking at co-localization of melatonin and oxytocin receptors would also be of interest. Our data indicate cross-talk between the melatonin and oxytocin receptor signaling pathways leading repression of OTR transcription. The nature of this cross-talk could be better understood by determining whether the receptors co-localize, thus placing the two similar signaling pathways within close proximity facilitating the cross-talk, or if melatonin’s effect is not specific to the melatonin/oxytocin signaling pathways.

Another area of interest would be investigating the mechanism of regulation underlying the similar gene expression profiles we observed in MT2R and OTR across the gestational timeline. Further investigation into the regulation of MT1R would also be of interest as it is also present in the tissues we examined and its repression of adenylate cyclase activity and cyclic AMP levels would also have pro-contractile effects in the myometrium. To date the mechanisms regulating the expression of both receptors remains largely unknown in humans (Kimura, Tanizawa, Mori, Brownstein and Okayama 1992; Gimpl and Fahrenholz 2001; Witt-Enderby, Bennett, Jarzynka, Firestine and Melan 2003). Insights into the factors regulating these receptors could provide additional information regarding the transition of the uterus from a quiescent to an active state.

The last and probably the most ambitious future direction involves melatonin’s interplay with the sheer volume of regulatory factors present in the uterus of a pregnant woman. These factors range from
endocrine factors, progesterone, estrogen, endothelins, inflammatory cytokines, to stimulated stretch receptors (Smith 2007). Any or all of these factors may interact with melatonin signaling in the uterus. This last aim points to the complexity and ever expanding field of factors that are being shown to modulate some aspect of the physiology of the pregnant human myometrium.
APPENDIX A

HUMAN SUBJECTS APPROVAL

Office of the Vice President For Research
Human Subjects Committee
Tallahassee, Florida 32306-2742
(850) 644-8633• FAX (850) 644-4392

REAPPROVAL MEMORANDUM

Date: 9/6/2007

To:
James Olcese
MC 4300

Dept.: BIOMEDICAL SCIENCE

From: Thomas L. Jacobson, Chair

Re: Reapproval of Use of Human subjects in Research:
  Myometrial Melatonin Receptors and Preterm Labor

Your request to continue the research project listed above involving human subjects has been approved
by the Human Subjects Committee. If your project has not been completed by 8/14/2008 please
request renewed approval.

You are reminded that a change in protocol in this project must be approved by resubmission of the
project to the Committee for approval. Also, the principal investigator must report to the Chair promptly,
and in writing, any unanticipated problems involving risks to subjects or others.

By copy of this memorandum, the Chairman of your department and/or your major professor are
reminded of their responsibility for being informed concerning research projects involving human
subjects in their department. They are advised to review the protocols of such investigations as often
as necessary to insure that the project is being conducted in compliance with our institution and with
DHHS regulations.

Cc:
HSC No. 2007 636-R
APPENDIX B

INFORMED CONSENT FORM

Patient Consent Form

Sponsor: Florida State University College of Medicine

Principal Investigator: Suzanne Bush M.D.

Background

There is a hormone in your brain that is called melatonin. This hormone helps your body regulate when you sleep and when you wake up. Some people call this your “biological clock”. There is some new evidence suggests that melatonin can influence the function of the female uterus, more specifically, the muscle layers of the uterus (these layers are called the “myometrium”).

Purpose

In this project we are studying how the uterus reacts to melatonin during pregnancy versus when it is not pregnant. This information may help us to have new insights into the control of myometrial function, especially during pregnancy. The goal of this research is to find ways to stop preterm births. This is the number one killer of newborns. We also want to help women in labor who may have trouble having their baby.

Procedure

For this research we need to look at a small sample of muscle from the uterus. We will keep the muscle cells in a special broth that keeps them alive for several days. We are asking for a 5-10 gram sample from you, so that the effects of melatonin on these cells can be looked at in our lab. Taking this small sample will have no ill effects on how your uterus works.

The samples you give will be taken by a licensed Doctor in the course of a medically-needed hysterectomy or caesarean section. The collection of this sample will cause no additional pain or discomfort.

