THE BEHAVIOURAL ANTECEDENTS AND NEURAL MECHANISMS OF NON-PHOTIC PHASE SHIFTING IN SYRIAN HAMSTERS

by

Ian C. Webb
M.A., Simon Fraser University, 2002
B.Sc. (Hons.), Memorial University of Newfoundland, 1999

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

In the
Department of
Psychology

© Ian C. Webb 2007

SIMON FRASER UNIVERSITY

Fall 2007

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.
APPROVAL

Name: Ian Webb

Degree: Doctor of Philosophy (Department of Psychology)

Title of Thesis: The Behavioral Antecedents and Neural Mechanisms of Non-Photic Phase Shifting in Syrian Hamsters

Chair: Dr. Michael Schmitt
Assistant Professor

Dr. Ralph Mistlberger
Senior Supervisor
Professor

Dr. Neil Watson
Supervisor
Professor

Dr. Elliott Marchant
Supervisor
Professor
Malaspina University-College

Internal Examiner: Dr. Charles Krieger
Professor
School of Kinesiology

External Examiner: Dr. Shimon Amir
Professor
Concordia University

Date Approved: October 22, 2007
Declaration of
Partial Copyright Licence

The author, whose copyright is declared on the title page of this work, has granted to
Simon Fraser University the right to lend this thesis, project or extended essay to
users of the Simon Fraser University Library, and to make partial or single copies only
for such users or in response to a request from the library of any other university, or
other educational institution, on its own behalf or for one of its users.

The author has further granted permission to Simon Fraser University to keep or
make a digital copy for use in its circulating collection (currently available to the
public at the “Institutional Repository” link of the SFU Library website
<www.lib.sfu.ca> at: <http://ir.lib.sfu.ca/handle/1892/112>) and, without changing
the content, to translate the thesis/project or extended essays, if technically
possible, to any medium or format for the purpose of preservation of the digital work.

The author has further agreed that permission for multiple copying of this work for
scholarly purposes may be granted by either the author or the Dean of Graduate
Studies.

It is understood that copying or publication of this work for financial gain shall not be
allowed without the author’s written permission.

Permission for public performance, or limited permission for private scholarly use, of
any multimedia materials forming part of this work, may have been granted by the
author. This information may be found on the separately catalogued multimedia
material and in the signed Partial Copyright Licence.

While licensing SFU to permit the above uses, the author retains copyright in the
thesis, project or extended essays, including the right to change the work for
subsequent purposes, including editing and publishing the work in whole or in part,
and licensing other parties, as the author may desire.

The original Partial Copyright Licence attesting to these terms, and signed by this
author, may be found in the original bound copy of this work, retained in the Simon
Fraser University Archive.

Simon Fraser University Library
Burnaby, BC, Canada

Revised: Summer 2007
STATEMENT OF ETHICS APPROVAL

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

(a) Human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

(b) Advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

(c) as a co-investigator, in a research project approved in advance,

or

(d) as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Bennett Library
Simon Fraser University
Burnaby, BC, Canada
ABSTRACT

In Syrian hamsters, circadian rhythms can be phase shifted by light at night or by behavioural arousal during the day (usual sleep period). Previous work in this lab has defined arousal procedures that have differential clock resetting effects; arousal stimulated by running in a novel wheel (WC) or by gentle handling (SD) can induce large phase advance shifts, whereas arousal by physical restraint (SLR), by confinement to a platform over water, or by caffeine administration have no phase shifting effects. Pharmacological and immunocytochemical experiments were conducted to identify the neural basis for the differential effects of arousal procedures on circadian rhythms. A preliminary experiment evaluated whether modafinil, a pharmaceutical that produces arousal without stimulating activity or anxiety, can induce or modulate phase shifts. This agent, like caffeine, did not induce phase shifts, and thus represents an additional tool to identify neural correlates of clock resetting. Two extensive mapping experiments were then conducted, using c-fos and double-labeling immunocytochemistry for hypocretin or tryptophan hydroxylase. Double-labelling for c-fos and hypocretin revealed that SD and WC, both of which induce shifts, and SLR, which does not, all were associated with significant c-fos expression in hypocretin cells. Thus, activation of the hypocretin system is not sufficient to induce non-photic shifts. All three procedures also increased c-fos expression in the intergeniculate leaflet, revealing no simple relationship between activation of this structure and circadian clock resetting. Examination of the serotonergic raphe nuclei and the noradrenergic locus coeruleus (LC) indicated that both WC and SD
failed to increase \textit{c-fos} expression in the serotonergic and non-serotonergic cells in these areas. Stressful restraint, however, significantly elevated the expression of \textit{c-fos} in rostral DRN serotonin neurons and in the LC. Therefore, it appears that activation of these areas is neither necessary nor sufficient for non-photic shifts. Instead, the current results suggest that the rostral DRN and the LC may be involved in a stress-induced inhibition of phase shifts to arousal. Further behavioural and pharmacological experiments indicated that, if stress blocks phase shifts to arousal, it likely does so at the input stage and via a mechanism separate from glucocorticoid receptor activation.

\textbf{Keywords:} Circadian; Entrainment; c-Fos; Nonphotic; Syrian hamster; Modafinil; Stress

\textbf{Subject Terms:} Circadian rhythms; Hamsters; Sleep-wake cycles
DEDICATION

In memory of Dr. Barry Beyerstein, a philosopher at heart, a scholar, and a gentleman.
ACKNOWLEDGEMENTS

This dissertation would not have been possible without the help of many colleagues and friends. First and foremost, I thank my parents for both financial and emotional support. Thank you to Dr. Ralph Mistlberger for guidance, invaluable advice, and an in depth knowledge of the literature. I owe a great debt of gratitude to all members of the Mistlberger lab, past and present, who lent a hand with various behavioural manipulations, histology, drug administration, data analysis, beer drinking, etc. These include Danica Patton, Glenn Yamakawa, Glenn Landry, James Handel, Dierdre Tse, Cathy Su, and Rhiannon Mear. I also thank Dr. Neil Watson, Dr. Michael Antle, and Dwayne Hamson for valuable practical advice on the black art of immunohistochemistry. Thanks to Michael Pollock for his expertise in sleep scoring and electroencephalography. Finally, thank you to the staff at the ARC for their cooperation and their help with animal care. This work was funded by operating grants to Dr. Ralph Mistlberger from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Institute of Health Research, and by an NSERC doctoral scholarship to the author.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPROVAL</td>
<td>II</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>III</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>V</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>VI</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>VII</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>XV</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>XXIV</td>
</tr>
<tr>
<td>GLOSSARY</td>
<td>XXV</td>
</tr>
<tr>
<td><strong>CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW</strong></td>
<td>1</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>2</td>
</tr>
<tr>
<td>Early Behavioural Studies</td>
<td>3</td>
</tr>
<tr>
<td>The Activity Phase Response Curve (PRC)</td>
<td>5</td>
</tr>
<tr>
<td>Activity Dose-Response Studies</td>
<td>5</td>
</tr>
<tr>
<td>The Phase Shifting Effects of Triazolam</td>
<td>6</td>
</tr>
<tr>
<td>The Phase Shifting Effects of Dark Pulses</td>
<td>7</td>
</tr>
<tr>
<td>The Behavioural Antecedents of Non-Photic Phase Shifting</td>
<td>8</td>
</tr>
<tr>
<td>Novelty</td>
<td>8</td>
</tr>
</tbody>
</table>
Adrenocortical Activation................................................................. 9
Motivation....................................................................................... 11
Arousal............................................................................................ 11
Locomotor Activity........................................................................ 14
Other Stimuli................................................................................... 15

Behaviorally Induced Non-Photic Clock Resetting in Other Species........ 15

Pathways and Neurotransmitters Mediating Non-Photic Phase Shifting .... 17

The Geniculohypothalamic Tract ...................................................... 17
Neuropeptide Y............................................................................... 19
GABA ............................................................................................ 21
Enkephalins................................................................................... 24
Serotonergic Mediation of Non-Photic Clock Resetting .................. 25
Hamsters ....................................................................................... 26
Mice .............................................................................................. 31
Rats ................................................................................................ 32
Other Afferent Pathways................................................................. 34

The Molecular Biology of Entrainment ........................................... 35
Interactions Between Photic and Non-Photic Zeitgebers .................. 39
LL Induced Potentiation of Non-Photic Phase Shifts ....................... 43

The Current Experiments.................................................................. 46

CHAPTER 2: MODAFINIL AND CIRCADIAN RHYTHMS IN SYRIAN HAMSTERS: ASSESSMENT OF THECHRONOBIOLOGIC POTENTIAL OF A NOVEL ALERTING COMPOUND ................................................................. 49
CHAPTER 3: DIFFERENTIAL EFFECTS OF AROUSAL PROCEDURES ON CIRCADIAN RHYTHMS IN HAMSTERS: NEURAL CORRELATES IN THE MIDBRAIN RAPHE AND LOCUS COERULEUS

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

Animals

Behavioural Manipulations

Perfusion and Fixation

Immunocytochemistry

Cell Counting

Data Analysis

RESULTS

Characteristics of TrpOH and c-Fos Immunoreactivity

Spatial Pattern of c-Fos Expression in the Dorsal Raphe Nucleus

c-Fos Expression in the Median Raphe Nuclear Region

c-Fos Expression in the Locus Coeruleus

DISCUSSION

Implications for Serotonergic Mediation of Non-Photic Shifting

Stress –Induced Activation of the Serotonergic and Noradrenergic Systems

Inhibition of Arousal-Induced Phase Shifting by Stress

Methodological Issues

Conclusions
CHAPTER 4: DIFFERENTIAL EFFECTS OF AROUSAL PROCEDURES ON CIRCADIAN RHYTHMS IN SYRIAN HAMSTERS: NEURAL CORRELATES IN THE HYPOCRETIN SYSTEM AND THE INTERGENICULATE LEAFLET ...

ABSTRACT

INTRODUCTION

Animals and Housing.

Drugs

General Procedures

Immunocytochemistry

Cell Counting

Statistical Analysis

RESULTS

Characteristics of Hcrt-1 and c-Fos Immunoreactivity

Spatial Pattern of c-Fos, Hcrt-1, and Double-Labeled Cells Following Each Arousal Procedure

c-Fos Expression in the IGL Following Each Arousal Procedure

DISCUSSION

Technical Considerations

Conclusions

FIGURES

CHAPTER 5: STRESS, GLUCOCORTICOIDS, AND NON-PHOTIC PHASE SHIFTING IN SYRIAN HAMSTERS
Experiment 1: Does short-term constant light affect the phase response to physical restraint during the light period? ................................................................. 166

Experiment 2: Does short-term constant light affect the phase response to physical restraint during the dark period? ................................................................. 166

Phase Shift Analysis ................................................................................ 167

RESULTS ..................................................................................................... 167

Experiment 1: Short-term LL does not promote phase advance shifts to SLR during the light period ......................................................................................... 167

Experiment 2: Short-term LL does not potentiate phase shifts to SLR early in the dark period ......................................................................................... 167

DISCUSSION .............................................................................................. 168

APPENDIX B: SYSTEMIC ADMINISTRATION OF YOHIMBINE DOES NOT PERTURB CIRCADIAN PHASE IN SYRIAN HAMSTERS................................. 172

INTRODUCTION ....................................................................................... 172

METHODS ................................................................................................ 173

Animals ................................................................................................. 173

Drugs ..................................................................................................... 173

Procedure ............................................................................................. 174

Phase Shift Measurement ....................................................................... 174

RESULTS ................................................................................................ 174

DISCUSSION ......................................................................................... 174
LIST OF FIGURES

FIGURE 1. Effects of vehicle and modafinil on behavioural state following administration at ZT6. (A) Effects of increasing dosages of modafinil on the percentage of behavioural state over a 6 h period following administration. (B) Effects on wakefulness, (C) slow-wave sleep, and (D) paradoxical sleep over a 12 h period following administration. Data shown as mean +/- SEM where appropriate. The shading indicates the dark period of the LD cycle. .......................................................... 66

FIGURE 2. Wheel running activity records of representative hamsters illustrating phase shifts in response to (A) vehicle, (B) 150 mg/kg modafinil, or (C) 300 mg/kg modafinil administered at ZT6. Also shown are phase shifts in response to (D) one night of constant light [LL] followed by constant darkness, (E) vehicle, (F) or 300 mg/kg modafinil administration at ZT4, and (G) LL followed by vehicle (H) or 300 mg/kg modafinil administration at ZT4 with novel wheel confinement from ZT6-9. Each horizontal line represents a 24 h period with wheel revolutions plotted in 10 min bins from left to right. Wheel running is indicated by vertical deflections and shading marks the dark period of the LD cycle. The circle and diamond symbols represent vehicle and drug injections, respectively, and the ‘V’ markers designate the beginning and end of novel wheel confinement. ........................................................................................................ 67
FIGURE 3. MEAN PHASE SHIFTS IN RESPONSE TO VEHICLE, 150 MG/KG MODAFINIL, AND 300 MG/KG MODAFINIL ADMINISTERED AT ZT6. ALSO ILLUSTRATED ARE SHIFTS IN RESPONSE TO ONE NIGHT OF CONSTANT LIGHT [LL] FOLLOWED BY CONSTANT DARKNESS [DD], VEHICLE [VEH], OR 300 MG/KG MODAFINIL ADMINISTRATION AT ZT4 WITH AND WITHOUT NOVEL WHEEL CONFINEMENT [WC] FROM ZT6-9. DATA SHOWN AS MEAN +/- SEM. ** = CONDITIONS SIGNIFICANTLY DIFFERENT AT P < 0.001.

FIGURE 4. MEAN WHEEL REVOLUTIONS OVER SIX 3 H PERIODS FOLLOWING ONE NIGHT OF CONSTANT LIGHT [LL] AND SUBSEQUENT EXPOSURE TO CONSTANT DARKNESS [DD], VEHICLE [VEH], OR 300 MG/KG MODAFINIL ADMINISTRATION AT ZT4, WITH OR WITHOUT NOVEL WHEEL CONFINEMENT [WC] FROM ZT6-9. DATA SHOWN AS MEAN +/- SEM. *** = SIGNIFICANTLY DIFFERENT FROM APPROPRIATE VEHICLE OR DRUG ALONE CONTROL CONDITION AT P < 0.001. ††† = SIGNIFICANTLY DIFFERENT FROM VEH + WC CONDITION AT P < 0.001.


FIGURE 6. WHEEL RUNNING ACTIVITY RECORDS OF REPRESENTATIVE HAMSTERS ILLUSTRATING PHASE SHIFTS IN RESPONSE TO (A) CONSTANT DARKNESS, (B) VEHICLE, OR (C) 300 MG/KG MODAFINIL ADMINISTERED AT ZT12. ALSO SHOWN ARE SHIFTS IN RESPONSE TO (D) A 15 MIN LIGHT PULSE [LP] AT ZT13 PRECEDED BY VEHICLE, OR (E) 300 MG/KG MODAFINIL ADMINISTERED AT ZT12 AND (F) A 15 MIN LP AT ZT13 PRECEDED BY VEHICLE, OR (G) 300MG/KG MODAFINIL ADMINISTRATION AT ZT6.
LAST FIVE PANELS ILLUSTRATE PHASE SHIFTS IN RESPONSE TO (H) CONSTANT DARKNESS, (I) VEHICLE OR (J) 300 MG/KG MODAFINIL ADMINISTRATION AT ZT17 AND (K) A 15 MIN LP AT ZT18 PRECEDED BY VEHICLE OR (L) 300 MG/KG MODAFINIL AT ZT17. THE CIRCLE, DIAMOND, AND SQUARE SYMBOLS REPRESENT VEHICLE INJECTIONS, DRUG INJECTIONS, AND LIGHT PULSES, RESPECTIVELY. ............................ 70

FIGURE 7. MEAN PHASE SHIFTS IN RESPONSE TO CONSTANT DARKNESS [DD], VEHICLE [VEH] OR 300 MG/KG MODAFINIL, WITH OR WITHOUT EXPOSURE TO A 15 MIN LIGHT PULSE [LP]. (A) DD, VEH OR MODAFINIL ADMINISTERED AT ZT12 WITH OR WITHOUT A LP AT ZT13, (B) DD, VEH OR MODAFINIL ADMINISTERED AT ZT6 WITH A LP AT ZT13. (C) DD, VEH OR MODAFINIL ADMINISTERED AT ZT17 WITH OR WITHOUT A LP AT ZT18. DATA SHOWN AS MEAN +/- SEM. *= CONDITIONS SIGNIFICANTLY DIFFERENT AT P < 0.05; ** = CONDITIONS SIGNIFICANTLY DIFFERENT AT P < 0.01; *** = CONDITIONS SIGNIFICANTLY DIFFERENT AT P < 0.001. ................................................................................... 71

FIGURE 8. PHOTOGRAPHS OF THREE OF THE BEHAVIORAL AROUSAL PROCEDURES USED IN THE CURRENT STUDY. A. SLEEP DEPRIVATION BY GENTLE HANDLING. B. WHEEL CONFINEMENT. C. STRESS-LOADED RESTRAINT ................................................................. 94


FIGURE 10. REPRESENTATIVE PHOTOMICROGRAPHS ILLUSTRATING THE EFFECTS OF THE BEHAVIOURAL MANIPULATIONS ON c-FOS EXPRESSION IN TrpOH-IR NEURONS IN THE DRN. (A) THE DRN AT APPROXIMATELY -8.0MM RELATIVE TO BREGMA. ALSO
SHOWN IS DOUBLE LABELLING OF c-Fos-IR/TrpOH-IR FOLLOWING (B) DD (C) WC
(D) SD AND (E) SLR. NUCLEAR c-Fos-IR APPEARS BLACK AND TrpOH-IR APPEARS
LIGHT BROWN. THE BLACK ARROWS INDICATE c-Fos-IR AND THE BLUE ARROWS
TrpOH-IR.

FIGURE 11. THE EFFECTS OF BEHAVIOURAL MANIPULATIONS ON c-FOS EXPRESSION IN
SPECIFIC SUBPOPULATIONS OF TrpOH-IR NEURONS IN THE DRN. THE SHADED BARS
REPRESENT THE NUMBER OF DOUBLE-LABELLED c-Fos-IR/TrpOH-IR NEURONS AND
THE OPEN BARS REPRESENT THE NUMBER OF SINGLE LABELLED TrpOH-IR NEURONS.
THE PERCENTAGES INDICATE THE PROPORTION OF TrpOH-IR THAT ALSO SHOWED c-
Fos-IR FOLLOWING THE BEHAVIOURAL MANIPULATIONS. DATA ARE SHOWN AS MEANS
± SEM. *** = P < .05 VS. ALL OTHER CONDITIONS, ### = P < .01 VS. ALL OTHER
CONDITIONS, * = P < .05 VS. DD.

FIGURE 12. EFFECTS OF THE BEHAVIOURAL MANIPULATIONS ON c-FOS EXPRESSION IN
TrpOH-IMMUNONEGATIVE NEURONS IN SPECIFIC SUBREGIONS OF THE DRN. DATA
ARE SHOWN AS MEANS ± SEM. * = P < .05; ** = P < .01; *** = P < .001.

FIGURE 13. THE EFFECTS OF BEHAVIOURAL MANIPULATIONS ON c-FOS EXPRESSION IN
TrpOH-IR NEURONS IN THE MnR AND PMnR. THE SHADED BARS REPRESENT THE
NUMBER OF DOUBLE-LABELLED c-Fos-IR/TrpOH-IR NEURONS AND THE OPEN BARS
REPRESENT THE NUMBER OF SINGLE LABELLED TrpOH-IR NEURONS. THE
PERCENTAGES INDICATE THE PROPORTION OF TrpOH-IR THAT ALSO SHOWED Fos-IR
FOLLOWING THE BEHAVIOURAL MANIPULATIONS. DATA ARE SHOWN AS MEANS ±
SEM. * = P < .05 VS. DD AND SD.
FIGURE 14.  THE EFFECTS OF BEHAVIOURAL MANIPULATIONS ON C-FOS EXPRESSION IN TrpOH-immunonegative neurons in the MnR and PMnR. DATA ARE SHOWN AS MEANS ± SEM. * = P = .05; ** = P < .01. 100

FIGURE 15.  REPRESENTATIVE PHOTOMICROGRAPHS ILLUSTRATING C-FOS EXPRESSION IN THE LC AT APPROXIMATELY -10.04MM RELATIVE TO BREGMA FOLLOWING (A) DD (B) WC (C) SD AND (D) SLR. NUCLEAR c-FOS-IR APPEARS BLACK AND TrpOH-IR APPEARS LIGHT BROWN. 101

FIGURE 16.  THE EFFECTS OF BEHAVIOURAL MANIPULATIONS ON C-FOS EXPRESSION IN THE LC. DATA ARE SHOWN AS MEANS ± SEM. *** = P < .001 VS. ALL OTHER CONDITIONS. 102

FIGURE 17.  ILLUSTRATION OF THE COUNTING BOXES USED FOR SPATIAL ANALYSIS. EACH BOX MEASURED 600 MM X 800 MM. THE DOTS REPRESENT Hcrt-1 IMMUNOREACTIVE NEURONS. V = 3RD VENTRICLE; VMH = VENTROMEDIAL HYPOTHALAMIC NUCLEUS, OT = OPTIC TRACT. 126

FIGURE 18.  A REPRESENTATIVE PHOTOMICROGRAPH ILLUSTRATING c-Fos (BLACK ARROW) AND Hcrt-1 (RED ARROW) IMMUNOREACTIVITY. DOUBLE-LABELLED CELLS ARE INDICATED BY THE PRESENCE OF TWO ARROWS. 127

Fos-ir following the manipulations. Data are shown as means ± SEM. * = significantly different from DD; A = significantly different from MOD; B = significantly different from CAFF; C = significantly different from SLR; D = significantly different from SD; E = significantly different from WC.

