

The Role of Serotonin-2C Receptors in the Rat Circadian System

Tamara Jayne Varcoe

**Research Centre for Reproductive Health
Discipline of Obstetrics and Gynaecology
University of Adelaide, Adelaide,
Australia**

A thesis submitted to the University of Adelaide in fulfilment of the requirements for admission to the degree of Doctor of Philosophy

November 2007

TABLE OF CONTENTS

Abstract.....	vii
Declaration.....	ix
Acknowledgements.....	x
Publications arising from these studies.....	xi
Table of figures.....	xiii
Abbreviations.....	xvi

CHAPTER 1. LITERATURE REVIEW 1

1.1 INTRODUCTION.....	2
1.1.1 <i>Circadian Rhythms</i>	2
1.1.2 <i>The Mammalian Circadian System</i>	2
1.1.3 <i>Light</i>	3
1.1.4 <i>Input Pathways Transmitting Light Information</i>	3
1.1.5 <i>Suprachiasmatic Nucleus</i>	4
1.1.6 <i>SCN Cell Autonomy</i>	5
1.1.7 <i>Relevance to Human Health</i>	5
1.2 MOLECULAR CLOCKWORK WITHIN SCN CELLS.....	6
1.2.1 <i>Positive Control</i>	7
1.2.2 <i>Negative Control</i>	7
1.2.3 <i>Associated Clock and Clock Controlled Genes</i>	8
1.2.4 <i>Output Mechanisms</i>	10
1.3 PHASE SHIFTS.....	10
1.3.1 <i>Per Genes and Phase Shifts</i>	11

1.3.2	<i>Signal Transduction Pathways</i>	12
1.4	SEROTONIN.....	16
1.4.1	<i>Organisation of Serotonergic Input to the SCN</i>	16
1.4.2	<i>Serotonin Receptor Expression in the SCN</i>	17
1.4.3	<i>Functional Significance of Serotonin Receptor Expression</i>	20
1.4.4	<i>Serotonin and the Modulation of Light Input</i>	20
1.4.5	<i>Serotonin and the Non-photic Control of Circadian Rhythms</i>	22
1.4.6	<i>Serotonin and the Transmission of Photic Information at Night</i>	24
1.4.7	<i>Animal Models</i>	27
1.4.8	<i>5-HT_{2C} Receptor Structure</i>	28
1.4.9	<i>5-HT_{2C} Receptor Distribution and Function</i>	29
1.5	SUMMARY.....	29
1.6	HYPOTHESES AND AIMS.....	30
 CHAPTER 2. MATERIALS AND METHODS		32
2.1	ANIMALS AND ETHICS.....	33
2.2	<i>IN VIVO</i> SAMPLE COLLECTION AND PREPARATION.....	33
2.3	<i>IN VITRO</i> SAMPLE COLLECTION AND PREPARATION.....	35
2.4	RNA EXTRACTION, REVERSE TRANSCRIPTION AND PCR.....	35
2.4.1	<i>Extraction of RNA using TriReagent</i>	35
2.4.2	<i>RNA Extraction Using Ambion RNAqueous Kits</i>	37
2.4.3	<i>DNase Treatment and Reverse Transcription</i>	37
2.4.4	<i>Primer Design</i>	38
2.4.5	<i>Polymerase Chain Reaction (PCR)</i>	41
2.4.6	<i>Quantitation of Data</i>	41
2.4.7	<i>βactin as a Housekeeper</i>	42

2.4.8 Sequencing Primers.....	46
2.5 RADIOIMMUNOASSAY TO ANALYSE PLASMA MELATONIN CONCENTRATION	48
2.6 URINARY MELATONIN METABOLITE COLLECTION AND PROCESSING	49
2.7 CORE BODY TEMPERATURE MONITORING	50
2.8 SCN2.2 CELL CULTURE	51
2.8.1 Cell Culture	51
2.8.2 Immunohistochemistry.....	51
2.9 DATA ANALYSIS	53