Benefits

There are no direct benefits to you for participating in this study. However, in the future other women may benefit from this research in terms of better medical care.

Risks

The risks involved are not any more than those with the usual hysterectomy or caesarean section.

Alternatives

If you choose to not participate in this study, your doctor will still perform your medically needed hysterectomy or caesarian section. He will however not take any samples for use in this study.
Refusal to Participate and Withdrawal from the Study

You may at any time stop being a part of this study and ask that your personal data be removed from our files without any loss of benefits to which you may otherwise get. You are not giving up your rights to any legal claims or rights.

Cost Compensation

Taking part in this study is voluntary and there is no payment for your being in this study.

You and/or your insurance company will be responsible for paying for the medically needed hysterectomy or caesarian section and all charges and services related to those procedures. You will be billed for all applicable co-payments, deductibles or other items not paid by your insurance company.

Confidentiality

Your test results will be stored in a coded, secret format that will ensure your privacy. The results of this study may be published in the future, but, all results will be published in group format only. No single person will be identified by their results. Study results will be stored on a secure computer server at the FSU College of Medicine to which only the study researchers will have access. These results will be kept ten years after which they will be destroyed. The data we get during the course of this study be kept private, to the extent allowed by law.

If you will help us with this study, please sign this consent form at the bottom of this page. You have the right to ask and be provided with answers to any inquiry regarding the project. You may contact the project director, Dr. James Olcse, Florida State College of Medicine, 2300-E, (850) 645-1479 (Email: james.olcese@med.fsu.edu), and/or The Sacred Heart IRB at 850-416-4279

Thank you for your cooperation and support,

________________________________________  __________________________
Patient’s Signature                                  Date

________________________________________  __________________________
Physician                                           Date

________________________________________  __________________________
Witness                                             Date
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

EDUCATION:

**Florida State University College of Medicine**, Tallahassee, FL (Aug 2004-Present)

**Florida Atlantic University**, Boca Raton, FL (Jul 2004)
Bachelor of Science in Chemistry with Biochemistry, Magna Cum Laude, GPA 3.779

**Palm Beach Community College**, Lake Worth, FL (Dec 2002)
Associate of Arts Degree with Presidential Distinction, GPA 3.947

Research Assistant/Graduate Student
Florida State University College of Medicine, Tallahassee, FL, (Aug 2005-Present)
**Topic:** *Uterine Contractility and Regulation of Oxytocin Receptor Expression in Human Myometrium*

Research Assistant/Directed Independent Study Student
Florida Atlantic University, Boca Raton, FL, (Jun 2003-Jul 2004),
Supervisor: Dr. Russell Kerr
**Topic:** *Extraction, identification, purification and quantification of bioactive Kallolide compounds from the marine coral Pseudopterogorgia bipinatta*

PUBLICATIONS:

Peer Reviewed Publications


- James Sharkey, Casey Cable, James Olcese (submitted) Melatonin sensitizes human myometrial cells to oxytocin in a PKCα/ERK-dependent manner, J Clin Endocrinol Metab.

Published Abstracts


TEACHING EXPERIENCE:
Small Group Facilitator
Florida State University College of Medicine, Tallahassee, FL (Sept 2007-Present)
Courses: Clinical Microanatomy, Pathology, Clinical Physiology and Medical Biochemistry

Study Group Subject Tutor
Multicultural Association of Pre-Health Students, Tallahassee, FL (Aug 2005-May 2007)
Courses: General Physics I and II with and without Calculus, General Chemistry I and II, Biochemistry I and II, Medical Biochemistry and Clinical Physiology

Mentor

PROFESSIONAL SOCIETIES:
Endocrine Society:
- Student Fellow

HONORS:
Florida Atlantic University:
- Dean’s List (2 Semesters)
- Carey E. and Rosamund Jackson Award for Excellence in Undergraduate Chemistry (2004)

Palm Beach Community College:
- President’s Academic Honors List (3 Semesters)

US Navy:
- Navy Achievement Medal for Outstanding Performance of Duties (1999)

OTHER WORK EXPERIENCE:
Engineering Laboratory Technician