One symbol represents p < .05, two symbols represent p < .01, three symbols represent p < .001. Uppercase letters indicate differences in the percentage of double-labelled cells while lower case letters indicate differences in the number of single-labelled Hcrt-1 cells.

Figure 20. Effects of the behavioural and pharmacological manipulations on c-fos expression in Hcrt-1-ir cells and Hcrt-1 immunonegative neurons.

A. Effects of behavioural and pharmacological manipulations on the number of single labelled Hcrt-1 cells, and the percentage of double-labelled cells. The shaded bars represent the number of double-labelled c-Fos-ir/Hcrt-1-ir neurons and the open bars represent the number of single labelled Hcrt-1-ir neurons. The percentages indicate the proportion of Hcrt-1r cells that also showed Fos-ir following the manipulations. B. Effects of the behavioural and pharmacological manipulations on c-fos expression in Hcrt-1-immunonegative neurons * = significantly different from DD; A = significantly different from MOD; B = significantly different from CAFF; C = significantly different from SLR; D = significantly different from SD; E = significantly different from WC.

One symbol represents p < .05, two symbols represent p < .01, three symbols represent p < .001. Uppercase letters in panel A indicate differences in the
PERCENTAGE OF DOUBLE-LABELLED CELLS WHILST LOWER CASE LETTERS INDICATE
DIFFERENCES IN THE NUMBER OF SINGLE LABELLED HCRT-1 CELLS. DATA ARE SHOWN
AS MEANS ± SEM........................................................................................................................................... 129

FIGURE 21. EFFECTS OF THE BEHAVIOURAL AND PHARMACOLOGICAL MANIPULATIONS
ON C-FOS EXPRESSION IN HCRT-1-IMMUNONEGATIVE NEURONS BY BRAIN AREA. DATA
ARE SHOWN AS MEANS ± SEM. THE SHADED BARS REPRESENT THE NUMBER OF
DOUBLE-LABELLED C-FOS-IR/HCRT-1-IR NEURONS AND THE OPEN BARS REPRESENT
THE NUMBER OF SINGLE LABELLED HCRT-1-IR NEURONS. THE PERCENTAGES INDICATE
THE PROPORTION OF HCRT-IR THAT ALSO SHOWED C-FOS-IR FOLLOWING THE
MANIPULATIONS. DATA ARE SHOWN AS MEANS ± SEM. * = SIGNIFICANTLY DIFFERENT
FROM DD; A = SIGNIFICANTLY DIFFERENT FROM MOD; B = SIGNIFICANTLY
DIFFERENT FROM CAFF; C = SIGNIFICANTLY DIFFERENT FROM SLR; D =
SIGNIFICANTLY DIFFERENT FROM SD; E = SIGNIFICANTLY DIFFERENT FROM WC. ONE
SYMBOL REPRESENTS P < .05, TWO SYMBOLS REPRESENT P < .01, THREE SYMBOLS
REPRESENT P < .001. ........................................................................................................................................... 130

FIGURE 22. C-FOS EXPRESSION IN THE INTERGENICULATE LEAFLET (IGL) FOLLOWING
THE VARIOUS AROUSAL PROCEDURES. A. PHOTOMICROGRAPH ILLUSTRATING C-FOS
AND HCRT-1 IMMUNOREACTIVITY IN THE IGL OF A CONTROL AND A STRESS-LOADED
RESTRAINT TREATED ANIMAL. B THE NUMBER OF C-FOS POSITIVE CELLS OBSERVED IN
THE IGL FOLLOWING EACH TREATMENT. THE ASTERISKS INDICATE A SIGNIFICANT
DIFFERENCE FROM THE DD, MOD, AND CAFF GROUPS WITH ** = P < .01 AND *** = P
< .001..... ........................................................................................................................................ 131
FIGURE 23. PHASE SHIFTS IN RESPONSE TO WHEEL CONFINEMENT WITH OR WITHOUT
SUBSEQUENT PHYSICAL RESTRAINT. (A) A REPRESENTATIVE ACTOGRAM FROM
ANIMAL HSTWC11 SHOWING PHASE SHIFTS IN RESPONSE TO WHEEL CONFINEMENT
(WC) FROM ZT6-9 WITH AND WITHOUT SUBSEQUENT PHYSICAL RESTRAINT (SLR).
EACH HORIZONTAL LINE REPRESENTS A 24 H PERIOD WITH WHEEL REVOLUTIONS
PLOTTED IN 10 MIN BINS FROM LEFT TO RIGHT. WHEEL RUNNING IS INDICATED BY
VERTICAL DEFLECTIONS AND SHADING MARKS THE DARK PERIOD OF THE LD CYCLE.
THE ‘V’ MARKERS DESIGNATE THE BEGINNING AND END OF NOVEL WHEEL
CONFINEMENT AND THE UNFILLED BOX REPRESENTS THE RESTRAINT PROCEDURE. (B)
MEAN PHASES SHIFTS TO WHEEL CONFINEMENT WITH AND WITHOUT SUBSEQUENT
PHYSICAL RESTRAINT. DATA SHOWN AS MEAN +/- SEM........................................ 146

FIGURE 24. THE EFFECTS OF WHEEL CONFINEMENT (WC) WITH AND WITHOUT
SUBSEQUENT RESTRAINT STRESS (WC + SLR) ON TOTAL DAILY WHEEL RUNNING
ACTIVITY (A) PERCENT CHANGE IN TOTAL DAILY ACTIVITY FOLLOWING WC AND
WC+SLR RELATIVE TO AN AVERAGE 5-DAY BASELINE ON THE MANIPULATION DAY
AND THREE DAYS OF CONSTANT DARK. (B) NUMBER OF WHEEL REVOLUTIONS BY
VARIED TIME BLOCKS ACROSS THE MANIPULATION DAY. * = P < .05, ** = P < .01... 147

FIGURE 25. PHASE SHIFTS IN RESPONSE TO VEHICLE, MIFEPRISTONE, AND PHYSICAL
RESTRAINT WITH AND WITHOUT PRE-TREATMENT WITH MIFEPRISTONE. A. TWO
REPRESENTATIVE ACTOGRAMS FROM ANIMALS C3P6 AND C4P2 SHOWING PHASE
SHIFTS IN RESPONSE TO VEHICLE (VEH), MIFEPRISTONE (MIFE), AND PHYSICAL
RESTRAINT (SLR) WITH AND WITHOUT PRE-TREATMENT WITH MIFEPRISTONE.
VEHICLE INJECTIONS ARE REPRESENTED BY UNFILLED DIAMONDS, MIFEPRISTONE
INJECTIONS BY FILLED DIAMONDS, AND PHYSICAL RESTRAINT BY UNFILLED BOXES. B.
MEAN PHASE SHIFTS TO VEHICLE, MIFEPRISTONE, AND PHYSICAL RESTRAINT WITH AND
WITHOUT PRE-TREATMENT WITH MIFEPRISTONE. GROUP NUMBERS ARE SHOWN ABOVE
EACH BAR. NOTE THAT DATA FOR BOTH THE VEH AND MIFE CONDITIONS FOR GROUP
1 AND 2 HAVE BEEN POOLED FOR THIS FIGURE. DATA SHOWN AS MEAN +/- SEM.....

FIGURE 26. PERCENT CHANGE IN TOTAL DAILY WHEEL RUNNING RELATIVE TO AN
AVERAGE 5-DAY BASELINE INDUCED BY VEHICLE AND MIFEPRISTONE WITH AND
WITHOUT RESTRAINT STRESS OVER THE MANIPULATION DAY AND THREE DAYS OF
CONSTANT DARKNESS. ........................................................................................................ 149

FIGURE 27. A SCHEMATIC REPRESENTATION OF THE CIRCADIAN SYSTEM AND THE
STRESS ATTENUATION MODEL. THE PLUS SYMBOLS INDICATE PUTATIVE EXCITATORY
INPUTS, THE MINUS SYMBOLS INHIBITORY INPUTS, AND THE QUESTION MARKS INPUTS
OF UNKNOWN INFLUENCE. ACCORDING TO THE MODEL, AROUSAL DURING THE
SUBJECTIVE DAY EXCITES THE IGL LEADING TO THE RELEASE OF ONE OR MORE
EXCITATORY NEUROTRANSMITTERS IN THE SCN, THUS RESULTING IN A PHASE
ADVANCE. INESCAPABLE STRESS CONCURRENT WITH AROUSAL, HOWEVER, LEADS TO
INCREASED EXCITATION OF THE LC AND/OR THE DRN, ULTIMATELY RESULTING IN
THE RELEASE OF 5-HT IN THE IGL AND A SUBSEQUENT DECREASE IN IGL OUTPUT TO
THE SCN.................................................................................................................................. 162
LIST OF TABLES

Table 1. Effects of vehicle and modafinil on behavior following administration at ZT6. The mean percentage of time spent engaged in various behaviors in the home cage over a 3 h period following modafinil or vehicle administration at ZT6. Data shown as mean (+ SEM).................. 72
### GLOSSARY

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,7-DHT</td>
<td>5,7-dihydroxytryptamine; a serotonergic neurotoxin</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine; serotonin; a monoaminergic neurotransmitter synthesized by the cells comprising the raphe nuclei</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-n-propylamino)tetralin; an agonist at serotonin 1A/7 receptors</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone; a hormone secreted from the anterior pituitary that stimulates secretion of glucocorticoids from the adrenal cortex</td>
</tr>
<tr>
<td>c-fos</td>
<td>An immediate early gene whose expression largely correlates with neuronal activation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System; the brain and spinal cord</td>
</tr>
<tr>
<td>cry</td>
<td>Cryptochrome; a core clock gene postulated to play a role in the circadian timing mechanism</td>
</tr>
<tr>
<td>CT</td>
<td>Circadian Time; a time scale based upon division of the endogenous period of a circadian rhythm into 24 equal parts</td>
</tr>
<tr>
<td>DD</td>
<td>Constant Darkness; a period of continuous darkness over the 24h day</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal Raphe Nucleus; a prominent group of serotonin synthesizing cells located in the midbrain</td>
</tr>
<tr>
<td>Entrainment</td>
<td>The process of synchronizing an endogenous rhythm to an external environment cycle</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric Acid; the ubiquitous inhibitory neurotransmitter of the mammalian nervous system</td>
</tr>
<tr>
<td>GHT</td>
<td>Geniculohypothalamic Tract; a collection of axons running from the intergeniculate leaflet to suprachiasmatic nucleus</td>
</tr>
<tr>
<td>Hcrt</td>
<td>Hypocretin; refers to two neuropeptides (hcrt-1 and -2) that are expressed in lateral hypothalamic neurons</td>
</tr>
</tbody>
</table>
IMMUNOCYTOCHEMISTRY; a histological technique utilizing antibodies to visualise cellular components

INTRACEREBROVENTRICULAR; a route of administration via application to the ventricles of the brain

INTERGENICULATE LEAFLET; a relatively small neural area located between the dorsal and ventral lateral geniculate nuclei that has been implicated in non-photic phase shifting

LOCUS COERULEUS; a noradrenergic nucleus located in the brainstem

LIGHT/DARK; refers to the scheduled daily cycle of light and dark presentation

CONSTANT LIGHT; a period of continuous light over the 24h day

MEDIAN RAPEHE NUCLEUS; a group of serotonin synthesizing cells located in the midbrain

NOREPINEPHRINE; a monoaminergic neurotransmitter synthesized by the cells comprising the locus coeruleus

N-METHYL-D-ASPARTATE; the ubiquitous excitatory neurotransmitter of the mammalian nervous system

NON-PHOTIC; Refers to a class of zeitgebers separate from the light/dark cycle

NEUROPEPTIDE Y; a neuromodulator

PERIOD; a core clock gene postulated to play a role in the circadian timing mechanism

THE INTERVAL BETWEEN SUBSEQUENT PHASES IN A CYCLE

ANY GIVEN POINT IN A CYCLE

A change in the phase of a cycle such that it occurs at an earlier (advance) or later time point (delay). See Fig. 6D and 6J for example of photically induced phase delays and advances, respectively.

PHASE RESPONSE CURVE; a graph illustrating the relationship between the timing of stimulus presentation and the magnitude and direction of the phase shift produced by that stimulus

RETINOHYPOTHALAMIC TRACT; a bundle of axons travelling from the retina to the suprachiasmatic nucleus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN</td>
<td>Suprachiasmatic Nucleus; a nucleus in the anterior hypothalamus that is the locus of the mammalian circadian clock controlling daily rhythms of behaviour and physiology</td>
</tr>
<tr>
<td>SD</td>
<td>Sleep Deprivation by Gentle Handling; a phase shifting procedure whereby the animal is deprived of sleep via tactile stimulation</td>
</tr>
<tr>
<td>SLR</td>
<td>Stress-loaded Restraint; a modified restraint procedure that includes compressed air stimulation to maintain arousal</td>
</tr>
<tr>
<td>Tau</td>
<td>The endogenous period of a rhythm expressed under constant conditions</td>
</tr>
<tr>
<td>WC</td>
<td>Wheel Confinement; a phase shifting procedure that involves locking the animal in a large Wahmann wheel</td>
</tr>
<tr>
<td>Zeitgeber</td>
<td>An environmental time cue that can entrain an endogenous rhythm, e.g., light</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber Time; a time scale based upon the period of an environmental cycle (e.g., ZT0-24 for the environmental light/dark cycle)</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

Abstract

Endogenously generated circadian rhythms are primarily synchronized to the external environment via photic stimulation. However, in Syrian hamsters, various behavioural procedures that increase locomotor activity and/or arousal (e.g., wheel confinement, sleep deprivation by gentle handling) can phase advance circadian rhythms when applied during the light period. Although the critical non-photic stimulus remains to be fully elucidated, the evidence to date suggests that some minimal amount of locomotion is necessary to perturb circadian phase. Alternatively, arousal may be key, but for this to be true, an inhibitory action of stress must be invoked. The primary neural afferents that carry non-photic information to the suprachiasmatic nucleus have been identified. Activation of the thalamic intergeniculate leaflet appears both necessary and sufficient with neuropeptide Y, GABA, and enkephalins potentially transferring non-photic information to the clock. The serotonin system may also contribute to this phenomenon but the body of evidence is inconsistent and equivocal at best. At the molecular level, non-photic clock resetting is associated with suppressed clock gene expression, particularly of *per1* and *per2*. Recent studies have focused upon the interaction between photic and non-photic zeitgebers and have indicated that these entraining agents are mutually inhibitory when applied simultaneously. Short-term exposure to constant light, however, greatly potentiates shifts to non-photic stimuli.
Introduction

By virtue of the rotational nature of the cosmos, life on our planet is rhythmic. The rotation of the earth on its own axis, the moon’s orbit around the earth, and our own planet’s trajectory around the sun produce regular daily, monthly, and yearly variations in a multitude of terrestrial parameters. Clearly, the ability to anticipate these environmental vicissitudes and to prepare in advance of these changes would offer an enormous advantage to any organism. In fact, virtually all organisms studied to date, with the exception of some fossorial creatures, show rhythmic variations in both physiology and behaviour that mirror the solar day. These endogenously generated biological rhythms persist in the absence of environmental time cues (zeitgebers) but under constant conditions lose synchrony with the external world and free run with a periodicity close to, but not equal to, the major geophysical cycles. Rhythms with a periodicity close to the 24h light/dark (LD) cycle have been termed circadian (literally, ‘about a day’).

Convergent evidence gathered over several decades indicates that the hypothalamic suprachiasmatic nucleus (SCN) is the locus of the master circadian pacemaker driving daily rhythms of physiology and behaviour. Ablation of this structure eliminates most circadian rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972) while electrical stimulation results in photic-like phase shifts (Rusak and Groos, 1982). As well, many SCN neurons show a daily rhythm in neural firing rate, both in vivo and in vitro, with a periodicity of about 24h (Inouye and Kawamura, 1979; Green and Gillette, 1982; Shibata et al., 1982; Welsh et al., 1995). Further, SCN transplants restore rhythmicity in SCN ablated animals with a periodicity characteristic of the donor tissue (Lehman et al., 1987; Ralph et al., 1990).
The SCN is synchronized to local time primarily by the environmental LD cycle and receives photic input via two separate pathways. The first is a direct monosynaptic pathway from specialized photoreceptive retinal ganglion cells (Gooley et al., 2001; Hannibal and Fahrenkrug, 2002; Hattar et al., 2002), which has been termed the retinohypothalamic tract (RHT; Moore and Lenn, 1972). The second, an indirect pathway also originating in the retina, arises from the thalamic intergeniculate leaflet (IGL; Harrington et al., 1985; Moore and Card, 1994). The SCN, however, also receives direct afferents from cell groups known to be involved in behavioural arousal (e.g., serotonin, norepinephrine; Meyer-Bernstein and Morin, 1996; Moga and Moore, 1997) suggesting that non-photic cues, particularly those associated with behavioural state, may influence circadian phase.

Although Aschoff (1960) first proposed at the historic Cold Spring Harbour symposium the idea that locomotor activity could influence the clock, classic models of circadian regulation did not include the possibility of behavioural input. It is now widely accepted, however, that experimental manipulations affecting locomotor activity and/or arousal are capable of exerting considerable influence upon the mammalian circadian pacemaker. This seems to be particularly true for Syrian hamsters, the model species in which non-photic clock resetting has been most extensively characterized.

**Early Behavioural Studies**

Although several early studies demonstrated that access to a wheel could alter circadian period (Aschoff et al., 1973), Mrosovsky and Salmon (1987) were the first to establish that locomotor activity can affect the phase of the clock. It was discovered that
reentrainment to an 8h LD phase advance is remarkably accelerated by 3h of confinement to a novel running wheel during the mid light period. Further study revealed that acceleration of reentrainment also could be accomplished by 3h of exposure to an estrous female (Honrado et al., 1996). However, reentrainment with this method was faster when the animal had access to a wheel. Moreover, the amount of wheel running, and the amount of wakefulness in locked wheels, was negatively correlated with the time to reentrain. The observed phase advance shifts were well beyond the maximal effects of light on the clock and suggested that locomotor activity, arousal, or some correlate could affect the pacemaker. Temporal dissociation of the effects of light and induced wheel running indicated that the maximal phase advance produced by 3h of wheel confinement during the mid subjective day was ~3h. The large advances evident in the reentrainment studies appeared to be the result of an additive effect of induced activity and light (Reebs and Mrosovsky, 1989b).

Further systematic investigations revealed that overt activity rhythms could be phase advanced (~2h) and entrained by a 2h wheel confinement (WC) during the mid light period (Reebs and Mrosovsky, 1989a). However, shift magnitude was much smaller with this manipulation as compared to earlier studies and there was great individual variability in response. Similar results have been reported for the transfer of animals normally housed without running wheels to novel cages with running wheels for 1h (Wickland and Turek, 1991b; Wickland and Turek, 1994). Also, hamsters were shown to entrain to a 30 min opportunity to forage for food in an open field, although they did not consume food at that time (Rusak et al., 1988).
The Activity Phase Response Curve (PRC)

Similar to photic effects on circadian phase, the magnitude and direction of running-induced phase shifts is dependant upon the timing of activity. However, the activity PRC is quite different from that produced by photic stimuli. Light presented early in the subjective night produces phase delays while presentation in the late subjective night produces advances (Decoursey, 1960). In contrast, activity generates advances when it occurs during the day and little or no delays during the night (Reebs et al., 1989; Reebs and Mrosovsky, 1989a). Maximal phase advances (~3h) are observed during the mid-to-late subjective day (CT6-9; Reebs and Mrosovsky, 1989a).

Examination of the early behavioural data reveals striking similarities between the WC PRC and those produced by other non-photic stimuli capable of inducing phase shifts. These include cage changes, social interaction, benzodiazepines, dark pulses, and saline injections (although the saline injection PRC has slightly later peak; Turek and Losee-Olson, 1986; Mrosovsky, 1988; Mead et al., 1992; Mistlberger et al., 2002). All of these manipulations produce PRCs with maximal advances in the mid-to-late subjective day and small delays (less consistently) during the subjective night. The common characteristics of these non-photic PRCs suggest, as discussed below, that some non-specific aspect of each of these manipulations is responsible for inducing the phase shifts. It also implies the existence of common physiological mechanisms.