CHAPTER 3. ACUTE EFFECTS OF 5-HT_{2C} RECEPTOR

ACTIVATION *IN VIVO*.....54

3.1 INTRODUCTION.....	55
3.2 METHODS AND RESULTS	57
3.2.1 Clock Gene Rhythmicity in 12L:12D Conditions	57
3.2.2 The acute effects of light exposure or DOI administration on clock gene expression in the SCN.....	60
3.2.3 The effects of RO-60 0175 on clock gene expression at ZT16 and ZT22	64
3.2.4 The effects of DOI or RO-60 0175 on clock gene expression at CT10	66
3.3 DISCUSSION	67

CHAPTER 4. CHRONIC EFFECTS OF 5-HT_{2C} RECEPTOR

ACTIVATION *IN VIVO*.....73

4.1 INTRODUCTION.....	74
4.2 METHODS AND RESULTS	76

4.2.1	<i>Phase shifting effects of 5-HT_{2C} receptor activation on clock gene expression in the SCN 24 hours after treatment</i>	76
4.2.2	<i>Phase shifting effects of 5-HT_{2C} receptor activation on clock gene expression in the SCN and in peripheral tissue 3 days after treatment</i>	79
4.2.3	<i>Phase shifting effects of 5-HT_{2C} receptor activation on rhythms of core body temperature</i>	85
4.2.4	<i>Specificity of activation: the effects of specific 5-HT_{2C} receptor agonists and antagonists on aMT.6S rhythms</i>	87
4.3	DISCUSSION	89

CHAPTER 5. ACUTE EFFECTS OF 5-HT_{2C} RECEPTOR

ACTIVATION *IN VITRO* 94

5.1	INTRODUCTION.....	95
5.2	METHODS AND RESULTS	97
5.2.1	<i>Serotonin receptor expression in culture</i>	97
5.2.2	<i>Temporal expression of clock and clock controlled genes in culture</i>	99
5.2.3	<i>Dose response relationship between glutamate and c-fos expression in vitro</i>	101
5.2.4	<i>Phase dependent effects of glutamate administration in vitro</i>	103
5.2.5	<i>The effect of 5-HT_{2C} receptor activation on c-fos and Per1 expression in the cultured SCN</i>	105
5.2.6	<i>The effect of glutamate and DOI on c-fos and Per1 expression at various circadian times</i>	107
5.3	DISCUSSION	112

CHAPTER 6. ANALYSIS OF 5-HT RECEPTOR EXPRESSION

IN THE SCN2.2 CELL LINE..... 118

6.1	INTRODUCTION.....	119
6.2	METHODS AND RESULTS	121
6.2.1	<i>Analysis of Serotonin Receptor Expression Using PCR.....</i>	<i>121</i>
6.2.2	<i>Analysis of 5-HT_{1B} receptor immunoreactivity</i>	<i>123</i>
6.2.3	<i>The acute effect of 5-HT_{1B} and 5-HT_{2A} receptor activation on c-fos and Per1 expression.....</i>	<i>125</i>
6.2.4	<i>Analysis of NeuN immunoreactivity</i>	<i>129</i>
6.3	DISCUSSION.....	131
	CHAPTER 7. CONCLUSIONS AND SUMMARY	134
	CHAPTER 8. REFERENCES.....	143

ABSTRACT

The suprachiasmatic nucleus receives dense serotonergic projections from the raphe nuclei and this input has been implicated in the modulation of circadian rhythms. This input appears to have many functions including the transmission of non-photic information during the day and the modulation of photic information at night. However, it has emerged that this input may also be involved in the transmission of light information with activation of 5-HT_{2C} receptors at night having a photo-mimetic effect. The studies described in this thesis aim to clarify the role of 5-HT_{2C} receptors in the control of circadian rhythms in the rat model and compare their actions to light.