Activity Dose-Response Studies

In addition to being dependent upon time of day, the magnitude of phase shifts produced by WC is related to the duration or amount of the activity (Reebs and
Mrosovsky, 1989b; Wickland and Turek, 1991b; Wickland and Turek, 1994). One-hour wheel confinements tend not to produce shifts as large as 3 or 5 hr pulses. However, 3h pulses are sufficient to produce maximal effects (Reebs and Mrosovsky, 1989b). Also, the number of wheel revolutions during 1h and 3h wheel confinements predicts the magnitude of the resulting phase shift (Bobrzynska and Mrosovsky, 1998) and the relationship appears to be sigmoidal (Janik and Mrosovsky, 1993). Small advances (50 min) typically occur below 4000 wheel revolutions and maximal advances (~3h) are observed above 5000 revolutions. However, large advances (~2h) are possible with lower amounts (<1500 revolutions) of wheel running activity (Wickland and Turek, 1991b).

The Phase Shifting Effects of Triazolam

The short-acting benzodiazepine triazolam can phase shift locomotor rhythms in hamsters and the resultant PRC is similar to that for other non-photic events (Turek and Losee-Olson, 1986; Turek and Van Reeth, 1988; Maywood et al., 1997). Systemic administration at CT6 in constant light (LL) or at zeitgeber time (ZT) 6 in LD can produce 1-2h phase advances (Turek & Losee-Olson, 1986). Injections of triazolam also have been reported to accelerate reentrainment to an 8h phase advance of the LD cycle (van Reeth and Turek, 1987). However, this is not a robust effect (Mrosovsky and Salmon, 1990).

Surprisingly, rather than producing sedation, triazolam induces locomotor activity in the hamster (Wickland and Turek, 1991a; Maywood et al., 1997). Therefore, the possibility exists that the advances produced by this agent are secondary to an increase in activity, as opposed to a direct pharmacological effect upon the circadian clock. In fact, a
number of studies suggest that this is the case. Phase shifts to triazolam are inhibited by confinement to a tube or nest box (Van Reeth and Turek, 1989; Mrosovsky and Salmon, 1990). The restraint procedures appear to prevent phase shifts by directly blocking activity as these manipulations produce no shifts themselves (Van Reeth and Turek, 1989; Van Reeth et al., 1991; Mistlberger et al., 2003). Further support for the role of activity comes from the observations that the amount of time spent wheel running after triazolam administration is positively related to the magnitude of the phase shift and that the failure of triazolam to induce shifts in older hamsters (16-28 mos) is likely due to inactivity (Mrosovsky and Salmon, 1990; Van Reeth et al., 1992; Mrosovsky and Biello, 1994).

**The Phase Shifting Effects of Dark Pulses**

Dark pulses in LL also can phase shift overt activity rhythms and the effect appears to be partly dependant upon locomotor activity. Hamsters show increased activity following a dark pulse and the resulting PRC is similar to that for WC (Ellis et al., 1982; Aschoff and von Goetz, 1988). Predictably, phase advances to dark pulses in the mid subjective day are blocked by physical restraint (Reebs et al., 1989; Van Reeth and Turek, 1989) and locking wheels during a pulse can attenuate the resultant shift (Reebs et al., 1989; Mistlberger et al., 2002). In addition, the failure of dark pulses to induce shifts in older hamsters also may be attributable to inactivity (Van Reeth, 1992; Mrosovsky and Biello, 1994). That being said, locomotor mediation of dark pulse-induced shifting appears to be dependant upon CT. Dwyer and Rosenwasser (2000a) have shown that dark pulses in the early subjective day elicit phase shifts that are independent of activity.
Therefore, it appears that dark pulse-induced phase shifting is mediated by activity in the mid to late subjective day and by a photic mirror image mechanism during the early subjective day (Rosenwasser and Dwyer, 2001).

The Behavioural Antecedents of Non-Photic Phase Shifting

Wheel confinement, cage changes, social interaction, triazolam, dark pulses, and saline injections can phase advance locomotor activity rhythms (Mrosovsky, 1988; Reebs and Mrosovsky, 1989a; Van Reeth and Turek, 1989; Mead et al., 1992; Mistlberger et al., 2002). As discussed earlier, these procedures produce similar PRCs suggesting that some common element is responsible for clock resetting. The evidence cited above points to locomotor activity as an important factor. However, pinpointing the precise critical variable has proven difficult. Wheel confinement, for example, is a complex manipulation involving “waking the animal up, arousing it, altering its motivation”, varying the novelty of the environment and inducing locomotor activity (Mrosovsky and Biello, 1994). Any of these aspects may be crucial. What follows is a brief review of the variables that have been considered to be of importance (See Mrosovsky, 1996a for an excellent review).

Novelty

It may be that novelty, under some circumstances, contributes to non-photic clock resetting. Shifts as large as 12h, for example, have been reported after simultaneous transfer into a novel wheel and DD (Gannon and Rea, 1995). However, as Mrosovsky, (1996a) points out, novelty may just be an effective way of producing arousal or activity.
Systematic manipulations of novelty, although few in number reveal little, if any, effect. Reebs and Mrosovsky (1989a), for example, transferred hamsters to a novel running wheel from home cages containing either a running wheel or a gnawing bar. In this case, the wheel was more novel for the animals previously housed with a gnawing bar. The results indicated no differences in entrainment, suggesting that novelty is not a critical variable. The fact that spontaneous midday homecage wheel running induced by food deprivation also can reset the clock further supports this contention (Mistlberger et al., 2006).

**Adrenocortical Activation**

Several lines of evidence suggest that adrenocortical activation may induce non-photic phase shifting (Hastings et al., 1995). This hypothesis was given some credence by the observation that saline injections induce an elevated cortisol response only in those animals that shift in response to this stimulus (Sumova et al., 1994). As well, saline injections are associated with an increase in cortisol only at phases capable of producing phase advances (i.e., not during the saline PRC dead zone) (Sumova et al., 1994). Furthermore, exposure to a novel environment or a cage change elevates plasma ACTH and/or corticosterone in rats (Buijs et al., 1997; Dishman et al., 1998) and sleep deprivation by gentle handling, a procedure capable of producing large phase advances (Antle and Mistlberger, 2000), increases cortisol levels in hamsters (Mistlberger et al., 2003). Moreover, inescapable foot shock, resident-intruder interactions, and open field exposure, classic stress manipulations, have been reported to phase advance hamster...
locomotor activity rhythms when presented during the mid-subjective day (Mistlberger et al., 2003; Cain et al., 2004).

Other evidence, however, indicates that hypothalamic-pituitary-adrenal (HPA) activation likely does not underlie non-photic clock resetting. First, adrenalectomised hamsters do not entrain to daily cortisol infusion and retain the ability to shift in response to non-photic stimuli (Turek, unpublished results.; as cited in Mrosovsky, 1995(Albers et al., 1985). In addition, continuous infusion of cortisol does not appear to influence hamster rhythmicity (Albers et al., 1985) and acute administration of dexamethasone does not perturb circadian phase (Janik et al., 2001). As well, stressful physical restraint procedures that restrict locomotion and elevate cortisol levels do not induce phase shifts during the mid light period (Van Reeth et al., 1991;Mistlberger et al., 2003). Confinement to a tube or to a pedestal over water, for example, do not phase advance locomotor activity rhythms, despite producing sustained arousal (Van Reeth & Turek, 1989; Mistlberger et al., 2003). Furthermore, icv administration of corticotrophin releasing factor (CRF) at doses that reduce the amplitude of locomotor activity rhythms do not produce phase shifts in hamsters (Seifritz et al., 1998) and metyrapone, a corticosteroid synthesis inhibitor, does not block phase shifts to sleep deprivation (Mistlberger et al., 2003). These results, taken together, suggest that activation of the HPA alone does not mediate non-photic phase shifting. On the contrary, below we will consider a counter hypothesis that correlates of stress may actually inhibit phase shifts to behavioural arousal.
Motivation

The motivational context in which locomotor activity occurs may be an important factor in behaviourally induced clock resetting. Manipulations that induce an animal to run when it would prefer not to tend not to produce shifts. For example, old hamsters (16-28 mos) that fail to wheel run (and fail to shift) in response to non-photic stimuli also fail to shift when induced to run by low ambient temperatures or exposure to an estrous female (Van Reeth, 1992; Mrosovsky and Biello, 1994). Similarly, ‘sluggards’, animals that do not run when confined to a novel wheel, also do not shift when induced to run for thermoregulatory reasons (Janik and Mrosovsky, 1993). These data, however, do not provide strong support for the motivational hypothesis. As Mistlberger et al. (1996, p. 209) point out, the animals used in the above studies were never shown to shift in response to any stimulus, “so it is unclear whether the failure to shift is specific to thermoregulatory running or to these individuals”. More recent evidence demonstrates that cold pulses, when presented without a novel wheel, can produce significant phase shifts that are dependant upon locomotor activity (Mistlberger et al., 1996). Similarly, midday wheel running induced by food deprivation can phase advance locomotor activity rhythms (Mistlberger et al., 2006). Therefore, motivational context does not appear to be critical.

Arousal

All effective non-photic stimuli are necessarily associated with arousal as these manipulations interrupt the hamsters’ normal rest period. A role for arousal was originally highlighted by the finding that manual injections of saline in the late (CT8-10)
subjective day can produce phase advances of ~1h (Hastings et al., 1992; Mead et al., 1992; Sumova et al., 1994; Cutrera et al., 1996; Sumova et al., 1996; Maywood et al., 1997). The authors put forth the argument that the arousal associated with handling is the key factor, as animals injected remotely via a cannula do not phase shift (Hastings et al., 1992). However, these animals show a bout of activity following manual injections in the late subjective day, a response not observed in remotely injected animals (Hastings et al., 1992). Therefore, the increased locomotor activity following this procedure cannot be ruled out as a potential mediator. Also, other labs did not see a phase shifting response to saline injections, and apparently such shifts are no longer observed in the original lab (Hastings, personal communication).

More convincing evidence for the role of arousal comes from sleep deprivation procedures that involve minimal locomotion. For example, sleep deprivation by gentle handling (SD), when carried out in the mid subjective day, can produce large phase advances in hamsters (Antle and Mistlberger, 2000). Moreover, the magnitude of phase shifts produced by this procedure is inversely related to the number of interventions needed to maintain waking. These results suggest that arousal may be sufficient to produce phase shifts. However, locomotor activity was not completely restricted in these studies. The animals were estimated to have travelled ~80m over the 3h period, small as compared to the 2.5 km that hamsters run on average during 3 h in a novel wheel but nonetheless possibly critical for inducing phase shifts.

Several recent studies now suggest that arousal, in and of itself, is not sufficient to elicit phase shifts. Arousing manipulations that restrict locomotor activity are not effective. For example, as outlined above, immobilization or confinement to a platform
over water do not induce shifts when applied during the midday (Van Reeth et al., 1991; Mistlberger et al., 2003). Pharmacological studies also have suggested that arousal alone does not perturb circadian phase. Caffeine, systemically administered in the mid-subjective day, does not induce phase shifts in hamsters despite a potent stimulation of arousal (Antle et al., 2001).

The current body of evidence, however, is also consistent with an alternative explanation. It may be that arousal alone is sufficient to induce shifts of a non-photic nature but, under some circumstances, the stress associated with these manipulations can block phase shifting. This hypothesis is supported by several pieces of information. First, as noted above, stressful procedures that restrict ambulation do not induce phase advance shifts despite high levels of arousal (Mistlberger et al., 2003). As well, administration of the cortisol synthesis inhibitor metyrapone increases phase shifts to the 3h SD procedure (Mistlberger et al., 2003). Moreover, there is a negative correlation between the number of interventions needed to maintain wakefulness during SD, a manipulation associated with elevated cortisol levels (Mistlberger et al., 2003), and the size of the resulting phase advance (Antle & Mistlberger, 2000).

As noted above, however, some stressful procedures (e.g., resident-intruder interactions, open field exposure) have been reported to produce phase advances when presented during the light period (Cain et al., 2004; Mistlberger et al., 2003). At first glance, these observations appear to be at odds with a stress attenuation hypothesis. However, these stressful manipulations do not restrict locomotor activity and it may be that the locomotion induced by these procedures mitigates the effects of stress. It is well known, for example, that locomotor activity can reduce the impact of stress on the
organism. In rats, voluntary wheel running decreases learned helplessness behaviours, prevents many of the stress-induced changes in immune function, and blunts the monoaminergic response to stress (Dishman et al., 1995; Dishman et al., 1997; Soares et al., 1999; Dishman et al., 2000). As well, the magnitude of phase shifts induced by the resident-intruder interactions and the open field are related to the amount of locomotor activity expressed during these manipulations (Mistlberger et al., 2003).

**Locomotor Activity**

The evidence cited thus far also points to locomotor activity as a critical stimulus. The common feature of all effective non-photic manipulations is the ability to produce arousal in association with some level of motor activity. Manipulations that prevent locomotion but sustain arousal (e.g., physical restraint, confinement to a pedestal over water) do not induce shifts (Mistlberger et al., 2003). Similarly, during the mid-subjective day, restricting locomotor activity attenuates phase shifts to triazolam and to dark pulses (e.g., Reeds et al., 1989; Van Reeth and Turek, 1989; Dwyer and Rosenwasser, 2000a; Rosenwasser and Dwyer, 2002). As well, the magnitude of phase shifts induced by many non-photic manipulations is positively related to measures of locomotor activity (Mrosovsky and Salmon, 1990; Mistlberger et al., 1996; Bobrzynska and Mrosovsky, 1998; Mistlberger et al., 2003). Therefore, it is suggested that locomotor activity is pivotal to non-photic phase shifting by behavioural (and by some pharmacological) means and, although continuous wheel running (what we might call exercise) is not needed, some minimal amount of activity may be necessary.
Other Stimuli

A number of other potential non-photic mediators appear to have been ruled out. Temperature increases induced by passive warming do not phase shift locomotor activity rhythms in hamsters (Mrosovsky, unpublished observations; as cited in Mrosovsky, 1996a). As well, at midday, hamsters subjected to 3h of horizontal rotation while restrained do not shift (Mistlberger et al., 2003). Melatonin also appears to have been eliminated. Pinealectomized animals phase shift in response to triazolam injection and systemic administration of melatonin does not result in clock resetting during the middle of the subjective day, nor does it potentiate shifts to WC (van Reeth et al., 1987; Antle et al., 2002).

Behaviorally Induced Non-Photic Clock Resetting in Other Species

Activity and/or arousal appears to be a significantly less potent zeitgeber in other rodent species and non-photic clock resetting occurs only under specific conditions in these animals. Daily forced treadmill running or scheduled access to the homecage wheel have been reported to entrain the free-running rhythms of both blind and sighted mice (Edgar and Dement, 1991; Marchant and Mistlberger, 1996; Laemle and Ottenweller, 1999). As well, enucleated rats weakly entrain to daily forced treadmill running (Mistlberger, 1991). However, mice show only negligible phase advances in response to acute pulses of sleep deprivation, novel wheel confinement, or treadmill running (Marchant and Mistlberger, 1996; Challet et al., 2001; Cheng et al., 2004) and acute interactions with an estrous female or a dominant male do not phase shift circadian rhythms in the rat (Meerlo and Daan, 1998). Several of the neurochemicals associated
with non-photic resetting, however, have been reported to phase shift rhythms in both mice and rats (See Pathways and Neurotransmitters Mediating Non Photic Phase Shifting). As well, three days of wheel confinement (4h, CT6-10) induces phase delays in mice (Challet et al., 2000), however this appears to be largely due to a lengthening of tau.

Non-photic entrainment also has been observed in several diurnal animals and, in some cases, the PRC appears quite similar to that for nocturnal rodents. That is, in diurnal species, non-photic stimuli also induce phase shifts during the light period despite this being the organism’s active period. The European ground squirrel (Spermophilus citellus), for example, can entrain to daily scheduled wheel confinement presented at the end of the subjective day (Hut et al., 1999). Similarly, systemic administration of triazolam has been reported to induce phase advances in the squirrel monkey (Saimiri sciureus) when administered during the mid-subjective day and to induce delays during the late subjective night (Mistlberger et al., 1991). Daily investigator induced locomotor activity (1h) in the late subjective night or in the early day has been reported to produce phase delays in the common marmoset (Callithrix jacchus; (Glass et al., 2001). This procedure also can entrain free running activity rhythms, however, it only does so for a fixed period of time (~20 days; Glass et al., 2001). Taken together, these results suggest that the gating of non-photic shifts is more closely related to the phase of the pacemaker itself as opposed to the phase of the rest-activity cycle.

The crepuscular rodent Octodon degus also can entrain to daily scheduled wheel access (Kas and Edgar, 1999) and reentrainment to a 6h phase advance or delay of the LD cycle is expedited by exposure to a conspecific (Goel and Lee, 1995). Interestingly,
the latter effect appears to be mediated by olfactory cues (Goel and Lee, 1997; Governale and Lee, 2001).

**Pathways and Neurotransmitters Mediating Non-Photic Phase Shifting**

The behavioral analysis of non-photonic phase shifting has provided some important clues to the biological mechanisms underlying this phenomenon. For instance, the differential shape of the photic and non-photonic PRCs suggests mediation by neural pathways and transmitters separate from those of the RHT. Moreover, the commonality in non-photonic PRCs across a wide array of behavioral and neurochemical manipulations points to the existence of a convergent SCN pathway and/or mechanism. In fact, two major SCN afferents have now been implicated in non-photonic phenomena: 1) the GHT which is immunoreactive for neuropeptide Y (NPY) among other neuromodulators (Harrington et al., 1985; Moore and Card, 1994) and 2) an ascending serotonergic pathway originating in the pontine raphe nuclei (Meyer-Bernstein and Morin, 1996).

**The Geniculohypothalamic Tract**

The GHT arises from the IGL of the thalamus, a thin elongated nucleus receiving direct retinal afferents that is situated between the dorsal and ventral lateral geniculate nuclei (Hickey and Spear, 1976; Pickard, 1985; Morin et al., 1992). This relatively small structure is responsive to light (Harrington and Rusak, 1989; Thankachan and Rusak, 2005; Muscat and Morin, 2006), relays photic information to the SCN (Zhang and Rusak, 1989), modulates circadian period under LL (Harrington and Rusak, 1986; Pickard et al.,
1987) and contributes to entrainment under skeleton photoperiods (Edelstein and Amir, 1999). The IGL also sends prominent projections to the pretectum and is therefore likely involved in subcortical non-visual light processing (Morin and Blanchard, 1995; Morin and Blanchard, 1997). Several lines of evidence, however, suggest that the GHT is the final common pathway for non-photic information being conveyed to the circadian clock.

First, electrical stimulation of the IGL produces phase shifts similar to those induced by non-photic stimuli (Rusak et al., 1989). Conversely, lesioning the components of the GHT attenuate or block the phase shifting effects of many non-photic manipulations (Johnson et al., 1988; Biello et al., 1991; Janik and Mrosovsky, 1994; Wickland and Turek, 1994; Maywood et al., 1997; Schuhler et al., 1999). In many instances, however, IGL lesions induce hypoactivity, thus leaving open the possibility that the lack of shifting is due to decreased ambulation. Further study, however, has shown that mice sustaining complete IGL lesions fail to entrain to daily sessions of forced treadmill running (Marchant et al., 1997). Other evidence implicating the IGL in non-photic shifting includes the observation that effective non-photic manipulations markedly increase the expression of \textit{c-fos} (a marker of neuronal activity; (Morgan and Curran, 1991) in IGL neurons (Janik and Mrosovsky, 1992; Edelstein and Amir, 1995; Janik et al., 1995; Mikkelsen et al., 1998; Muscat and Morin, 2006).

The body of evidence implicating the GHT in non-photic phenomena is internally consistent and compelling. However, one recent study has cast doubt on the assumption that the GHT is the final common pathway for non-photic signals seeking access to the circadian clock. Morin & Muscat (2006) have reported that, although many IGL neurons express \textit{c-fos} following wheel confinement, this immediate early gene is rarely expressed
in neurons projecting to the SCN. Therefore, it remains a possibility that the IGL neurons involved in non-photic processing do not have direct access to the clock. However, this finding needs replication and extension.

Regardless of the remaining questions concerning the exact nature of non-photic clock inputs, a great deal of research has focused upon identification of the IGL neurotransmitters involved in non-photic signalling. The IGL in various species has been reported to contain NPY (Card and Moore, 1982; Harrington et al., 1985; Smale et al., 1991; Morin and Blanchard, 2001), GABA (Moore and Speh, 1993; Morin and Blanchard, 2001), enkephalins (Moore and Speh, 1993; Morin and Blanchard, 1995; Morin and Blanchard, 2001), and neurotensin (Morin and Blanchard, 2001), all of which project to the SCN in varying degrees (Morin and Blanchard, 2001). What follows is a comprehensive review of the evidence implicating each of these neuromodulators in non-photic phase shifting.

**Neuropeptide Y**

Neuropeptide Y was the first neurotransmitter to be localized to the IGL (Card et al., 1983; Harrington et al., 1985) and to date has been the most studied with regard to non-photic regulation. Approximately 50% of IGL neurons that project to the hamster SCN are immunoreactive for NPY (Morin & Blanchard, 2001) and a convergent body of evidence suggests that non-photic phase shifting is specifically mediated by the NPYergic GHT projection. Midday wheel confinement markedly increases *c-fos* expression in IGL neurons immunoreactive for NPY (Janik et al., 1995) and increases release of this peptide within the SCN (Glass et al., 2005). In addition, intra-SCN administration of NPY in hamsters and in mice results in a PRC similar to that produced
by non-photic behavioural manipulations (Huhman and Albers, 1994; Biello and Mrosovsky, 1996; Maywood et al., 2002; Soscia and Harrington, 2005). Moreover, the phase advances produced by this peptide are independent of locomotor activity (Biello et al., 1994; Mrosovsky, 1995). In vitro, NPY, when applied during the mid-subjective day, dose-dependently phase advances the firing rate of SCN neurons (Medanic and Gillette, 1993; Shibata and Moore, 1993; Golombek et al., 1996; Biello et al., 1997b; Harrington and Schak, 2000). Furthermore, blocking the effects of NPY inhibits activity-induced phase shifting. Injection of anti-NPY at CT 4, for example, prevents shifts produced by confinement to a novel wheel (Biello et al., 1994).

Several labs have spent considerable time identifying the NPY receptors involved in non-photic SCN signal transduction and it now appears that the Y2 subtype is responsible. Relatively selective Y2 receptor agonists produce phase advances during the mid-subjective day both in vivo (Huhman et al., 1996) and in vitro (Golombek et al., 1996; Harrington and Hoque, 1997; Gribkoff et al., 1998) but administration of Y1 or Y5 receptor antagonists is without effect (Golombek et al., 1996; Huhman et al., 1996; Gamble et al., 2005). As well, intra-SCN administration of NPY fails to produce shifts in Y2 receptor knockout mice (Soscia and Harrington, 2005). The NPYergic mechanisms for non-photic shifting may differ between species, however, as Y2 receptor mRNA is undetectable in the rat SCN (Larsen and Kristensen, 1998).

As pointed out above, there is some reason to believe that the IGL neurons that convey non-photic information to the clock do so via an indirect pathway. The evidence cited thus far suggests that the activation of SCN Y2 receptors is both necessary and sufficient for non-photic clock resetting. Given that the IGL appears critical for non-
photic shifts, that this structure is largely composed of NPY cells, that the vast majority of IGL NPY neurons express c-fos following wheel confinement, and that 50% of IGL neurons project to the SCN, it seems reasonable to assume that IGL NPY neurons projecting to the SCN mediate non-photic phenomena. However, the SCN also may receive NPY inputs from a site other that the IGL as complete lesions of this structure do not totally eliminate SCN NPY immunoreactivity (Harrington et al., 1985; Johnson et al., 1989; Weber et al., 1998). Therefore, it remains a possibility that the IGL neurons activated during non-photic procedures project to some unknown structure whose activation in turn either directly or indirectly results in SCN NPY release. Clearly this hypothesis is in violation of the principle of parsimony and further research is needed to elucidate the exact nature of NPYergic inputs to the clock.

GABA

γ-aminobutyric acid (GABA), the ubiquitous inhibitory neurotransmitter of the mammalian nervous system, has been proposed to play a role in the synchronization of SCN neurons (Colwell, 2000; Liu and Reppert, 2000; Shirakawa et al., 2000) and also may contribute to the control of circadian phase. This neurochemical is present in virtually all SCN and IGL neurons (Aguilar-Roblero et al., 1993; Moore and Speh, 1993; van den Pol and Dudek, 1993), including those that comprise the GHT (Morin & Blanchard, 2001; Moore & Card, 1994). GABA$_A$ receptors have been detected both pre and post synaptically in the SCN with alpha 1-5, beta 1-3, and gamma 1-3 subunits expressed in mice or rats (Gao et al., 1995; O'Hara et al., 1995; Belenky et al., 2003).

Numerous GABA$_A$ receptor positive allosteric modulators have been shown to perturb circadian phase in hamsters or in mice. Systemic administration of
benzodiazepines (triazolam, midazolam, chlordiazepoxide), barbiturates (pentobarbital, phenobarbital) or muscimol has been reported to induce phase advances when administered at midday (Turek and Losee-Olson, 1986; Ebihara et al., 1988a, b; Turek, 1988; Biello et al., 1991; Tominaga et al., 1992; Maywood et al., 1997; Vansteensel et al., 2003; Legan et al., 2005). However, the extent to which shifts in response to each of these agents are dependent upon pharmacologically induced hyperactivity has not been thoroughly investigated. As outlined above, triazolam can increase locomotion in hamsters (e.g., Wickland and Turek, 1991b; Maywood et al., 1997) and confinement to a restraint tube or to a nesting box attenuates shifts in response to this compound (Van Reeth and Turek, 1989; Mrosovsky and Salmon, 1990). Chlordiazepoxide, however, induces phase shifts in hamsters in the absence of increased locomotor activity (Biello and Mrosovsky, 1993; Meyer et al., 1993) and shifts to this agent occur despite confinement to a nesting box (Biello & Mrosovsky, 1993). Similar results also have been obtained for pentobarbital in mice (Ebihara and Hayakawa, 1990). Therefore, some GABA potentiators may induce phase shifts via a direct pharmacological effect, as opposed to a secondary increase in locomotor activity.

Several lines of evidence suggest that GABA may act directly in the IGL to induce non-photic clock resetting. IGL lesions, for example, block shifts in response to chlordiazepoxide or triazolam (Johnson et al., 1988; Biello et al., 1991) and muscimol injections into this structure can induce phase shifts without increasing locomotion (Smith et al., 1989). However, as outlined above, GABA_A receptors have been localized to the SCN (Gao et al., 1995; O’Hara et al., 1995; Belenky et al., 2003) and it may be that GABA, released from IGL neurons or other SCN afferents, may contribute to non-photic
shifting. This contention is supported by the observations that intra-SCN injection of muscimol can induce phase shifts \textit{in vivo} (Smith et al., 1989; Ehlen et al., 2006) and application can phase advance the peak of SCN neural activity \textit{in vitro} (Tominaga et al., 1994). Functional tract tracing studies are needed to distinguish between these possibilities.

There are conflicting reports regarding the ability of GABA\textsubscript{b} receptor agonists to perturb circadian phase both \textit{in vivo} and \textit{in vitro} (Smith et al., 1990; Biggs and Prosser, 1998; Bergeron et al., 1999; Liu and Reppert, 2000; Novak et al., 2004) and the presence of GABA\textsubscript{b} receptors in the SCN has not been confirmed. Although, intra-SCN administration of GABA\textsubscript{b} agonists have been reported to block photic phase delays (Novak et al., 2004) and to inhibit SCN cells \textit{in vitro} (Gribkoff et al., 2003).

Interestingly, SCN GABA\textsubscript{a} receptor activation appears necessary for NPY mediated clock resetting. Phase shifts to NPY are blocked by bicuculine both \textit{in vivo} and \textit{in vitro} (Biello and Mrosovsky, 1996; Huhman et al., 1997; Gribkoff et al., 1998). Tetrodotoxin also blocks NPY induced shifts but not GABA\textsubscript{a} receptor mediated advances, suggesting that GABA directly influences the neurons that control circadian phase, while NPY requires depolarisation and the subsequent activation of GABA\textsubscript{a} receptors to perturb the clock (Huhman et al., 1997). Intra-SCN administration of bicuculine has also been reported to block shifts in response to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, a 5-HT\textsubscript{1A/7} receptor agonist; (Mintz et al., 1997). The extent to which GABA\textsubscript{a} receptors mediate shifts in response to other behavioural manipulations or neurochemicals is unknown, however, and deserves further investigation.
Enkephalins

The enkephalins are neuropeptides that bind to three subtypes of G-protein coupled receptors (μ, δ, and κ; (Satoh and Minami, 1995; Reisine et al., 1996) and, although all three receptors have been detected in the SCN (Desjardins et al., 1990; Byku et al., 2000), there is no general consensus on the specific subtypes expressed in this area (Desjardins et al., 1990; George et al., 1994; Mansour et al., 1994; Arvidsson et al., 1995; Ding et al., 1996). Enkephalinergic IGL cells have been described in several species (Mantyh and Kemp, 1983; Card and Moore, 1989; Smale et al., 1991; Conley and Friederich-Ecsy, 1993; Moore and Speh, 1993; Morin and Blanchard, 1995) and, in hamsters but not in rats, these neurons appear to contribute to the GHT (Card and Moore, 1989; Morin and Blanchard, 1995). However, it should be noted that lesions of the IGL have been reported to decrease enkephalin reactivity in the rat SCN (Takatsuji and Tohyama, 1989).

In vivo, systemic administration of fentanyl (a relatively selective μ receptor agonist; (Chen et al., 1993; Meijer et al., 2000; Vansteensel et al., 2003; Vansteensel et al., 2005), BW373U86, or SNC-80 (both δ receptor agonists; (Byku and Gannon, 2000a, b) have been reported to induce large phase advances in hamsters when administered during the mid to late light period and similar results have been obtained in mice following morphine (also a relatively selective μ receptor agonist) injection (Marchant and Mistlberger, 1995). The kappa receptor agonist U50488H and morphine have been reported to be without effect in hamsters, however (Byku and Gannon, 2000a). The reasons for the discrepant results for μ agonists in hamsters are unclear but may relate to differences in pharmacology or the timing of administration.
Like most GABA effectors, the degree to which shifts in response to each of the opioid receptor agonists are dependent upon pharmacologically induced hyperactivity is unknown. Morphine, BW373U86, and SNC-80 increase ambulation in mice or hamsters immediately following administration (Marchant and Mistlberger, 1995; Byku and Gannon, 2000a, b) and blocking this activity in mice inhibits morphine-induced shifts (Mistlberger & Marchant, 1995). In contrast, fentanyl does not induce hyperactivity and facilitates sleep in hamsters (Meijer et al., 2000; Vansteensel et al., 2005), thus suggesting a direct activation of the mechanisms mediating non-photic resetting.

Assuming a direct pharmacological effect, it is unclear where these opioid receptor agonists may act to perturb circadian phase. Fentanyl has been shown to inhibit hamster SCN neuronal firing in vitro (Vansteensel et al., 2005) but the clock appears unresponsive to the enkephalins or to morphine (Cutler et al., 1999a). As well, intra-SCN injection of met-enkephalin in the hamster does not perturb circadian phase (Hugh Piggins, unpublished results; as cited in Cutler et al., 1999). Clearly, further study is needed to fully elucidate the role of this system, if any, in non-photic clock resetting. Central injections of the various opioid agonists and antagonists may prove useful in this regard.

**Serotonergic Mediation of Non-Photic Clock Resetting**

The serotonin system consists of a series of distributed brain stem and pontine nuclei (Jacobs and Azmitia, 1992), only two of which, the median (MnR) and dorsal raphe nuclei (DRN), project to the forebrain (Tork, 1990). In rodents, the physiological substrate exists for serotonergic input to the circadian system. The SCN and the IGL are
almost exclusively innervated by fibres originating in the MnR and DRN, respectively 
(Meyer-Bernstein and Morin, 1996; Moga and Moore, 1997; Hay-Schmidt et al., 2003). 
As well, SCN 5-HT1A, 1B, 2A, 2C, 5A and 7 receptors have been described in several 
rodent species (Prosser et al., 1993; Roca et al., 1993; Wright et al., 1995; Heidmann et 
al., 1998; Moyer and Kennaway, 1999; Oliver et al., 2000; Belenky and Pickard, 2001; 
Neumaier et al., 2001; Sprouse et al., 2004a; Duncan et al., 2005). Single cell recordings 
and microdialysis have revealed that 5-HT neurons are differentially active across the 
arousal spectrum with maximal firing rates observed during arousal with ambulation and 
relative quiescent concomitant with REM sleep (Jacobs and Azmitia, 1992; Jacobs and 
Fornal, 1997, 1999). The activity profile of these neurons makes this cellular population a 
potential candidate for the mediation non-photic phase resetting and, although a great 
deal of evidence suggests that this neurotransmitter is involved in phase control, there are 
inconsistencies and conflicting reports in the literature (See Mistlberger et al., 2000 and 
Morin, 1999 for reviews). As many of the divergent findings may be due to species-
specific anatomical and physiological differences, research conducted in the three most 
common rodent models will be discussed separately.

Hamsters

Correlational evidence suggests that 5-HT may mediate non-photic phase shifting 
in hamsters. In this species, SCN 5-HT release varies daily with a nadir during the mid-
subjective day and a peak at the onset of the subjective night (Dudley et al., 1998). 
Recent studies have indicated that, during the mid light period, intra-SCN 5-HT is 
increased by ~ 50% after 3h of wheel running and by as much as 170% following 3h of 
sleep deprivation by gentle handling (Dudley et al., 1998; Grossman et al., 2000; Glass et
al., 2003). In contrast, there is an activity-induced suppression of SCN 5-HT release during the subjective night (Dudley et al., 1998).

Attempts to establish a causal link between serotonin and non-photic clock resetting have largely focused upon the ability of serotonergic agents to perturb circadian phase. Arguing against a role for 5-HT in non-photic shifting are the observations that drugs or manipulations capable of increasing SCN 5-HT do not always produce shifts. For example, during the early to mid-subjective day, systemic administration of WAY100635 (a 5-HT$_{1A}$ antagonist) increases SCN 5-HT titers but fails to induce phase shifts or potentiate shifts to WC (Antle et al., 2000). Comparable results have been obtained for systemic administration of L-tryptophan (200% increase in SCN 5-HT) and fluoxetine (a 5-HT reuptake inhibitor; Mistlberger et al., unpublished observations). Therefore, it appears that an increase in SCN 5-HT at midday is not always sufficient to perturb circadian phase.

Lesioning and depletion studies have provided conflicting results regarding serotonergic mediation of clock resetting. In hamsters, intra-SCN injections of the serotonergic neurotoxins 5,7-DHT or p-chloroamphetamine have been reported to attenuate or block shifts in response to systemic triazolam or 8-OH-DPAT (Cutrera et al., 1994b; Penev et al., 1995; Meyer-Bernstein and Morin, 1998; Schuhler et al., 1998). By the same token, however, intra-SCN 5,7-DHT does not attenuate shifts in response to WC (Bobrzynska et al., 1996a) and has been reported to have no effect on triazolam induced acceleration of reentrainment to a LD shift (Smale et al., 1990). It may be that divergent pathways carry non-photic information to the clock and that these tracts are differentially activated by the varied non-photic procedures, a notion supported by the
finding that lesions of the superior colliculus block phase shifts to triazolam but not to WC (Marchant and Morin, 1999). It must be noted, however, that, due to incomplete lesioning, recovery of function, and changes in locomotor activity, the results of lesioning studies must be interpreted with caution.

The bulk of the literature regarding serotonergic control of phase in hamsters is centred on 8-OH-DPAT (a 5-HT$_{1A/7}$ agonist). This agent has been repeatedly shown to produce small phase advances (~30-60 min) when injected systemically during the subjective day (Cutrera et al., 1994a; Bobrzynska et al., 1996b; Cutrera et al., 1996; Mintz et al., 1997; Challet et al., 1998; Schuhler et al., 1998; Schuhler et al., 1999; Horikawa et al., 2000; Ehlen et al., 2001; Gannon, 2003) and these shifts appear to be independent of locomotor activity (Bobrzynska et al., 1996b). The phase resetting action of 8-OH-DPAT appears to be largely mediated by extra-SCN sites as shifts in response to intra-SCN microinjection tend to be small or undetectable (Mintz et al., 1997; Challet et al., 1998; Antle et al., 2003; Knoch et al., 2006) unless receptors are supersensitized by pre-treatment with a 5-HT synthesis inhibitor (Ehlen et al., 2001). However, intra-SCN 5,7-DHT attenuates shifts in response to systemic 8-OH-DPAT (Schuhler et al., 1998) suggesting that, under normal in vivo conditions, SCN serotonergic afferents may partially contribute to phase shifts by this compound. In animals pre-treated with a 5-HT synthesis inhibitor, systemic injections of ritanserin (a 5-HT$_{2A/5A/7}$ antagonist) or intra-SCN administration of DR4004 (a 5-HT$_7$ antagonist) block phase advances produced by intra-SCN administration of 8-OH-DPAT or 5-HT (Ehlen et al., 2001).

Mintz and colleagues (1997) have suggested that systemically administered 8-OH-DPAT binds to DRN 5-HT$_{1A}$ autoreceptors thereby inhibiting DRN cells. This, in
turn, disinhibits IGL neurons of unknown phenotype, increases SCN neurotransmitter release, and thus results in a non-photic like phase advance (Mintz et al., 1997). This model has received empirical support from the subsequent observations that IGL lesions block shifts to 8-OH-DPAT (Schuhler et al., 1999), electrical stimulation of the DRN induces serotonin release in the IGL (Grossman et al., 2004), and 5-HT inhibits IGL neuronal firing in rats (Blasiak and Lewandowski, 2003; Blasiak et al., 2006). However, it is unclear if 8-OH-DPAT directly interacts with serotonin receptors in the IGL to influence circadian phase as there are conflicting reports regarding the ability of intra-IGL injections to induce shifts (Mintz et al., 1997; Challet et al., 1998). As well, serotonergic deafferentation studies have shown that 5,7-DHT microinjection into the IGL does not affect shifts in response to this agent (Schuhler et al., 1999). Arguing further against a role for 5-HT₁₅ receptors in non-photic phenomena are the observations that systemic administration of WAY100135 (a 5-HT₁₅ antagonist) does not attenuate WC induced shifting in hamsters (Antle et al., 1998). Systemic or intra-SCN administration of metergoline (a 5-HT₁₂/₁₅ antagonist) or NAN-190 (a 5-HT₁₅ antagonist) produces similar results (Antle et al., 1998).

Recent work from the Glass lab suggests that 8-OH-DPAT may act in the DRN or MnR to perturb circadian phase via 5-HT₇ receptor activation. It has been proposed that phase shifts to this agent and to behavioural manipulations result from a DRN or MnR 5-HT₇ receptor mediated GABAergic disinhibition of serotonergic output to the SCN (See Dudley et al., 1999; Glass et al., 2000; Glass et al., 2003). Briefly, this model is supported by the following observations. Intra-DRN or MnR 8-OH-DPAT injection or electrical stimulation of these areas induces phase advances during the midday and increases SCN
5-HT release, an effect that can be blocked by 5-HT_7 antagonists (Mintz et al., 1997; Meyer-Bernstein and Morin, 1999; Glass et al., 2000; Glass et al., 2003; Duncan et al., 2004). Furthermore, intra-DRN administration of muscimol (a GABA_a agonist) or DR4004 (a 5-HT_7 antagonist) attenuates, but does not entirely block, shifts in response to wheel confinement (Glass et al., 2003). As well, intra-DRN metergoline blocks SCN 5-HT increases induced by 3h of sleep deprivation (Glass et al., 2003). It also has been reported, however, that systemic retanserin (a 5-HT_5a/7 antagonist), NAN-190 (a 5-HT_1a/7 antagonist) or metergoline (a 5-HT_1/2a/7 antagonist) do not attenuate WC induced shifting in hamsters (Antle et al., 1998). Therefore, the involvement of 5-HT_7 receptors in behaviourally induced non-photic resetting is questionable.

It must be noted that the models proposed by Mintz et al. (1997) and Glass et al. (2003) are not mutually exclusive and both may have some merit. 5-HT_7 receptor activation in the DRN may also inhibit GABAergic projections to the IGL, for example, thereby disinhibiting IGL SCN output in a manner similar to that outlined by Mintz et al. (1997). Confirmation of such details will require further investigation, however. Given the apparent necessity of the IGL for shifts of a non-photic nature, to gain a better insight into the roles of the DRN and MnR in these phenomena, it may be instructive to carry out raphe stimulation studies in IGL lesioned animals.

As well, it should also be emphasized that the failure of 5-HT_1a/7 antagonists to attenuate shifts in response to behavioural manipulations should not be considered fatal blows to the models outlined above. As evidenced by the discussion thus far, different pathways and neurotransmitters contribute to non-photic phase resetting and it is likely that the phase shift resulting from a behavioral manipulation is sum product of different
neurochemicals acting at the level of the SCN or elsewhere. This point is further underscored by the observations that electrolytical or chemical lesions of some neural structures can attenuate or inhibit phase shifts to some stimuli while leaving shifts to other manipulations relatively intact (Meyer-Bernstein, & Morin, 1998; Marchant & Morin, 1999). Therefore, failure of a specific receptor antagonist to block phase shifts to a behavioral manipulation should not be considered strong evidence against a role for that receptor in phase control. The activation of redundant non-photic pathways may simply be sufficient to induce the resultant phase shift. That being stated, the validity of both models is challenged by the finding that chemically induced DRN lesions do not influence phase shifts to triazolam or to WC (Meyer-Bernstein, & Morin, 1998).