The acute effects of 5-HT_{2C} receptor agonist administration on clock gene expression were investigated in the rat SCN. Systemic administration of the 5-HT_{2A/2C} agonist DOI to rats during early night induced *c-fos*, *Per1* and *Per2* expression in a manner similar to light. This response was time of day dependent with maximal induction occurring in the early night, and no response during the day. The role of 5-HT_{2C} receptors in this response was confirmed with the use of the selective 5-HT_{2C} receptor agonist RO-60 0175.

The effect of 5-HT_{2C} receptor activation on the phase of expression of various circadian rhythms including temperature, melatonin and clock gene expression in the SCN and periphery was examined. Both DOI administration and light exposure at night phase delayed rhythms of melatonin and temperature. Similarly, the selective 5-HT_{2C} receptor agonist RO-60 0175 phase delayed rhythms of 6-sulphatoxymelatonin, a response which was antagonised by the 5-HT_{2C} receptor antagonist SB-242084. The expression of functional and clock genes within the pineal was also phase delayed following both light and 5-HT_{2C} receptor agonist administration. However, the phase of expression of clock genes within the

SCN or liver did not shift in response to either a single nocturnal light pulse or agonist administration.

To investigate the site of action of 5-HT_{2C} receptor agonists, rat SCN explants were maintained in culture allowing exposure of agonists to denervated tissue. The acute effect of DOI administration at various circadian times on *c-fos* and *Per1* expression was assessed. 5-HT_{2C} receptor activation significantly increased *Per1* expression when administered during early subjective night, but had no effect during either subjective day or late subjective night, similar to that observed *in vivo*.

Finally, the suitability of immortalised rat SCN cells for investigation of the intracellular actions of 5-HT_{2C} receptors in the circadian system was assessed. Using RT-PCR the expression of various serotonin receptors in the SCN2.2 cell line was compared with that observed in punches of adult rat SCN. The mRNA for 5-HT_{1B} and 5-HT_{2A} receptor was expressed in both the SCN2.2 cell line and the adult rat SCN. However, 5-HT_{2C} receptor mRNA along with 5-HT₃ receptor, 5-HT_{5A} receptor and 5-HT₇ receptor mRNA was expressed in the adult rat SCN tissue but not the SCN2.2 cells. These significant differences in serotonin receptor expression limit the usefulness of this cell line for further investigation.

Together these experiments further implicate 5-HT_{2C} receptors in the control of circadian rhythms. The role of these receptors appears limited to early night, with activation showing photo-mimetic responses. Furthermore, the location of action appears to be post-synaptic within the SCN, altering the core clock genes, which in turn phase delay various circadian rhythms.

DECLARATION

This work contains no material which has been accepted for the award for any other degree or diploma in any university or any other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I further grant my consent to the University of Adelaide to make this thesis available for loan and photocopying once accepted for the degree.

The author acknowledges that copyright of published works contained within this thesis (as listed on page xi) resides with the copyright holder/s of those works.

Tamara Jayne Varcoe

November 2007

ACKNOWLEDGEMENTS

I would like to thank my supervisor Associate Professor Dave Kennaway for not only providing me with the opportunity to undertake research within his laboratory, but actively encouraging me to do so. I have learnt many skills which will be invaluable throughout my career including persistent optimism, even in the face of seemingly overwhelming odds.

I would also like to thank the members of the Circadian Physiology Research Group who were always willing to provide advice and assistance, including late night trips to the animal house. In particular, Robert Moyer for providing expert advice about immunohistochemistry, Athena Voultios for her advice on the peculiarities of PCR, Mark Salkeld for assisting with SCN microdissections, and Annie Connelly for her help with the 6-sulphatoxymelatonin experiments. Thankyou also to staff and students of the Discipline of Obstetrics and Gynaecology, in particular Helen Irving-Rodgers with advice regarding fluorescence immunohistochemistry and Lyn Harland for her molecular biology expertise.

These studies were conducted with the financial assistance of the National Health and Medical Research Council through a grant to D.J. Kennaway. I would also like to thank the Australian Government for providing financial support through an Australian Postgraduate Award.