**Mice**

In mice, activation of 5-HT\textsubscript{1A} receptors \textit{in vitro} appears sufficient to produce non-photic like advances. During the subjective day, application of 5-HT or 8-OH-DPAT can phase advance the peak firing rate of SCN tissue from both wild type and 5-HT\textsubscript{7} knockout mice (Prosser et al., 1993; Sprouse et al., 2005; Prosser et al., 2006). As well, these shifts are inhibited by metergoline or WAY-1000635 (Sprouse et al., 2005). Unlike shifts to 8-OH-DPAT, however, \textit{in vitro} shifts to 5-HT in 5-HT\textsubscript{7} knockout mice are blocked by retanserin, thus suggesting that, in this species, 5-HT\textsubscript{5A} receptors are also involved in the control of circadian phase (Sprouse et al., 2005).

\textit{In vivo}, there have been conflicting reports regarding the ability of 8-OH-DPAT to induce phase shifts in mice (Antle et al., 2003; Horikawa and Shibata, 2004). However, intra SCN 5,7,-DHT administration impairs entrainment to daily scheduled treadmill running or to restricted homecage wheel access (Edgar et al., 1997; Marchant et
The reasons for the discrepant findings in the in vivo pharmacological studies are unclear. Differences between the in vivo and in vitro findings, however, may be the result of receptor upregulation or increased sensitivity induced in the slice by serotonergic deafferentation, a result supported by the observation that increasing serotonergic activity can block in vitro shifts to 5-HT or 8-OH-DPAT (Prosser et al., 2006).

Rats

Increased serotonergic activity is also sufficient to shift the rat SCN in vitro (See (Prosser, 2000) for a review). Quipazine, 5-HT, 5-CT (a 5-HT1A/1B/1D/5/7 agonist), buspirone, RU24969 (a 5-HT1A agonist), 8-OH-DPAT and fluoxetine (in the presence of L-tryptophan) have been reported to induce phase advances in SCN neural firing rates when applied during the subjective day (Prosser et al., 1990; Medanic and Gillette, 1992; Shibata et al., 1992; Lovenberg et al., 1993; Prosser et al., 1993; Sprouse et al., 2004a; Sprouse et al., 2004b; Sprouse et al., 2006). In rat tissue, 5-HT, 8-OH-DPAT or quipazine induced shifts in firing rhythms are inhibited by 5-HT7 receptor antagonists (ritanserin, mesulergine, SB-269970, metergoline, NAN-190) but not by selective 5-HT1A antagonists (pindolol, WAY-100,635, UH301), thus suggesting mediation by the former receptor (Lovenberg et al., 1993; Prosser et al., 1993; Sprouse et al., 2004a). 5-HT5A receptors may also play a role as retanserin (a 5-HT2A/5A/7 antagonist), but not mesulergine (a 5-HT2A/2C/7 antagonist), blocks 5-HT-induced in vitro shifts (Sprouse et al., 2004b).

In vivo, systemic or icv injection of 8-OH-DPAT has been reported to produce small phase advances in rats when administered in the mid-to-late subjective day (Edgar
et al., 1993). There are conflicting reports regarding the ability of systemic quipazine to perturb circadian phase, however (Edgar et al., 1993; Kohler et al., 1999).

In contrast to both mice and hamsters, results from several laboratories have indicated that 5-HT may also convey photic information to the clock during the active period. Systemic administration of quipazine during the dark period has been reported to produce photic like phase shifts in locomotor activity rhythms and melatonin secretion, and to induce SCN c-fos expression (Kennaway et al., 1996; Moyer et al., 1997; Kohler et al., 1999). Comparable effects also have been observed in vitro (Prosser et al., 1990; Prosser et al., 1993; Kalkowski and Wollnik, 1999). These effects are mimicked by 5-HT2A/2C receptor agonists and blocked by antagonists with affinity for the 5-HT2C receptors (Kennaway and Moyer, 1998; Ferguson and Kennaway, 2000; Kennaway et al., 2001; Varcoe et al., 2003). Similar to light (See the section on the Molecular Biology of Entrainment), these agonists also increase per 1 and 2 expression in the SCN at ZT16 (Varcoe et al., 2003). As these 5-HTC antagonists have no effect upon photically induced changes in melatonin secretion or SCN c-fos induction (Kennaway, 1997), it has been suggested that a retino-raphe-SCN pathway (present in rats but not in the hamster, (Morin, 1994; Shen and Semba, 1994; Kawano et al., 1996), may mediate these effects. Serotonin release may ultimately modulate glutamate signalling in the SCN as the effects of 5-HTC agonists on melatonin secretion and SCN c-fos expression are blocked by MK801 (Kennaway and Moyer, 1999).
Other Afferent Pathways

Orphanin FQ/nociceptin (OFQ/N), the endogenous ligand for the opiate-like receptor NOP-1 (Meunier et al., 1995; Reinscheid et al., 1995), a G protein-coupled receptor that does not bind opiates with high affinity (Bunzow et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994), may also contribute to non-photic phase resetting in rodents. This receptor is expressed in the rat and mouse SCN (Allen et al., 1999; Houtani et al., 2000), and in vitro application of OFQ/N or W-212393 (a NOP-1 agonist) suppresses rat SCN neurons (Allen et al., 1999). Systemic injection of W-212393, a NOP-1 agonist, dose dependently induces phase advances in body temperature rhythms in rats, and in locomotor activity rhythms in mice, when administered during the mid-subjective day (Teshima et al., 2005; Miyakawa et al., 2007). Injection of OFQ/N into the hamster SCN appears to be without effect, however, (Allen et al., 1999). The extent to which these shifts are dependent upon locomotor activity is unknown and, clearly, further investigation into the effect of these compounds on circadian phase is warranted.

Neurotensin cells also have been detected in the hamster IGL and appear to project to the SCN (Morin & Blanchard, 2001). In vitro, midday application of this neuropeptide to the rat SCN induces large phase advances in neuronal firing (Meyer-Spasche et al., 2002). To my knowledge, no in vivo tests have been carried out, however.

Recently neuromedin U and neuromedin S, novel peptides localized to the ventrolateral rat SCN (Nakahara et al., 2004; Mori et al., 2005), have been reported to induce non-photic like phase shifts when administered icv during the midday (Nakahara et al., 2004; Mori et al., 2005). Interestingly, icv injection of Neuromedin U or Neuromedin S induces SCN c-fos or per 1 expression during the light period (Nakahara
et al., 2004; Mori et al., 2005), results opposite to that seen for other non-photic stimuli (See the next section).

The Molecular Biology of Entrainment

Convergent evidence suggests that the mammalian circadian clock’s timing mechanism consists of interlocking positive and negative transcriptional feedback loops resulting in a rhythmic expression of several core clock components. To date, the putative clock genes include period (per) 1 and 2, cryptochrome (cry) 1 and 2, clock, bmal1 and rev-erb a (See Reppert and Weaver, 2001, 2002; Ko and Takahashi, 2006 for reviews). Briefly, the postulated mechanism of oscillation may be described as follows. During the subjective night, the basic helix-loop-helix PAS transcription factors Clock and Bmal1 dimerize in the cytoplasm, translocate to the nucleus, and, via E-box sequence binding, promote the expression of per, cry, and rev-erb a (Gekakis et al., 1998; Hogenesch et al., 1998; Kume et al., 1999; van der Horst et al., 1999; Bunger et al., 2000; Preitner et al., 2002). The Per and Cry proteins, in turn, accumulate in the cytoplasm during the light period, form a multimeric complex, and translocate to the nucleus where they inhibit the activity of Clock/Bmal1, thus attenuating their own transcription (Kume et al., 1999; Shearman et al., 2000; Sato et al., 2006). As well, the Rev-erb α protein inhibits bmal1 transcription, thereby forming an additional negative feedback loop (Preitner et al., 2002; Guillaumond et al., 2005). The Per/Cry complex, however, also inhibits Rev-erb α and, therefore, lifts the inhibitory drive on bmal1 transcription, forming a positive feedback loop that reinitiates the cycle (Preitner et al., 2002). Overall, this system constitutes an auto regulatory self-sustaining oscillator with a period of ~24h. It should be noted,
however, that the periodicity of this system is partially due to posttranslational modifications. Casein kinase 1ε and Casein kinase 1δ, for example, are involved in the phosphorylation of core clock components and mutations in these genes result in shortened circadian periods (Ralph and Menaker, 1988; Xu et al., 2005).

As outlined previously, photic information transduced by melanopsin expressing retinal ganglion cells (Gooley et al., 2001; Hattar et al., 2002; Hannibal et al., 2002) reaches the SCN directly via the RHT (Moore & Lenn, 1972) and indirectly via the IGL (Pickard, 1985; Zhang and Rusak, 1989). Light presented early in the dark period produces phase delay shifts while light applied during the late night induces phase advances (DeCoursey, 1960). Regardless of the timing of light exposure, however, photic stimulation during the subjective night results in a rapid expression of per1 or 2 (but not per3 nor cry1 or 2) suggesting that the regulation of SCN Per levels is pivotal for photically mediated clock resetting (Shearman et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998; Field et al., 2000). This contention is further supported by the observation that Per 1 antisense attenuates phase resetting induced by light or glutamate (GLU) both in vivo and in vitro (Akiyama et al., 1999).

The signal transduction pathways responsible for photically induced gene suppression have been partially elucidated and it appears that slightly divergent cascades are associated with photically induced phase delays and advances (See Gillette and Mitchell, 2002 for a review). Photic stimulation early or late in the subjective day results in a release of GLU from the RHT that is transduced by SCN NMDA receptors (Rea et al., 1993; Ding et al., 1994; Mintz and Albers, 1997). During the early light period, NMDA receptor activation increases intracellular Ca$^{2+}$ levels thus initiating a cascade of
events including the activation of various protein kinases, increased NO production, phosphorylation of the transcription factors CREB, ERK, and ELK-1 and, ultimately, the induction of per 1 or 2 expression (Ginty et al., 1993; Ding et al., 1994; Golombek and Ralph, 1994; Weber et al., 1995; Obrietan et al., 1999; Travnickova-Bendova et al., 2002; Coogan and Piggins, 2004). In the late dark period, when photic or glutamatergic stimulation results in a phase advance, the activation of SCN NMDA receptors initiates a similar signalling cascade with the additional steps of guanylyl cyclase activation and ryanodine receptor mediated increases in intracellular Ca\(^{2+}\) levels (Weber et al., 1995; Mathur et al., 1996; Ding et al., 1998). Photic stimulation during the dark period also induces the expression of c-fos in the SCN but its transcription appears unnecessary for photically-induced shifts (Colwell and Foster, 1992; Honrado et al., 1996).

Conversely, non-photic stimuli, which induce phase advances during the mid light period, when Per and Cry levels are maximal, appear to rapidly reset the clock through the suppression of clock genes. Wheel confinement (Maywood et al., 1999; Maywood and Mrosovsky, 2001; Yannielli et al., 2002), dark pulses (Fukuhara, 2004; Mendoza et al., 2004), 8-OH-DPAT (Horikawa et al., 2000; Duncan et al., 2005), in vitro NPY application (Fukuhara et al., 2001), triazolam, and intra-SCN NPY (Brewer et al., 2002; Maywood et al., 2002) or muscimol (Ehlen et al., 2006; Gamble et al., 2006) have been reported to suppress the expression of SCN per 1 or 2 in mice, rats, or hamsters. Decreasing SCN per 1 expression via the use of antisense induces non-photic like shifts suggesting that reduced per gene expression is a cause rather than a consequence of clock resetting (Hamada et al., 2004).
The signal cascades that mediate non-photic suppression of the per genes remain to be fully elucidated. These manipulations, however, are associated with decreased levels of phosphorylated CREB and ERKs, and suppressed c-fos expression in the SCN (Janik and Mrosovsky, 1992; Mead et al., 1992; Mikkelsen et al., 1998; Grossman et al., 2000; Coogan and Piggins, 2005). Increasing the levels of cAMP, and application of cAMP analogues or pituitary adenylate cyclase activating polypeptide (PACAP) have been reported to phase advance the firing rate of the rat or hamster SCN in vitro (Gillette and Prosser, 1988; Prosser and Gillette, 1989; Hannibal et al., 1997; Harrington and Hoque, 1997). In vivo, however, when applied during the subjective day, PACAP appears to produce only transient phase advances followed by stable phase delays (Piggins et al., 2001). These divergent findings are likely due to differences in the in vitro and in vivo environments and suggest that, under natural conditions, cAMP does not contribute to non-photic phase resetting. Further in vivo manipulations of SCN cAMP levels are required to support this contention, however. Nitric oxide donors (SN1 and SNAP) also induce non-photic like advances in the rat SCN in vitro (Starkey, 1996).

Several intracellular mechanisms that may contribute to the gating of non-photic shifts also have been identified. Casein kinase 1ε, for example, may be involved in the regulation of both the magnitude and the timing of phase shifts to non-photic stimuli in hamsters. Tau mutant hamsters tend to show larger phase shifts in response to non-photic stimuli at midday and a responsiveness to WC (Mrosovsky et al., 1992), NPY (Biello and Mrosovsky, 1996), and 8-OH-DPAT (Colecchia et al., 1996) earlier in the light period, particularly at CT1 where large phase advances have been observed. A similar role was initially postulated for the Ras G-protein Dexras1 as, in contrast to wildtypes, Dexras1
knockout mice show large phase advances to 3h of WC during the subjective day (Cheng et al., 2004). A recent study, however, has shown that, in these mice, shifts in response to the DD transition are of an equivalent size to that seen following WC (Dallmann and Mrosovsky, 2007), thus downplaying the role of this molecule. Interestingly, three days of 4h WC induces phase advances in Clock knockout mice, a response opposite to that observed in wild types (Challet et al., 2000).

Interactions Between Photic and Non-Photic Zeitgebers

The phase of a rhythm at any given point during the circadian day is the sum product of all zeitgebers that influence its controlling oscillator. Given that photic and non-photic zeitgebers do not occur in isolation, it is therefore essential to study the potential interactions of these two stimuli if a full appreciation of natural entrainment is to be gained. Double pulse experiments, where the two categories of entraining agents are presented in a single circadian cycle, have revealed that light can attenuate shifts in response to non-photic manipulations and vice-versa (See Challet and Pevet, 2003 and Yannielli and Harrington, 2004 for reviews).

Phase advance shifts to varied behavioural or chemical manipulations including wheel confinement (Mrosovsky, 1991), sleep deprivation (Grossman et al., 2000), systemic, intra-IGL, or intra-SCN 8-OH-DPAT (Penev et al., 1997; Challet et al., 1998; Ehlen et al., 2001), intra-SCN NPY (Biello and Mrosovsky, 1995; Maywood et al., 2002), intra-SCN muscimol (Mintz et al., 2002), and systemic triazolam (Joy and Turek, 1992), or fentanyl (Vansteensel et al., 2005) are attenuated or blocked by concomitant or subsequent light exposure. In vitro, optic chiasm stimulation or the activation of SCN
AMPAs or NMDA receptors attenuate NPY or 5-HT induced phase shifts (Biello et al., 1997a; Prosser, 2001) and, at the molecular level, NPY or WC induced reductions in SCN expression of per 1 and 2 are blocked by photic stimulation (Maywood and Mrosovsky, 2001; Maywood et al., 2002).

On the other hand, activity and/or arousal induced by WC in hamsters or by morphine injection in mice attenuate phase advance shifts to light, but not delays (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998; Mistlberger and Holmes, 1999; Lall and Biello, 2002; Edelstein et al., 2003). Acute sleep deprivation or 3 days of exposure to a novel wheel, however, have been reported to attenuate photically induced phase delays (Mistlberger et al., 1997; Challet et al., 2001; Christian and Harrington, 2002). The reasons for differential effects upon photic phase advances and delays are unclear but may relate to the disparate intracellular signal cascades underlying the two types of shifts (See the section on the Molecular Biology of Entrainment).

Non-photic attenuation of light-induced shifts also can be mimicked in vivo by the neurochemicals associated with the GHT or the raphe nuclei. Intra-SCN administration of NPY has been reported to attenuate light-induced phase advances but its effects upon delays remain controversial (Weber and Rea, 1997; Lall and Biello, 2003; Gamble et al., 2005). Similarly, increasing SCN 5-HT levels via direct injection (Weber et al., 1998), or through the administration of tryptophan (Glass et al., 1995) or fluoxetine (Challet et al., 2001) can decrease the size of phase shifts to light and these effects can be mimicked by intra-SCN or systemic administration of 5-HT$_{1A/7}$ or 5-HT$_B$ agonists in mice or hamsters (Rea et al., 1994; Pickard and Rea, 1997; Weber et al., 1998). However, there appears to be some species-specific differences in the effectiveness of these agents (Antle et al.,
Delta and mu opioid receptor agonists also have been reported to attenuate phase shifts to light (Tierno et al., 2002; Vansteensel et al., 2005). GABAergic modulation of photic phase shifting appears complex (Ralph and Menaker, 1989). However, a number of studies have reported that agents increasing activity at GABAa or GABAb receptors can attenuate phase delays or advances induced by light or intra-SCN NMDA (Colwell et al., 1993; Gillespie et al., 1997; Gillespie et al., 1999; Mintz et al., 2002).

*In vitro*, NPY application also can attenuate the phase shifting effects of glutamate, NMDA, and *in vivo* light pulses (Biello et al., 1997a; Yannielli and Harrington, 2000, 2001; Brewer et al., 2002). These inhibitory effects appear to be mediated by Y5 receptors as Y5 specific agonists attenuate photic phase advances and delays *in vivo* (Lall and Biello, 2003; Gamble et al., 2005) and NMDA induced shifts *in vitro* (Yannielli et al., 2004). As well, Y5 receptor antagonists block NPY induced inhibition of NMDA and light induced shifts (Lall & Biello, 2003; Yanielli et al., 2004).

Serotonergic inhibition of shifts to light appear to be due to both a blockade of neurotransmitter release from the RHT and a decrease in the activity of photically responsive IGL and SCN neurons, mediated by 5-HT7, 5-HT1A and 5-HT1B receptors. The evidence for these actions is as follows. As outlined above, in hamsters, 5-HT1A/7 and 5-HT1B agonists have been reported to attenuate phase shifts to light (Rea et al., 1994; Pickard and Rea, 1997; Weber et al., 1998). In the SCN, the *in vivo* neural photic response is attenuated by 5-HT, 8-OH-DPAT, or 5-CT (Ying and Rusak, 1994, 1997) and this attenuation can be blocked by antagonists of the 5-HT7 receptor (Ying & Rusak, 1997). Similarly, *in vitro* field potentials induced in the hamster or murine SCN by optic nerve stimulation are blocked by the application of 5-HT, 8-OH-DPAT, and the 5-
HT1A/1B receptor agonist TFMPP (Rea et al., 1994; Pickard et al., 1999; Smith et al., 2001). The effects of TFMPP appear to be mediated by the 5-HT1B receptor which is localized to the RHT terminal (Pickard et al., 1999), as TFMPP does not reduce invoked SCN EPSCs in tissue from 5-HT1B knockout mice (Pickard et al., 1999; Smith et al., 2001), nor does it effect EPSCs induced by locally applied glutamate (Pickard et al., 1999). 5-HT and 8-OH-DPAT also reduce photic responsiveness in the IGL but, in this area, the effects appear to be due to 5-HT1A receptor activation (Ying et al., 1993). Further evidence for a role of 5-HT1A and 5-HT7 receptors in this phenomenon include the finding that systemic pre-treatment with metergoline blocks WC induced attenuation of photic phase advance shifts (Mistlberger and Antle, 1998).

At the molecular level, it appears that non-photic stimuli may decrease photically induced phase shifts by suppressing light-induced SCN per gene expression. Pretreatment with brotizolam (a GABAa potentiator) decreases photically induced SCN peri and 2 expression in the late dark period (Yokota et al., 2000). Subsequent studies, however, have indicated that non-photic attenuation of light induced shifting may be more closely associated with the suppression of peri 2. NPY application in vitro completely suppresses SCN peri 2 expression induced by an in vivo light pulse in the early night but only has transient effects upon peri 1 (Brewer et al., 2002). Similarly, peri 1 expression is unaffected by acute wheel confinement or 3 days of wheel confinement, procedures that decrease photic phase advances or delays, respectively (Edelstein et al., 2003; Christian & Harrington, 2002). The signal transduction pathways mediating these photic/non-photic genetic interactions remain to be identified.
Interestingly, it appears that the suppression of non-photic input potentiates photically induced phase shifts. Administration of anti-NPY to the SCN, for example, increases photic phase advance shifts in hamsters (Biello, 1995) and similar results have been obtained for systemic or intra-SCN administration of selective Y₅ receptor antagonists (Lall and Biello, 2003; Yannielli et al., 2004). However, in vitro, Y₅ antagonists do not potentiate phase shifts to NMDA (Yannielli et al., 2004), a difference likely due to deafferentation in the slice preparation. A reduction in SCN serotonergic tone also potentiates phase shifts to photic stimulation. The elimination of serotonergic input via chemical lesioning has been reported to enhance both photic phase delays and advances (Morin and Blanchard, 1991; Penev et al., 1993; Bradbury et al., 1997). As well, pre-treatment with 5-HT₁A receptor agonists (MKC-242) or compounds that are agonists at presynaptic 5-HT₁A receptors and antagonists at postsynaptic 5-HT₁A receptors (BMY7378,515535, MDL 73005) have been reported to enhance photic phase delays or advances (Moriya et al., 1998; Takahashi et al., 2002; Gannon, 2003). As these studies utilized systemic administration, their site of action remains unknown.

Little is known regarding the effects of blocking other GHT inputs on photic phase shifting and further investigation is needed. Intra-SCN administration of the GABAₐ antagonist bicuculine increases photic phase delays in hamsters (Gillespie et al., 1996). However, systemic administration of this compound has been reported to block photically induced phase delays (Ralph & Menaker, 1989).

II. Induced Potentiation of Non-Photic Phase Shifts
Recently, it has come to light (no pun intended) that phase shifts in response to effective non-photic manipulations are greatly increased by short-term exposure to constant light (LL). At ZT6, shifts to novel wheel confinement, sleep deprivation, intra-SCN NPY or systemic 8-OH-DPAT are potentiated (~2.5 fold) by one to three days of LL (Mistlberger et al., 2002; Knoch et al., 2004; Landry and Mistlberger, 2005; Knoch et al., 2006). In addition to producing an increase in shifting, short-term LL also gates phase shifts to sleep deprivation and 8-OH-DPAT at times when the clock is normally insensitive to non-photic stimuli (Knoch et al., 2004). Following two days of exposure to constant light systemic 8-OH-DPAT injections induce large Type-0 advances early in the subjective day and delays of a similar magnitude in the mid to late subjective night (Knoch et al., 2004; Knoch et al., 2006). These effects appear to be due to a direct effect of light itself as opposed to a light-induced decrease in ambulation as two days without a homecage wheel under DD conditions does not increase phase shifts in response to SD and increasing locomotor activity in LL via food deprivation does not attenuate the effect (Mistlberger et al., 2002; Knoch et al., 2004).

The marked suppression of locomotor activity produced by LL first focused attention upon changes in serotonergic inputs as a potential mechanism for the potentiation of non-photic shifts. LL suppresses SCN serotonin release during the subjective night (Knoch et al., 2004) and the restoration of SCN 5-HT levels by reverse microdialysis moderately attenuates the potentiation of phase shifts to systemic 8-OH-DPAT (Knoch et al., 2004). Moreover, lower doses of 8-OH-DPAT that are ineffective under LD conditions can produce large phase shifts following a period of LL (Knoch et al., 2006). In addition, LL potentiated shifts to systemic 8-OH-DPAT or sleep deprivation
at ZT 0 are blocked by 5-HT$_{1A}$ antagonists (Knoch et al., 2006). Short-term LL, however, does not induce changes in the number of 5HT$_{1A}$, 5HT$_{1B}$, or 5HT$_{7}$ serotonin receptors in the SCN suggesting that the mechanism may involve a change in receptor sensitivity and/or intracellular modifications downstream of ligand binding rather than an upregulation in receptor expression (Duncan et al., 2005; Knoch et al., 2006). The serotonergic contribution to LL-potentiated shifts appears to differ with time of day. Unlike systemic 8-OH-DPAT, intra-SCN injections only produce potentiated shifts at ZT 0 suggesting mediation by sites outside of the SCN during the mid-subjective day and night (Knoch et al., 2006). Further investigation with varied receptor antagonists across the 24-day may provide better insight into serotonergic mediation of this phenomenon.

The observation that NPY-mediated shifts are also potentiated by LL exposure suggest that changes occur in non-serotonergic clock inputs as well (Knoch et al., 2004). The nature of any non-serotonergic modifications remains to be explored, however.

Alternatively, rather than producing a change in specific neurochemical inputs, chronic light exposure may modify a core clock parameter rendering it more susceptible to perturbation by entraining stimuli. Circadian clocks have been successfully modelled as limit cycle oscillators that can exhibit small or large phase shifts dependent upon the amplitude of the oscillation (Johnson et al., 2003). Constant light dampens locomotor activity, SCN serotonin release, and $per_1$ expression in the SCN core (Knoch et al., 2004; Duncan et al. 2005) and, therefore, it possible that this decrease in pacemaker amplitude could render the clock more prone to large phase shifts. However, phase delays in response to intra-SCN NMDA are not potentiated by exposure to constant light.
(Landry & Mistlberger, 2005), suggesting that the changes induced by short-term light exposure are specific to the non-photic pathway.

Whatever the mechanism responsible for LL-induced potentiation the increased shifting is not apparent in SCN per gene expression. Short-term constant light exposure does not increase the extent of 8-OH-DPAT induced clock gene suppression (Duncan et al., 2005).

The Current Experiments

Although much work has been done concerning the nature of non-photic shifting in Syrian hamsters, important questions remain. For example, further research is needed to fully elucidate the critical non-photic stimulus. To date, the current body of work suggests two possibilities. First, it may that some minimal amount of locomotor activity is necessary for shifts of a non-photic nature. Alternatively, it may be that arousal alone is sufficient to perturb circadian phase but, for this to be true, an inhibitory action of stress must be invoked. To address the confounding influence of stress, it will be useful to develop a procedure that induces arousal without concomitantly increasing locomotor activity or activating the hypothalamic-pituitary-adrenal axis. To this end, we have utilized modafinil, an atypical alerting compound that does not increase ambulation or have anxiogenic effects in animal models (Edgar & Siedel, 1997; Simon et al., 1994). The first series of experiments in this dissertation are concerned with characterizing the behavioral effects of this agent in hamsters and examining its influence upon circadian phase, and on phase shifts to both photic and non-photic stimuli. Later experiments focus on the hypothesized stress-induced attenuation of phase shifts to arousal and examine the
time course of stress effects upon non-photic shifts and potential mechanisms of interaction. The appendices also outline several experiments examining the phase response to the anxiogenic agent yohimbine and the effect of short-term constant light exposure on phase shifts to immobilization.

The primary neural afferents that carry non-photic information to the circadian clock have been identified. In hamsters, activation of the IGL appears both necessary and sufficient for non-photic clock resetting with neuropeptide Y, GABA, and enkephalins potentially transferring non-photic information to the clock (See section on the Intergeniculate Leaflet). Inconsistencies abound in the serotonin literature, however, and further work is needed (Morin, 1999; Mistlberger et al., 2000). The evidence to date suggests that, in hamsters, 5-HT release in the SCN and the IGL correlates with waking (Dudley et al., 1998; Grossman et al., 2004), but is not necessary for shifts of non-photic nature (Bobrzynska et al., 1996b), and is not sufficient for clock resetting unless the system is already sensitized (Ehlen et al., 2001; Knoch et al., 2006).

Little is known, however, regarding how areas upstream of the IGL or the SCN are activated during non-photic manipulations. As a first attempt to find potential non-photic inputs to these areas and to identify the neural basis for the differential phase resetting actions of arousal procedures, hamsters were exposed to various behavioral and pharmacological arousal-inducing procedures (wheel confinement, sleep deprivation, stress-loaded restraint, caffeine, and modafinil) and, using immunocytochemical techniques, the spatial patterns of c-fos expression (a marker of cellular activation, Cohen and Curran, 1989) were examined in several neurochemical systems (hypocretin, serotonin, and norepinephrine) known to be involved in the control of locomotor activity
and/or arousal. Given the evidence that the IGL may be the final common non-photic pathway to the circadian clock, we also examined \textit{c-fos} expression in this area.
CHAPTER 2: MODAFINIL AND CIRCADIAN RHYTHMS IN SYRIAN HAMSTERS: ASSESSMENT OF THE CHRONOBIOTIC POTENTIAL OF A NOVEL ALERTING COMPOUND

Abstract

In Syrian hamsters, behavioural procedures for inducing arousal (e.g., running in a novel wheel or gentle handling) can shift circadian rhythms when applied during the usual sleep period (‘subjective day’) and can attenuate phase shifts to light during the active period (‘subjective night’). This raises the possibility that drugs that affect behavioural state may have ‘chronobiotic’ potential. We characterized the effects of modafinil, an atypical alerting compound, on circadian rhythms in male Syrian hamsters. EEG recordings and video observations confirmed that modafinil dose-dependently increases wakefulness at the expense of slow-wave and paradoxical sleep with no increase in locomotor activity per unit time awake. Despite inducing arousal, modafinil at these doses (150 or 300 mg/kg), administered in the subjective day or early or late in the subjective night, did not perturb circadian phase. Modafinil (300 mg/kg) also had no effect on phase shifts to light exposure either early or late in the night and did not alter the size of phase shifts induced by running in a novel wheel for 3 h during the mid-day. Modafinil (300 mg/kg) did, however, decrease by ~50% the amount of novel wheel-stimulated running, moving leftward the dose-response relation between wheel revolutions and shift magnitude. These results indicate that, in Syrian hamsters, modafinil

---

1 The following chapter has been published in The Journal of Pharmacology and Experimental Therapeutics under the co-authorship of Ralph E. Mistlberger and Michael S. Pollock.
alone has no significant chronobiotic efficacy. Nevertheless, this agent may increase the sensitivity of the circadian pacemaker to non-photic stimuli, and may thus have some potential as a tool for promoting clock resetting in combination with behavioural strategies.

**Introduction**

Convergent evidence indicates that the hypothalamic suprachiasmatic nucleus is the locus of the mammalian circadian clock driving daily rhythms of behaviour and physiology (Klein et al., 1991). While daily light-dark (LD) cycles are the most powerful cue ('zeitgeber') for synchronizing this endogenous pacemaker to local time, non-photic stimuli can also exert considerable influence on circadian timing (Mistlberger and Skene, 2004). In Syrian hamsters, for example, behavioural arousal during the usual sleep period ('subjective day' in nocturnal animals), stimulated by running in a novel wheel, social interactions, saline injection, or gentle handling, can induce large phase advance shifts of circadian rhythms (Mrosovsky, 1996a; Mistlberger et al., 2000). Stimulated running and short-term sleep deprivation have also been reported to attenuate phase shifts in response to photic stimuli (Yannielli and Harrington, 2004).

Given this evidence for regulation of circadian timing by arousal, it is reasonable to suspect that pharmaceutical agents capable of influencing behavioural state may have similar effects. Such 'chronobiotics' could have potential therapeutic applications in the treatment of circadian sleep disorders, jet lag, and shift work malaise. While several benzodiazepine hypnotics have been shown to shift circadian rhythms in Syrian hamsters and squirrel monkeys (Mrosovsky, 1996a), there are no reports yet of circadian clock resetting by stimulants. Previous studies, however, have suggested that
pharmacologically induced arousal may be insufficient. Caffeine stimulates arousal when administered to Syrian hamsters in the mid-subjective day but does not induce phase shifts (Antle et al., 2001). Here we characterize the effects of the atypical stimulant modafinil on circadian rhythms in male Syrian hamsters and examine its interaction with photic and non-photic zeitgebers.

Modafinil is a unique wake-promoting pharmaceutical that is structurally unrelated to the classic psychomotor stimulants. This agent has been reported to prolong wakefulness in several mammalian species, including humans, with little, if any, rebound sleep (McClellan and Spencer, 1998; Boutrel and Koob, 2004; Bonnet et al., 2005). Modafinil’s pharmacological and behavioural profiles appear quite distinct from that of other stimulants (Akaoka et al., 1991; Engber et al., 1998; Scammell et al., 2000). Unlike amphetamine, modafinil does not produce stereotypical movement (Duteil et al., 1990; Simon et al., 1994), nor does it increase locomotor activity above levels expected for a normal waking animal (Edgar and Seidel, 1997). Furthermore, modafinil appears to exhibit a low potential for abuse (Schwartz, 2005). Clinical studies have demonstrated its efficacy in the treatment of excessive daytime sleepiness associated with narcolepsy and a variety of other conditions (1998; 2000; Schwartz, 2005). Given its clinical efficacy, reported low incidence of side effects, and apparent low abuse liability, modafinil has become an increasingly popular treatment and, therefore, it is pertinent that the chronobiotic properties of this agent be assessed.
Methods

Animals

Young male Syrian hamsters (60-170g, Charles River, Montreal, Quebec, Canada) were individually housed in polypropylene cages (47 x 26 x 20 cm) with wire mesh bottoms. The cages were equipped with 17.5 cm diameter running wheels and daily rhythms of locomotor activity were continuously monitored via microswitches interfaced with a computer. The subjects were kept under a 14:10 LD (~350 lx:0 lx) cycle with food and water provided ad libitum. All manipulations were approved by the university animal care committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as outlined by the U.S. National Institutes of Health.

Drugs

Modafinil (2-[(diphenylmethyl)sulfinyl]acetamide) was generously supplied by Cephalon, Inc. (West Chester, PA.). The drug was suspended in a sterile solution of 0.25% methylcellulose (Sigma-Aldrich Canada Ltd.) immediately prior to i.p. administration. Injections of sterile 0.25% methylcellulose served as the vehicle control.

EEG Recordings and Behavioural Observations

To mimic other behavioral arousal procedures known to induce large phase shifts of circadian wheel running activity rhythms, pilot EEG recordings and behavioral observations were carried out to select a dose that would produce virtually complete wakefulness over a 3 h period. These recordings were performed as described previously (Mistlberger et al., 2003). Briefly, under sodium pentobarbital (50 mg/kg) anesthesia and
using standard stereotaxic techniques, stainless steel screws and subcutaneous electrodes were implanted into the skull and nuchal muscle, respectively. The electrodes were connected to a plastic headcap that was subsequently cemented to the skull using dental acrylic. Following 7 days of recovery, the animals were habituated to a recording cable in an electrically shielded recording chamber for a period of 3 days. Hamsters were then injected with vehicle ($n = 4$), 100 mg/kg ($n = 2$), 150 mg/kg ($n = 1$), 200 mg/kg ($n = 1$) or 300 mg/kg ($n = 6$) modafinil at zeitgeber time (ZT) 6 and recorded for 12 h. In standard chronobiology notation, time within a LD cycle is referred to as ZT (there are 24 zeitgeber hours in a solar day), and ZT12 is defined as lights-off. ZT6 is thus 6 h before lights-off, the middle of the rest phase of the daily rest-activity cycle in nocturnal rodents. EEG and EMG signals were amplified (Grass model 79D, Grass Instruments), digitized at a sampling rate of 250 Hz, and stored on a computer using AcqKnowledge software (Biopac, Goleta, CA). Behavioral state was scored in 10s epochs as wakefulness (W), slow-wave sleep (SWS), paradoxical sleep (PS), transitional sleep (TS), or low amplitude sleep (LS) using unfiltered lateral EEG, lateral EEG filtered for delta waves (1-3 Hz), midline EEG filtered for theta (5-15 Hz), and EMG (high pass filtered at 50 Hz), as described previously (Mistlberger et al., 2003). Each epoch was classified as whichever state filled the majority of the time.

A second group of hamsters ($n = 10$) were administered vehicle or 300 mg/kg modafinil in counterbalanced order and videotaped in their home cages using an overhead camera. The injections were given at ZT6 and video recordings were made until ZT9. An observer blind to the experimental conditions later recorded the amount of time each animal engaged in active wake (arousal with ambulation), quiet wake (arousal without
ambulation, head up, eyes open), behavioural sleep (quiescent, sleep posture, eyes closed), grooming, feeding, and drinking. A switch from one behavioural category to another was only deemed to occur following a 10 s occurrence of the new behaviour. Locomotor activity also was quantified via image analysis using the methods of (Antle and Mistlberger, 2000). Briefly, the path travelled by each animal was traced onto acetate transparencies, digitised, and loaded into Image J (NIH). The total area of the path line was calculated and divided by the average line width, thus giving an approximation of the total distance travelled during the 3 h period. Both the EEG recordings and the behavioural observations were carried out under a 14:10 LD schedule.

**Effects of Modafinil on Circadian Phase When Administered During the Light Period**

To determine if modafinil can mimic the phase shifting effects of behavioural arousal procedures, hamsters (n = 20) stably entrained to LD were administered vehicle or modafinil (150mg/kg or 300mg/kg) at ZT6. The lights were then turned off, and left off for 4 days. The injections were given in a counterbalanced order and the hamsters were re-entrained to LD for at least 7 days between treatments. Phase shifts were measured as outlined below.

**Effects of Modafinil on Circadian Phase Following One Night of LL and its Interaction with Shifts to Novel Wheel Confinement**

Phase shifts induced by behavioural arousal are substantially potentiated by brief (1-3 days) exposure to constant light (LL) (Knoch et al., 2004). Therefore, we reasoned
that any phase shifts in response to modafinil administration would likely also be amplified and more easily detected by this procedure. Accordingly, following one night of LL, hamsters (n = 10) were left undisturbed in their home cage or administered vehicle or 300mg/kg modafinil at ZT4 in counterbalanced order. The room lights were turned out at ZT6 in all conditions and remained out for 4 days.

Caffeine has been reported to attenuate shifts in response to running in a novel wheel (Antle et al., 2001). We therefore sought to determine how modafinil might interact with other non-photic stimuli. Following one night of LL, vehicle or 300mg/kg modafinil was administered at ZT4 in combination with confinement to a novel wheel at ZT6. The latter manipulation involved placing the hamsters in a novel 33 cm diameter Wahmann running wheel for 3 h. This procedure stimulates running in most hamsters, and induces large (2 h or more) phase advance shifts in those hamsters that run more or less continuously (Mrosovsky, 1996a). The room lights were turned out during wheel confinement, and remained off for 4 days after the animals were returned to their home cages.

**Effects of Modafinil on Phase Shifts to Photic Stimuli**

Novel wheel-induced running and short-term sleep deprivation can attenuate phase shifts in response to light (Ralph and Mrosovsky, 1992; Mistlberger et al, 1997; Mistlberger and Antle, 1998; Challet et al, 2001). Therefore, we assessed whether modafinil-induced arousal might have a similar effect. Hamsters (n = 10) were injected with 300 mg/kg modafinil or vehicle at ZT6 or ZT12 with or without a 15 min light pulse (~190 lx) beginning at ZT13 (i.e., 1 h after lights-off). A second group of animals (n =
10) were administered 300 mg/kg modafinil or vehicle at ZT17 with or without exposure to a 15 min light pulse at ZT18. For the latter condition, the injections were given under dim red light (DDred, ~ 1 lx).

For the light pulse experiments described above, the room lights went out at ZT12 as scheduled on the manipulation day and then remained off for 4 or 7 days. The conditions were counterbalanced for order and separated by at least 14 days, resulting in a minimum of 30 days between successive drug administrations.

Data Analysis

Phase shifts were measured by comparing the time of spontaneous activity onset on day 2, 3 or 6 of DD following the manipulation with the average time of activity onset during the 3 days prior to the manipulation day (the so-called Aschoff Type II procedure; (Mrosovsky, 1996b). A computer algorithm was used to identify the onset of the main period of daily wheel running. Phase shift and activity measures were evaluated by within-subjects ANOVA with post hoc Bonferroni comparisons, Tukey multiple comparisons, or paired t-tests where appropriate. Means are presented +/- the standard error of the mean.

Results

Modafinil Induces Arousal When Administered During the Mid-day

The exploratory EEG recordings showed that modafinil, administered during the mid-light (i.e., sleep) period, dose-dependently increased W at the expense of SWS and PS, as compared to vehicle alone (Fig. 1a). At 300 mg/kg, modafinil produced virtually
continuous wakefulness for 6 h and this dosage was therefore selected for the subsequent phase shift experiments. More detailed examination at 300 mg/kg indicated elevated W and decreased SWS, at least for the remainder of the light period, as compared to vehicle injection alone (Fig. 1b-c). PS appeared almost completely suppressed over the 12 h recording period (Fig. 1d).

Behavioral observations confirmed increased arousal over a 3 h period following drug administration (Table 1). Modafinil (300 mg/kg) significantly increased the percentage of total wakefulness ($t(8) = 8.740, p < 0.0001$) and quiet wakefulness ($t(8) = 9.317, p < 0.0001$), and significantly decreased the percentage of behavioral sleep ($t(8) = 8.717, p < 0.0001$) and grooming ($t(8) = 2.649, p < 0.05$), as compared to vehicle alone. Drug administration, however, did not affect the percentage of active wakefulness ($t(8) = 1.125, p > 0.05$) and only marginally increased the total linear distance traveled within the home cage ($22.8 +/- 6.2m$ vs. $9.5 +/- 2.3m$; $t(8) = 2.214, p = 0.058$). The amount of activity per minute awake did not differ between modafinil and vehicle ($0.17+/-.02 m/min$ vs. $0.18+/-.02 m/min$; $t(8) = 0.12, p = 0.9$). One outlier was excluded from this analysis for a lack of response to the drug (likely due to a misplaced injection).