Finally, I would like to thank Jim for his continual support throughout this long and sometimes arduous process, for making me call from work when I arrived late at night, and for looking after our son when I couldn't.

PUBLICATIONS AND ABSTRACTS ARISING FROM THESE STUDIES

Publications:

Varcoe, T.J., Kennaway, D.J. and Voultsios, A. Activation of 5-HT_{2C} receptors acutely induces *Per* gene expression in the rat suprachiasmatic nucleus at night. *Molecular Brain Research* 119: 192-200, 2003.

Varcoe, T.J. and Kennaway, D.J. (in preparation)

Activation of 5-HT_{2C} receptors acutely induces *Per* gene expression in the rat suprachiasmatic nucleus in vitro.

Abstracts:

Varcoe, T.J. and Kennaway, D.J. “5-HT_{2C} receptors and the transmission of photic information at night: site of action”. Australasian Chronobiology Society International Conference, From Molecular Clocks to Human Health, Adelaide, Australia, 2007

Varcoe, T.J. and Kennaway, D.J. “The role of 5-HT_{2C} receptors in the rat circadian system”. 1st Annual Meeting of the Australasian Chronobiology Society, Robe, South Australia, Australia, 2004.

Varcoe, T.J., Kennaway, D.J. and Voultsios, A. “The effects of 5-HT_{2C} receptor agonists on clock gene expression in the rat suprachiasmatic nucleus”. Japan 1st World Congress of Chronobiology, Sapporo, Japan, 2003

Varcoe, T.J., Kennaway, D.J. and Voultios, A. “Manipulating circadian rhythms with a serotonin agonist”. Australian Society for Medical Research. National Scientific Meeting, Adelaide, Australia, 2003.

Varcoe, T.J., Kennaway, D.J. and Voultios, A. “Serotonin receptors and circadian rhythms”. Australian Society for Medical Research. South Australian Annual Scientific Meeting, Adelaide, Australia, 2003

TABLE OF FIGURES

Figure 1-1 The Mammalian Circadian System.....	2
Figure 1-2 Molecular machinery generating circadian rhythms.....	8
Figure 1-3 Signal Transduction Pathways utilised following NMDA/AMPA receptor activation leading to CREB phosphorylation and <i>Per1</i> induction.....	14
Figure 1-4 Serotonergic input to the SCN	17
Figure 2-1 Demonstration of the SCN tissue collection procedure.....	34
Figure 2-2 Histology section of rat brain after removal of one SCN.	34
Figure 2-3 Amplification efficiency of the housekeeper gene β actin and the clock genes <i>Per1</i> , <i>Per2</i> , <i>Per3</i> , <i>Cry1</i> , <i>Cry2</i> , <i>Clock</i> and <i>Bmal1</i>	43
Figure 2-4 Amplification efficiency of the clock controlled genes <i>c-fos</i> , <i>Dec1</i> , <i>Dec2</i> , <i>Rev-erbα</i> , <i>DBP</i> and <i>PK2</i> , the neuropeptide <i>VIP</i> and the melatonin receptor <i>MT1</i>	44
Figure 2-5 Amplification efficiency of the serotonin receptors 5-HT _{1A} , 5-HT _{1B} , 5-HT _{2A} , 5-HT _{2C} , 5-HT ₃ , 5-HT _{5A} and 5-HT ₇	45
Figure 2-6 Agarose gel of PCR products.....	47
Figure 3-1 The temporal expression of clock (<i>Per1</i> , <i>Per2</i> , <i>Per3</i> , <i>Clock</i> , <i>Bmal1</i> , <i>Cry1</i> , <i>Cry2</i> , <i>Dec1</i> and <i>Dec2</i>), clock controlled (<i>Rev-erbα</i> , <i>DBP</i> and <i>Prokineticin2</i>), and other genes of interest (<i>c-fos</i> and 5-HT _{2C} receptor) in the rat SCN across 24 hours.....	59
Figure 3-2 The effect of light (400 lux, 15minutes) or DOI (2mg/kg) on <i>c-fos</i> , <i>Per1</i> and <i>Per2</i> expression in the rat SCN at ZT16 or ZT22.	62
Figure 3-3 The effect of light (400 lux, 15 minutes) at ZT16 and ZT22 on <i>Bmal1</i> , <i>Cry1</i> and <i>Clock</i> gene expression in the rat SCN.....	63
Figure 3-4 The effect of RO 60-0175 administration (10 mg/kg) on rat SCN <i>c-fos</i> , <i>Per1</i> , <i>Per2</i> , <i>Dec1</i> and <i>Dec2</i> expression 30 minutes after administration at ZT16 and ZT22.	65