**Modafinil Does Not Shift Circadian Phase When Administered Mid-day**

Modafinil, administered at ZT6, did not induce phase shifts significantly different from the vehicle control injections, at doses of 150 mg/kg ($t(8) = 1.631, p = 0.1416$) or 300 mg/kg ($t(8) = 0.8082, n.s.$; Figs. 2a-c & 3).
Modafinil Does Not Alter Circadian Phase or Shifts in Response to Wheel Confinement Following a Day of LL

After 1 day in LL, injections of vehicle or of modafinil (300 mg/kg) at ZT4, followed by DD, resulted in phase shifts of 91 +/- 11 min and 97 +/- 24 min, respectively, that were not significantly different from each other ($t_{(9)} = 0.1990$, n.s.) or from the no-injection control condition (95 +/- 21 min; $t_{(9)} = 0.1539$, n.s. vs. vehicle alone; Figs. 2d-f & 3). A 3 h bout of running stimulated by confinement to a novel wheel from ZT6-9, following treatment with either vehicle or modafinil (300 mg/kg) at ZT4, induced large phase advance shifts of 255 +/- 38 min and 210 +/- 39 min, respectively. The difference between drug and vehicle conditions was again not significant ($t_{(9)} = 1.678$, $p > 0.05$; Figs. 2g-h & 3).

The large phase advance shifts apparent in the control, drug and wheel confinement conditions following a night of LL could be secondary to a shorter circadian period. Nineteen hamsters in total were released into DD in the no-injection control condition following entrainment to LD and following a night of LL. Regression lines were fit to activity onsets during each day of DD to obtain an estimate of the circadian period following the initial shift. The circadian period did not differ between lighting conditions (23.7 +/- .33 h vs. 23.65 +/- .22 h, in the LD vs. LL conditions, respectively; $t_{(18)} =0.65$, $p=.521$).
Modafinil Suppresses Wheel Running Activity When Administered Mid-day Following One Night of LL

Inspection of the activity charts revealed that modafinil suppressed spontaneous wheel running activity, both during novel wheel confinement and during the following night in the home cage. To quantify this effect, the mean number of revolutions over an 18 h period following administration was compared across the DD, vehicle, and 300 mg/kg conditions with or without wheel confinement from ZT6-9. Also, to assess the time course of any drug effect, the 18 h period following drug administration was divided into six 3 h blocks (ZT6-9, ZT9-12, ZT12-15, ZT15-18, ZT18-21, and ZT21-24), and the mean number of wheel revolutions was compared across these time blocks.

The overall within two-way ANOVA revealed a significant effect of condition (F (4,36) = 6.259, p < 0.001), time of day (F (6,54) = 13.132, p < 0.0001), and a significant interaction (F (24,216) = 9.509, p < 0.0001). By comparison with the vehicle control conditions, modafinil injection at ZT4 was associated with a 51.5% decrease in 18 h cumulative wheel revolutions (t (9) = 3.560, p < 0.01), while modafinil combined with wheel confinement from ZT6-9 was associated with a 55.4% decrease (t (9) = 3.039, p < 0.05; Fig. 4). The number of wheel revolutions during wheel confinement was decreased by 53.3% following modafinil administration by comparison with vehicle (t (9) = 4.891, p < 0.001; Fig. 4). Modafinil also significantly suppressed wheel running by 64.6% in the home cage from ZT12-15, by comparison with the vehicle injection condition (t (9) = 4.316, p < 0.001; Fig. 4).

Given the significantly reduced level of running during wheel confinement in the modafinil treatment condition, it bears repeating that the phase shifts induced by wheel
confinement did not differ between vehicle control and modafinil conditions (Fig. 3c). A scatter plot clearly shows large phase advance shifts associated with lower levels of novel wheel-induced running in the modafinil condition (Fig. 5).

**Modafinil Does Not Perturb Circadian Phase When Administered During the Night and Does Not Alter Light-Induced Shifts**

A 15 min light pulse at ZT13 induced a significant phase delay shift in both the vehicle \( t(9) = 3.813, p < 0.01 \) and drug \( t(9) = 4.381, p < 0.001 \) conditions (Figs. 6a-e & 7a). The drug and vehicle control conditions did not differ significantly \( t(9) = 1.238, n.s. \). Similar phase delays were obtained for 15 min light pulses that were preceded by injections of modafinil or vehicle at ZT6, rather than ZT12 (Figs. 6f-g & 7b). Again there was no difference between drug and vehicle conditions \( q(5,45) = 1.156, p > 0.05 \).

At ZT18, a 15 min light pulse elicited a significant phase advance shift in both the vehicle \( t(9) = 3.472, p < 0.01 \) and drug conditions \( t(9) = 5.510, p < 0.001 \; \text{Figs. 6i-l & 7c} \). Differences between the drug and vehicle control conditions were again not significant \( t(9) = 2.301, p > 0.05 \).

**Discussion**

In agreement with studies of other mammalian species (Edgar and Seidel, 1997; McClellan and Spencer, 1998; Boutrel and Koob, 2004), modafinil administered to Syrian hamsters during the normal sleep period dose-dependently increased wakefulness at the expense of SWS and PS but only marginally increased locomotor activity. Despite a strong induction of arousal, modafinil, at these doses, administered in the light or the dark period, did not induce significant phase shifts by comparison with vehicle control
injections. Modafinil also did not induce phase shifts after a day in LL, a treatment that greatly potentiates phase shifts in response to at least some behavioural arousal procedures (Knoch et al., 2004). These results, in combination with the recent finding that modafinil does not eliminate excessive sleepiness during the night-shift (suggesting no significant circadian adaptation to night work; Czeisler et al., 2005), indicate that this agent has little, if any, resetting action on the mammalian circadian pacemaker. Modafinil also had no effect on phase shifts to sub-saturating light pulses presented either early or late in the night, suggesting that this compound is unlikely to interfere with photic entrainment when used therapeutically.

The absence of phase shifts in response to modafinil is concordant with our observations that two other wake-promoting drugs, caffeine and yohimbine, also do not induce phase shifts in Syrian hamsters when administered during the mid subjective-day (Antle et al., 2001); See Appendix B). Given that behavioural manipulations capable of shifting circadian rhythms strongly stimulate arousal, it seems surprising that these agents do not have similar clock resetting effects. These results, however, do build on evidence from recent behavioural studies indicating that arousal, per se, is not sufficient to induce phase shifts. Physical restraint (with intermittent compressed air stimulation) or confinement to a small pedestal over water, procedures that induce continuous waking and elevated cortisol, do not perturb circadian phase (Mistlberger et al., 2003). Arousal states are heterogeneous, and the constellation of neural and endocrine correlates unique to each of these states appears to have differential effects on the circadian pacemaker. Which of these correlates is critical for phase shifting has not been fully resolved, and it is conceivable that some correlates promote shifting, while others block shifting.
Given that restraint and pedestal confinement prevent locomotion and do not
induce shifts, neural and endocrine correlates of activity are of special interest as critical
clock resetting stimuli. High intensity activity ('exercise') is clearly not necessary for
clock resetting, given that arousal enforced by the sleep deprivation procedure of gentle
handling can induce large phase shifts despite minimal stimulation of locomotor activity
(Antle and Mistlberger, 2000). However, hamsters subjected to this procedure are free to
move about in their cages, and accumulate ~80 m of linear distance travelled in 3 h in
standard size cages (47 x 26 x 20 cm). This is much less activity than is accumulated by
hamsters confined to a novel wheel, which may run several kilometers or more in 3 h.
Nonetheless, a low level of forward locomotion sustained over 3 h may be both necessary
and sufficient to induce clock resetting. In the present study, modafinil potently
stimulated arousal but only marginally increased locomotor activity over a 3 h period, by
comparison with the vehicle control condition. The linear distance travelled averaged
only ~20 m, or about one quarter the distance measured in hamsters sleep deprived by
gentle handling (Antle and Mistlberger, 2000). The predominant behaviour displayed by
hamsters during modafinil-induced arousal in the usual sleep period can be described as
'restless fidgeting', with relatively little forward locomotion; the subjective impression is
of an animal that wants to sleep but cannot. A working hypothesis, therefore, is that
sensori-motor correlates of forward locomotion are necessary for clock resetting to
arousal, and that these correlates are not sufficiently present in the arousal state induced
by modafinil.

An alternative perspective is that behavioural stress procedures (e.g., confinement
to a restraint tube or a pedestal) and alerting compounds such as modafinil, caffeine and
yohimbine have in common neural correlates that block non-photic phase shifts. If so, then modafinil would be predicted to inhibit phase shifts induced by running in a novel wheel, a property already demonstrated for caffeine (Antle et al, 2001). Several studies have reported a sigmoidal relationship between the magnitude of running-induced phase shifts and the number of wheel revolutions generated during wheel confinement, with small advance shifts (~30 min) typically occurring below 4000 wheel revolutions in 3 h and maximal advances (~2 h) above 5000 revolutions (Janik and Mrosovsky, 1993; Bobrzynska and Mrosovsky, 1998). Unexpectedly, in the current study, phase shifts following wheel confinement were not decreased despite a modafinil-induced 50% reduction in activity during the procedure, and large advances were evident at well under 3000 revolutions. This novel finding distinguishes modafinil from caffeine, and weighs against the idea that modafinil activates neural pathways that inhibit phase resetting correlates of arousal or locomotor activity.

This result instead appears to suggest that modafinil may reduce the threshold for shifts induced by running in a novel wheel, thus shifting leftward the dose-response relation between wheel revolutions and shift magnitude. Alternatively, it may be that LL alone increased the sensitivity of the circadian pacemaker to the phase resetting correlates of stimulated activity, thus permitting normal size shifts despite the lower levels of running in the modafinil condition. One obvious prediction of this hypothesis is that the dose-response relation between running levels and shift magnitude should also be shifted left in the vehicle control condition. In the current study, none of the vehicle treated hamsters ran at intermediate levels, thus there are no data by which to directly evaluate
this prediction. Given the potential value of a compound that might potentiate the phase shifting effects of exercise, this issue warrants further attention.

Modafinil administration had pronounced effects on home cage running activity. It is important to note, however, that despite a modafinil-induced decrease of wheel running, particularly during the early night, ambulation was not absent following drug administration. Indeed, our behavioural observations, in agreement with previous investigations of other species (Simon et al., 1995; Edgar and Seidel, 1997), revealed no change in the level of activity per minute awake in the home cage following modafinil treatment, as compared to vehicle. In Syrian hamsters, amphetamine also has been reported to inhibit wheel running at doses that increase or do not affect locomotor activity in an open field (Peterson and Morin, 1983; Della Maggiore and Ralph, 2000). Therefore, it is worth highlighting that, in this species, the methodology used to assess pharmacological effects on locomotor activity may impact the experimental outcome.

The cellular actions of modafinil are not yet fully elucidated, although changes in dopaminergic and noradrenergic transmission have been implicated (Duteil et al., 1990; Lin et al., 1992; de Saint Hilaire et al., 2001; Saper and Scammell, 2004). Most recently, it has been suggested that modafinil may influence behaviour through a blockade of dopamine reuptake followed by subsequent dopaminergic stimulation of α1 adrenergic receptors (Wisor and Eriksson, 2005). Increased glutamatergic and histaminergic transmission, and decreased GABAergic transmission have also been reported in distinct brain regions following modafinil treatment (Ferraro et al., 1996; Ferraro et al., 1999; Ishizuka et al., 2003). Interestingly, modafinil has been shown to increase the release of cortical 5-HT both in vitro and in vivo (Ferraro et al., 2000; de Saint Hilaire et al., 2001),
with differential efficacy across brain areas. Non-photic shifts can be induced by NPY, 5-HT and GABA agonists, and can be blocked by glutamate agonists (Morin and Allen, 2006). Data on how modafinil affects transmission at these synapses within the circadian system are lacking.

In addition to its efficacy for the treatment of excessive daytime sleepiness, modafinil has been reported to enhance cognitive performance in both sleep-deprived (Bonnet et al., 2005) and normal volunteers (Turner et al., 2003; Baranski et al., 2004) and may have some utility in the treatment of a variety of psychological disorders (Rugino and Samsock, 2003; Ninan et al., 2004). It is likely, therefore, that utilization of this compound will only increase in the coming years. In summary, the current study suggests that acute administration of modafinil, despite potent stimulation of arousal, has little effect on the circadian pacemaker and is unlikely to perturb circadian phase or alter photic entrainment in individuals using this compound therapeutically. This agent, however, is administered chronically in the clinical environment and further study is required to rule out the possibility of long-term effects on circadian timing. The present results also suggest that modafinil may modulate the relationship between phase shifts and locomotor activity and may thus have some potential as a tool for promoting clock resetting in combination with exercise or other behavioural arousal procedures. Further, the current data add support to the idea that arousal, per se, is not sufficient to induce phase shifts of a non-photic nature and demonstrate that different states of arousal can have divergent effects on the mammalian circadian clock.
Figures

Figure 1. Effects of vehicle and modafinil on behavioural state following administration at ZT6. (A) Effects of increasing dosages of modafinil on the percentage of behavioural state over a 6 h period following administration. (B) Effects on wakefulness, (C) slow-wave sleep, and (D) paradoxical sleep over a 12 h period following administration. Data shown as mean +/- SEM where appropriate. The shading indicates the dark period of the LD cycle.
Figure 2. Wheel running activity records of representative hamsters illustrating phase shifts in response to (A) vehicle, (B) 150 mg/kg modafinil, or (C) 300 mg/kg modafinil administered at ZT6. Also shown are phase shifts in response to (D) one night of constant light [LL] followed by constant darkness, (E) vehicle, (F) or 300 mg/kg modafinil administration at ZT4, and (G) LL followed by vehicle (H) or 300mg/kg modafinil administration at ZT4 with novel wheel confinement from ZT6-9. Each horizontal line represents a 24 h period with wheel revolutions plotted in 10 min bins from left to right. Wheel running is indicated by vertical deflections and shading marks the dark period of the LD cycle. The circle and diamond symbols represent vehicle and drug injections, respectively, and the ‘v’ markers designate the beginning and end of novel wheel confinement.
Figure 3. Mean phase shifts in response to vehicle, 150 mg/kg modafinil, and 300 mg/kg modafinil administered at ZT6. Also illustrated are shifts in response to one night of constant light [LL] followed by constant darkness [DD], vehicle [Veh], or 300 mg/kg modafinil administration at ZT4 with and without novel wheel confinement [WC] from ZT6-9. Data shown as mean +/- SEM. ** = conditions significantly different at p < 0.001.

Figure 4. Mean wheel revolutions over six 3 h periods following one night of constant light [LL] and subsequent exposure to constant darkness [DD], vehicle [Veh], or 300 mg/kg modafinil administration at ZT4, with or without novel wheel confinement [WC] from ZT6-9. Data shown as mean +/- SEM. ** = significantly different from appropriate vehicle or drug alone control condition at p < 0.001. +++ = significantly different from Veh + WC condition at p < 0.001.
Figure 5. Scatter plot of resultant phase shifts versus the number of wheel revolutions stimulated by novel wheel confinement (WC) from ZT6-9 following one night of constant light and the administration of vehicle [Veh] or 300 mg/kg modafinil at ZT4.
Figure 6. Wheel running activity records of representative hamsters illustrating phase shifts in response to (A) constant darkness, (B) vehicle, or (C) 300 mg/kg modafinil administered at ZT12. Also shown are shifts in response to (D) a 15 min light pulse (LP) at ZT13 preceded by vehicle, or (E) 300 mg/kg modafinil administered at ZT12 and (F) a 15 min LP at ZT13 preceded by vehicle, or (G) 300 mg/kg modafinil administration at ZT6. The last five panels illustrate phase shifts in response to (H) constant darkness, (I) vehicle or (J) 300 mg/kg modafinil administration at ZT17 and (K) a 15 min LP at ZT18 preceded by vehicle or (L) 300 mg/kg modafinil at ZT17. The circle, diamond, and square symbols represent vehicle injections, drug injections, and light pulses, respectively.
Figure 7. Mean phase shifts in response to constant darkness (DD), vehicle (Veh) or 300 mg/kg modafinil, with or without exposure to a 15 min light pulse (LP). (A) DD, veh or modafinil administered at ZT12 with or without a LP at ZT13, (B) DD, veh or modafinil administered at ZT16 with a LP at ZT13. (C) DD, veh or modafinil administered at ZT17 with or without a LP at ZT18. Data shown as mean +/- SEM. * = conditions significantly different at p < 0.05; ** = conditions significantly different at p < 0.01; *** = conditions significantly different at p < 0.001.
Table 1. Effects of vehicle and modafinil on behavior following administration at ZT6. The mean percentage of time spent engaged in various behaviors in the home cage over a 3 h period following modafinil or vehicle administration at ZT6. Data shown as mean (± SEM).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Vehicle</th>
<th>Modafinil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active Wake</td>
<td>3.1 (1.8)</td>
<td>4.6 (1.3)</td>
</tr>
<tr>
<td>Quiet Wake</td>
<td>15.1 (3.3)</td>
<td>80.7 (4.4)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Behavioral Sleep</td>
<td>70.3 (4.2)</td>
<td>9.7 (3.6)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Grooming</td>
<td>6.4 (1.0)</td>
<td>2.9 (0.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Feeding</td>
<td>4.6 (1.0)</td>
<td>2.0 (0.7)</td>
</tr>
<tr>
<td>Drinking</td>
<td>0.4 (0.1)</td>
<td>0.2 (0.1)</td>
</tr>
<tr>
<td>Total Wake</td>
<td>29.6 (4.2)</td>
<td>90.4 (3.5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> = conditions significantly different at p < 0.001, <sup>b</sup> = conditions significantly different at p < 0.05.
CHAPTER 3: DIFFERENTIAL EFFECTS OF AROUSAL PROCEDURES ON CIRCADIAN RHYTHMS IN HAMSTERS: NEURAL CORRELATES IN THE MIDBRAIN RAPHE AND LOCUS COERULEUS

Abstract

In Syrian hamsters, circadian rhythms can be markedly phase shifted by arousal stimulated by continuous running or gentle handling (Bobrzenska & Morosovsky, 1998; Antle & Mistlberger, 2000), but not by inescapable stress (confinement to a restraint tube or pedestal over water; Mistlberger et al., 2003) or anxiogenic drugs (caffeine, yohimbine; Antle et al., 2001). The dorsal and median raphe nuclei (DRN, MnR) have been implicated in clock resetting by arousal (Morin, 1999; Mistlberger et al., 2000) and, in rats and mice, exhibit strong regionally specific responses to inescapable stress and anxiogenic drugs (Lowry, 2002; Abrams et al., 2005). To examine a possible role for the midbrain raphe nuclei in the differential effects of these arousal procedures on circadian rhythms, hamsters were aroused for 3 h in the mid-sleep period by confinement to a novel running wheel, gentle handling or physical restraint (with loud compressed air stimulation), and regional expression of c-Fos and tryptophan hydroxylase (TrpOH) was quantified immunocytochemically in the DRN, MnR and locus coeruleus (LC). Neither wheel running nor gentle handling altered c-fos expression in the DRN, MnR or LC, although a small increase was evident in the caudal interfascicular DRN region. In contrast, arousal by stress-loaded physical restraint significantly elevated c-fos expression in rostral DRN neurons and in the LC. These results suggest that activation of the rostral
DRN or the LC is neither necessary nor sufficient for shifts of a non-photic nature and suggest that stress-induced activation of some DRN or LC neurons may oppose clock resetting in response to arousal during the daily sleep period. More generally, these results complement evidence from other rodent species for functional topographic organization of the DRN.

Introduction

Convergent evidence indicates that the hypothalamic suprachiasmatic nucleus (SCN) is the locus of the master circadian pacemaker controlling daily rhythms of physiology and behaviour (Moore and Eichler, 1972; Moore, 1983; Ralph et al., 1990). This endogenous clock is primarily synchronized to the environmental light/dark (LD) via photic stimulation (Moore and Lenn, 1972; Rea, 1998). However, experimental manipulations affecting locomotor activity and/or arousal are also capable of exerting considerable influence upon circadian phase (Mrosovsky, 1996a; Mislberger et al., 2000). Although identification of the retinohypothalamic tract as the primary photic entrainment pathway occurred more than thirty years ago, a complete elucidation of non-photic pathways awaits. The thalamic intergeniculate leaflet (IGL) appears to be a pivotal structure (Rusak et al., 1989; Biello et al., 1991; Mikkelsen et al., 1998) but the afferent inputs that are necessary or sufficient to activate the IGL and shift the SCN during non-photic procedures have not yet been specified. Arguably this gap in our knowledge stems partly from the complex nature of non-photic stimuli and our inability to precisely determine what aspects of these procedures are crucial for clock resetting.