Figure 3-5 The effect of DOI (2 mg/kg) or RO-60 0175 (10 mg/kg) administration on <i>c-fos</i> , <i>Per1</i> and <i>Per2</i> expression in the rat SCN at CT10	66
Figure 4-1 The effects of DOI (2 mg/kg) or light (400 lux, 15 min) at CT16 on plasma melatonin levels on the night following treatment	77
Figure 4-2 The effect of DOI (2 mg/kg) or light (400 lux, 15 min) at CT16 on <i>Per1</i> expression in the SCN across the day and night following treatment	78
Figure 4-3 The effect of DOI (2 mg/kg) or light (400 lux, 15 min) at CT16 on <i>Per2</i> expression in the SCN across the day and night following treatment	78
Figure 4-4 The effect of DOI (2 mg/kg) or light exposure (400 lux, 15 minutes) at CT16 on plasma melatonin concentration 3 subjective nights after treatment.	80
Figure 4-5 The effect of DOI (2 mg/kg) or light exposure (400 lux, 15 minutes) at CT16 on <i>Per1</i> expression in the SCN 3 subjective nights after treatment.	81
Figure 4-6 The effect of DOI (2 mg/kg) or light exposure (400 lux, 15 minutes) at CT16 on AA- <i>N-Acetyl Transferase</i> expression in the SCN 3 subjective nights after treatment.	82
Figure 4-7 The effect of DOI (2 mg/kg) or light exposure (400 lux, 15 minutes) at CT16 on <i>Per1</i> expression in the pineal 3 subjective nights after treatment.	83
Figure 4-8 The effect of DOI (2 mg/kg) or light exposure (400 lux, 15 minutes) at CT16 on <i>Per1</i> expression in the liver 3 subjective nights after treatment.	84
Figure 4-9 The effect of DOI (2 mg/kg), light (2 lux, 15 min) and saline on core body temperature offset	86
Figure 4-10 The effect of RO-60 0175 (5 and 10 mg/kg) and SB-242084 (10 mg/kg) on the phase of aMT.6S excretion rhythms in the rat.	88
Figure 5-1 <i>5-HT_{2C} receptor</i> mRNA expression in SCN from 21 day old rats maintained in culture for up to 3 days	98
Figure 5-2 <i>Clock</i> , <i>MT1 receptor</i> , <i>5-HT_{1B} receptor</i> , <i>5-HT_{2C} receptor</i> , <i>5-HT_{5A} receptor</i> , <i>VIP</i> and <i>NMDA receptor1</i> mRNA expression in rat SCN in culture	98

Figure 5-3 The temporal expression of <i>Per1</i> , <i>Per2</i> , <i>Bmal1</i> , <i>Prokineticin2</i> , <i>5-HT_{2C}</i> receptor and <i>Rev-erbα</i> mRNA in the isolated rat SCN after 48 hours of culture	100
Figure 5-4 The effect of increasing doses of glutamate on <i>c-fos</i> expression 60 minutes after the initiation of treatment at CT16.....	102
Figure 5-5 Phase response curve of <i>c-fos</i> mRNA following application of glutamate (10 mM) to rat SCN <i>in vitro</i>	104
Figure 5-6 The effect of increasing dose of DOI on <i>c-fos</i> and <i>Per1</i> mRNA 60 or 120 minutes after the initiation of treatment at CT16.....	106
Figure 5-7 The effect of glutamate (10 mM) and DOI (1 mM) on <i>c-fos</i> and <i>Per1</i> expression in the cultured rat SCN	109
Figure 5-8 Comparative effects of DOI on <i>c-fos</i> and <i>Per1</i> expression in the rat SCN either <i>in vivo</i> or <i>in vitro</i>	111
Figure 6-1 Amplification curves showing the expression of the selected serotonin receptors, <i>Per1</i> and <i>MT1 receptor</i> in representative rat SCN and SCN2.2 cells	122
Figure 6-2 5-HT _{1B} receptor immunoreactivity in the SCN2.2 cell line.....	124
Figure 6-3 The effect of a 2 hour serum shock on <i>Per2</i> and <i>Bmal1</i> expression in SCN2.2 cells	126
Figure 6-4 The effect of CGS 12060 (1 μ M, 15 minutes) or DOI (1 μ M, 15 minutes) on <i>c-fos</i> and <i>Per1</i> expression in the SCN2.2 cell line 36 hours following a serum shock.....	128
Figure 6-5 NeuN staining in both the SCN2.2 cell line and adult rat brain.....	130
Figure 7-1 A model of the role of serotonin in phase delays.....	141
Figure 7-2 A model of the role of serotonin in phase advances.	142

ABBREVIATIONS

5,7-DHT	5,7-dihydroxytryptamine
5-HT	5-hydroxytryptamine (serotonin)
AA-NAT	Arylalkylamine N-acetyltransferase
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ARNT	aryl hydrocarbon receptor nuclear translocator
AVP	Arginine vasopressin
aMT.6S	6-sulphatoxymelatonin
bHLH	basic helix loop helix
BLAST	Basic local alignment search tool
BMAL1	Brain muscle ARNT like protein 1
CaM	Calcium calmodulin
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CK1 ϵ	Casein kinase 1 epsilon
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
CRY	Cryptochrome
CT	Circadian time
Ct	Cycle threshold
DAG	Diaminoglycerol

DAPI	4',6-diamidino-2-phenylindole
DBP	Albumin D-site binding protein
df	degrees of freedom
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOB	(-)-1-2, 5-dimethoxy-4-bromophenyl-2-aminopropane
DOI	1-(2,5-dimethoxy-4-iodophenyl)-propan-2-amine
DTT	Dithiothreitol
EPSC	Excitatory post synaptic current
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric acid
GHT	Geniculohypothalamic tract
IGL	Intergeniculate leaflet
IP ₃	Inositol tri-phosphate
MAP2	Microtubule associated protein 2
MAPK	Mitogen activated protein kinase
mCPBG	m-Chlorophenylbiguanidine
mRNA	messenger ribonucleic acid
MT1	Melatonin receptor 1
NAT	N-acetyltransferase
NeuN	Neuronal Nuclei
NMDA	N-methyl-D-aspartic acid
NOS	Nitrous oxide synthase
NPY	Neuropeptide Y
NSB	Non-specific binding
NSE	Neuron specific enolase

OC	Optic chiasm
PACAP	Pituitary adenylate cyclase-activating peptide
PAS	Per-ARNT-Sim
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pCREB	phosphorylated cAMP response element binding protein
pCPA	p-Chlorophenylalanine
PDE	Phosphodiesterase
PER	Period
PGP	Protein gene product 9.5
PK2	Prokineticin2
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PRC	Phase response curve
PVN	Paraventricular nucleus
RHT	Retinohypothalamic tract
RNA	Ribonucleic acid
ROR α	retinoic acid-receptor-related orphan receptor alpha
RORE	retinoic acid-receptor-related orphan receptor response element
RT-PCR	Reverse transcription polymerase chain reaction
RyR	Ryanodine receptor
SAD	Seasonal affective disorder
s/c	subcutaneous
SCN	Suprachiasmatic nucleus
TBE	Tris-borate-EDTA
TGF α	Transforming growth factor alpha

TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide
ZT	zeitgeber time