Early studies suggested that locomotor activity was critical for phase shifting in response to a range of non-photic stimuli, including cage changes, novel wheel
confinement, dark pulses and benzodiazepines (Reebs and Mrosovsky, 1989a; Turek and Losee-Olson, 1986; Van Reeth and Turek, 1989). The magnitude of phase shifts induced by these manipulations is positively related to measures of locomotor activity (Mrosovsky and Salmon, 1990; Mistlberger et al., 1996; Bobrzynska and Mrosovsky, 1998) and if activity is blocked or restricted, phase shifts to some of these procedures are absent or strongly attenuated (Reebs and Mrosovsky, 1989; Van Reeth and Turek, 1989). Subsequent work demonstrated vigorous physical activity to be unnecessary, as keeping the animals awake induced comparable phase advance shifts, despite relatively low levels of activity (Antle and Mistlberger, 2000). Thus, the critical variable appeared to be arousal or some consequence of sleep disruption per se. Further investigation, however, has indicated that behavioural arousal is not always sufficient for inducing phase shifts. Stressful arousal procedures that prevent ambulation (e.g., physical restraint, confinement to a platform over water) or anxiogenic drugs (Antle et al., 2001; See Appendix B) do not effect circadian phase despite the induction of arousal (Mistlberger et al., 2003).

The current body of data therefore supports two possible hypotheses regarding the nature of the critical non-photic stimulus. While vigorous ‘exercise’ is clearly not necessary, when hamsters have little or no opportunity to locomote, phase shifts never occur, despite arousal confirmed by behavioural, polygraphic and endocrine measures (Mistlberger et al., 2003). Therefore, it may be that non-specific arousal with some minimal level of locomotor activity is necessary for shifts of a non-photic nature. Alternatively, behavioural arousal alone may be sufficient to phase advance the circadian pacemaker, but for this to be true, an inhibitory action of stress and/or anxiety must be invoked.
The midbrain serotonergic raphe nuclei have been implicated in non-photic regulation of circadian rhythms (Morin, 1999; Mistlberger et al., 2000) and in the response to inescapable stress (Lowry, 2002). The SCN and IGL are innervated by the median raphe nucleus (MnR) and the dorsal raphe nucleus (DRN), respectively (Hay-Schmidt et al., 2003; Meyer-Bernstein and Morin, 1996; Moga and Moore, 1997) and effective non-photic manipulations are associated with increased 5-HT release in the circadian system (Dudley et al., 1998; Grossman et al., 2000; Grossman et al., 2004). As well, serotonergic agonists can modulate shifts to light pulses (Pickard & Rea, 1997; Weber et al., 1998), and under certain conditions can induce non-photic type phase shifts (Cutrera et al., 1996; Challet et al., 1998; Ehlen et al., 2001). Although most stressful and anxiogenic stimuli tend not to induce phase shifts (Antle et al., 2001; Mistlberger et al., 2003), subpopulations of serotonergic cells within the DRN are preferentially activated by inescapable stress (Grahn et al., 1999; Grahn et al., 2002; Abrams et al., 2004; Amat et al., 2005) and anxiogenic drugs (Abrams et al, 2005). Thus, it may be that some patterns of serotonergic activation are conducive to clock resetting, while others may oppose it.

As a first step to evaluate this hypothesis, hamsters were subjected to novel wheel confinement, sleep deprivation by gentle handling, or physical restraint and examined using immunohistochemical techniques for the expression of c-fos (an immediate early gene whose expression largely correlates with neuronal firing, see Cohen and Curran, 1989) in tryptophan hydroxylase (TrpOH, the rate limiting enzyme in 5-HT synthesis) expressing cells. Given the evidence for noradrenergic mediation of the serotonergic response to inescapable stress (Grahn et al., 2002), we also examined c-fos expression in the locus coeruleus (LC).
Materials and Methods

Animals

Young male Syrian hamsters (n = 24, 100-120 g, Charles River, Montreal, Quebec, Canada) were individually housed in polypropylene cages (47 x 26 x 20 cm) with wire mesh bottoms and pipes for light avoidance. The cages were equipped with 17.5 cm diameter running wheels and daily rhythms of locomotor activity were continuously monitored via microswitches interfaced with a computer. The subjects were kept under a 14:10 LD (~225 lx:0 lx) cycle with food and water provided ad libitum. All manipulations were approved by the university animal care committee and carried out in accordance with the guidelines prepared by the Canadian Council on Animal Care.

Behavioural Manipulations

Once stable entrainment was attained the hamsters were randomly divided into four groups and exposed to various non-photic arousal procedures with disparate effects upon circadian phase. Hamsters in the DD control condition were left undisturbed in their homecages. For the wheel confinement (WC) procedure, animals were confined to a novel 33 cm diameter Wahmann running wheel, a procedure that, as outlined above, stimulates running in most hamsters, and induces large (2 h or more) phase advance shifts in those hamsters that run more or less continuously (Mrosovsky, 1996a). Hamsters subjected to the sleep deprivation by gentle handling (SD) condition were continuously observed in the homecage and stimulated (tactile or auditory) upon adoption of a sleep posture. This procedure induces phase advance shifts equivalent to those seen following
WC (Antle & Mistlberger, 2000). For stress-loaded restraint (SLR), hamsters were placed into opaque polyvinylchloride tubes (15 cm long, 4 cm internal diameter) sealed on either side with rubber mesh and stimulated with compressed air (110 psi through 3/8 in. tubing, 107dB intensity) to prevent behavioural sleep. Despite the sustained arousal produced by this latter manipulation, the SLR procedure has surprisingly little effect upon circadian phase (Mistlberger et al., 2003). For illustrative purposes, images of the various behavioural manipulations are included in Fig. 8.

These three-hour manipulations began at zeitgeber time (ZT) 6 (i.e., six hours before dark onset) and the overhead room lights were turned out at the start of each procedure. The SD and SLR manipulations were carried out under very dim red light (~ 1 lx) and running wheels were locked during the DD and SD conditions.

**Perfusion and Fixation**

At ZT9, immediately following the behavioural manipulations, the animals were euthanized under dim red light with an overdose of sodium pentobarbital and perfused transcardially with 50 ml of 0.1M phosphate buffered saline (PBS, ph ~7.3) followed by 50 ml of 4% paraformaldehyde in PBS (PFA, ph ~7.3). Brains were removed, postfixed for 2 h in PFA, and cyroprotected in 20% sucrose in PBS overnight. The tissue was then sectioned (30μm thickness) in the coronal plane using a cryostat and stored at -20°C in de Olmos cyroprotectant (Watson et al., 1986) until immunocytochemical processing.
Immunocytochemistry

To visualize c-Fos, the sections were first washed in 0.3% Triton X-100 in PBS (PBSx, 3 X 5 min rinses) then incubated for 1h with 10% normal horse serum (NHS, Vector Laboratories, Ltd.) in PBSx containing a 1:5 dilution of avidin (Vector Laboratories, Ltd.). This was followed by a brief wash (3 min) in PBSx. Next the tissue was incubated with a goat anti-c-Fos polyclonal IgG (Santa Cruz Biotechnology, Inc., cat. # SC-52-G, 1:5000) in 1% NHS in PBSx containing a 1:5 dilution of biotin (Vector Laboratories, Ltd.). This antibody is raised against the N-terminus of the human c-Fos protein and reacts with a protein having a molecular weight of approximately 62kD (manufacturer’s technical information). Incubation with the primary antibody occurred at 4°C over a period of 36-48 h and was followed by rinsing with PBSx (3 X 5 min). The slices were then incubated with a biotinylated horse anti-goat antibody (Vector Laboratories, Ltd., 1:250 in 1% NHS in PBSx) for 1 h at room temperature and again rinsed in PBSx (3 X 5 min). Next, the tissue was exposed to 0.3% H2O2 in PBSx for 30 min followed by another series of rinses. The sections were then incubated with ABC (Standard Vectastain Elite Kit, Vector Laboratories, Ltd.) for 1 h at room temperature and washed in PBSx (3 X 5 min). Last, the stain was developed with 0.04% DAB in Tris buffer containing containing 0.1% H2O2 and 0.03% NiCl2 to produce a black nuclear stain.

To label TrpOH expressing cells, the sections were subsequently incubated for 1h in 10% normal rabbit serum (NRS, Vector Laboratories, Ltd.) in PBSx with a 1:5 dilution of avidin, followed by incubation with a sheep anti-TrpOH polyclonal IgG (Biogenesis, cat. # 9260-2505, 1:5000 in 5% NRS in PBSx containing a 1:5 dilution of biotin). This
antibody is raised against recombinant rabbit TrpOH isolated from E. coli and stains a single band corresponding to 55 kD (manufacturer's technical information). The tissue was incubated with this antiserum at 4°C for 36-48 h followed by rinses (PBSx, 3 X 5 min). This was followed by incubation with a biotinylated rabbit anti-sheep antibody (Vector Laboratories, Ltd., 1:250 in 5% NRS in PBSx) for 1 h at room temperature. Next, the sections were washed in PBSx (3 X 5 min) and incubated with ABC for 1 h at room temperature. Subsequent PBSx washes (3 X 10 min) were followed by development with DAB (0.04% DAB in Tris buffer containing 0.1% H₂O₂) to produce a brown cytoplasmic product. The tissue was then mounted on gelatin-coated microslides, allowed to dry, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped with Permount (Fisher Scientific).

**Cell Counting**

Four sections per animal (approximately −7.30 mm, −7.64 mm, −8.00 mm, and −8.80 mm posterior to bregma, (Paxinos and Watson, 1996) spanning the rostral and caudal aspects of the DRN and MnR nuclei were digitised using a standard light microscope (Nikon Eclipse 80i) connected to a digital camera (Retiga 2000R, QImaging Corporation). The digital images were overlaid with the corresponding figures from the Paxinos and Watson (1996) rat brain atlas to delineate the boundaries and subregions of the DRN and MnR nuclei. The DRN was divided as follows: at −7.3 mm into the dorsal (DRD) and the ventral subregions (DRV), at −7.64 mm and at −8.00 mm into the DRD, DRV, and two ventrolateral subregions (DRL); at −8.80 mm into the caudal (DRC) and interfascicular (DRI) aspects. Cells in the MnR also were counted at −7.30 mm, −7.64 mm,
and −8.00mm (See Fig. 9). The MnR region was divided into the MnR and paramedian raphe (PMnR) subregions at −7.64mm and −8.0mm to ensure encapsulation of all TrpOH-ir cells within the area. All TrpOH-ir, c-Fos-ir, and double-labelled cells falling within these areas were quantified with the aid of ImageJ (NIH) by an observer blind to treatment conditions. The number of c-Fos immunoreactive nuclei was also examined at three levels of the LC (approximately −9.68mm, −9.80mm, and −10.04mm posterior to bregma, Paxinos & Watson, 1996). Atlas overlays for the LC analysis were unnecessary as TrpOH positive cells clearly outlined the boundaries of this nucleus (See Fig. 15).

Data Analysis

Overall multifactorial mixed design ANOVAs were used to assess general effects with the behavioural manipulations as a between subjects factor and brain area as a within subjects factor. The Greenhouse-Geisser correction was used to correct for differences in sphericity across repeated measures and, where appropriate, the non-parametric post hoc Games-Howell test was used to assess treatment effects within each brain area. All analyses were carried out with SPSS 11.5 (SPSS Inc., Chicago, IL, USA) and the significance level was set at 0.05. The data are expressed as mean cell count ± SEM.

Results

Characteristics of TrpOH and c-Fos Immunoreactivity

The distribution of c-Fos and TrpOH-ir appeared consistent with that described previously for the rat (Weissmann et al., 1987; Iijima and Sato, 1991). Brown TrpOH-ir
was observed in the cytoplasm, dendrites and fibers in all of the raphe nuclei and in the LC while c-Fos positive cells appeared as dark-stained cell nuclei throughout the brain (See Figs. 10 & 15). Elimination of the one or both of the primary antibodies resulted in no specific staining.

Spatial Pattern of c-Fos Expression in the Dorsal Raphe Nucleus

As shown in Fig. 11, the percentage of c-Fos-ir/TrpOH-ir cells was dependant upon the behavioural manipulation (F[3, 19] = 28.96, p < .001) and the brain area examined (F[3.40, 64.65] = 6.20, p = .001). There was also a significant treatment X area interaction (F[10.208, 64.65] = 6.58, p < .001) indicating regional differences in the effects of behavioural manipulations on c-fos expression in TrpOH-ir cells. In general, posthoc analyses revealed that the SLR manipulation increased the percentage of double-labelled cells in the rostral DRN as compared to all other treatments (significant at -7.3mm DRD, -7.3mm DRV, -7.6mm DRL, -8.0mm DRD, and -8.0mm DRL; See Fig. 11). In contrast, the SLR condition did not increase c-fos expression in TrpOH cells at the most caudal levels of the DRN. However, at -8.8mm in the DRI, the SD manipulation increased the percentage of double-labelled cells as compared to the DD condition (Games-Howell mean difference = 8.54, p = .05).

Similarly, the number of single labelled c-Fos-ir cells differed across treatment (F[3, 19] = 16.46, p < .001) and brain area (F[2.94, 55.93] = 9.60, p < .001), and there was also a significant treatment X area interaction (F[8.83, 55.93] = 3.09, p < .01). As shown in Fig. 12, the general pattern of results mirrors that outlined above. In virtually all regions of the rostral DRN, the SLR manipulation increased the number of c-Fos-ir
neurons as compared to the control condition, and, in the majority of cases, increased it compared to the WC and/or SD manipulations. Again, in the most caudal aspects of the DRN (-8.80 mm), the SLR manipulations did not increase c-fos expression as compared to the other groups. In the DRI, however, the WC manipulation increased c-fos expression as compared to all other manipulations (p < .05 vs. DD, SD, and SLR).

As expected, the number of single labelled TrpOH-ir cells did not differ as a result of the behavioural manipulations (F[3, 19] = 2.03, p > .05) and there was no significant area X treatment interaction (F[14.81, 93.82] = 1.08, p > .05; See Fig. 11), indicating consistent selection of the slices for analysis and an accurate placement of the atlas overlays.

**c-Fos Expression in the Median Raphe Nuclear Region**

In contrast to the pattern of results seen for the DRN, the percentage of c-Fos-ir/TrpOH-ir neurons did not differ across brain area (F[2.33, 44.20] = 0.24, p > .05), nor was there a significant area by treatment interaction (F[6.98, 44.20] = 0.77, p > .05). There was, however, a significant main effect of treatment (F[3, 19] = 5.31, p < .01) but post hoc analyses revealed that none of the behavioural manipulations increased the percentage of doubled-labelled cells in the MnR or PMnR regions (See Fig. 13).

The number of single labelled c-Fos-ir cells, however, differed both across treatment (F[3, 19] = 5.31, p < .01) and brain area (F[2.25, 42.70] = 9.42, p < .001) but there was no significant treatment X area interaction (F[6.74,42.70] = 1.67, p > .05). Post hoc analyses indicated that the SLR manipulation increased c-fos expression in the
rostral aspects of the MnR and PMnR as compared to the DD condition (significant at -7.3mm and -7.6mm; See Fig. 14)

As expected, the number of TrpOH-ir cells did not differ as a result of treatment (F[3, 19] = 2.26 p > .05). There was, however, a significant effect of area (F[2.75, 52.26] = 76.71, p < .001) and a significant area X treatment interaction (F[8.25, 52.26] = 3.06, p < .01). Post hoc analyses revealed that the number of TrpOH cells observed in the MnR at -8.00 mm was significantly lower in the SLR treatment as compared to the DO and SD conditions (See Fig. 13).

c-Fos Expression in the Locus Coeruleus

The number of c-Fos-ir cells in the LC differed both across treatment (F[1, 19] = 228.2, p < .001) and brain area (F[1.79, 33.97] = 9.50, p < .001), and there was a significant treatment X area interaction (F[5.36, 33.97] = 6.72, p < .001). Posthoc analyses revealed that the SLR manipulation significantly increased c-fos expression in the LC at all rostral-caudal levels examined, as compared to all other manipulations (See Figs. 15 & 16).

Discussion

Overall, the current results suggest that activation of the rostral DRN or the LC, as indexed by c-fos expression, is not necessary for shifts of a non-photic nature. In general, both novel wheel confinement and sleep deprivation by gentle handling, effective non-photic procedures, failed to increase c-Fos-ir in the serotonergic and non-serotonergic cells of the rostral DRN, the MnR region, and the LC. In the caudal interfascicular DRN
region, however, sleep deprivation and wheel confinement slightly increased c-fos expression in serotonergic and non-serotonergic cells, respectively. c-fos expression in the serotonergic MnR, however, was not associated with any of the arousal procedures. In contrast, physical restraint, an arousing procedure that does not result in clock resetting, elevated c-fos expression in serotonergic and non-serotonergic DRN neurons, particularly in the most rostral aspects of the nucleus, and in the LC, thus suggesting that activation of these areas is also not sufficient for non-photic clock resetting. Instead, the current results suggest a potential role for both the rostral DRN and the LC in the inhibition of non-photic phenomena. Furthermore, it may be that activation of particular areas of the DRN promotes non-photic clock resetting while other regions are involved in the inhibition of phase shifts to arousal.

**Implications for Serotonergic Mediation of Non-Photic Shifting**

As briefly outlined in the introduction, the rostral ascending serotonin system has been implicated in phase resetting by non-photic means. However, its involvement in non-photic phenomena remains contentious. Serotonin levels in the SCN and IGL are elevated by effective non-photic procedures (Dudley et al., 1998; Grossman et al., 2000; Grossman et al., 2004), but the extent to which serotonin release in these areas mediates non-photic shifting is unknown. Increased SCN 5-HT at mid day is not always sufficient to perturb circadian phase (Antle et al., 2000) and, at the level of the clock, serotonergic agonists do not induce shifts unless 5-HT receptors are supersensitized with a 5-HT synthesis inhibitor (Ehlen et al., 2001) or by exposure to constant light (Knoch et al., 2006). As well, 5,7-dihydroxytryptamine lesions (Bobrzynska et al., 1996a; Meyer-
Bernstein and Morin, 1998) or serotonergic antagonists (Antle et al., 1998) do not attenuate phase shifts in response to many non-photic manipulations. Therefore, in many cases, it appears that the activation of serotonergic inputs is neither necessary nor sufficient for shifts of a non-photic nature.

The current results are consistent with this general conclusion in that two procedures for inducing non-photic shifts (WC and SD) were not associated with a change in c-fos expression in the rostral DRN or the MnR whereas the arousal procedure that does not induce shifts (SLR) was associated with c-fos expression in the rostral DRN and presumably with increased 5-HT release in the circadian system. Thus, in hamsters, activation of the rostral DRN also appears insufficient to induce non-photic clock resetting.

Interestingly, the pattern of c-fos expression following each arousal procedure was similar across all areas of the DRN with the exception of the caudal interfascicular region. Although the observed number of c-Fos positive cells in this area was small, sleep deprivation and wheel confinement increased expression in serotonergic neurons and non-serotonergic cells, respectively. As well, immobilization stress failed to increase c-fos expression in this region but did so in virtually all other areas of the DRN. Little is known regarding the functional specificity of interfascicular neurons but these cells appear to form a distinct neural subpopulation. Compared to the majority of DRN neurons, this caudal area shows a specific arrangement of afferent projections (Waterhouse et al., 1986; Datiche et al., 1995; Leander et al., 1998; Janusonis et al., 1999; Janusonis et al., 2003), different neurochemical phenotypes (Charara and Parent, 1998), and, in some rodents, a unique pattern of diurnal c-fos expression (Janusonis and Fite, 2001). The
increased activation observed during effective non-photic procedures and the lack of activity during physical restraint point to a potential role for these cells in non-photic phase control. However, additional study is needed to further elucidate the involvement of these neurons, if any, in non-photic phenomena.

Stress–Induced Activation of the Serotonergic and Noradrenergic Systems

The current results are in agreement with a large body of literature implicating the DRN and the LC in the response to exteroceptive psychogenic stressors (Abercrombie and Jacobs, 1987; Cullinan et al., 1995; Pacak and Palkovits, 2001; Sved et al., 2002; Berridge and Waterhouse, 2003). Within the DRN subpopulations of serotonergic cells appear to be preferentially activated by strong, inescapable stress, a response that is facilitated by NE input from the LC (Grahn et al., 1999; Grahn et al., 2002; Abrams et al., 2004; Amat et al., 2005). Our observations that c-fos expression in the LC and the rostral DRN are selectively increased by physical restraint are consistent with these findings. The pattern of stress-induced c-fos expression in the current study, however, was similar across all areas of the nucleus, save the most caudal aspects where expression remained at control levels. This general pattern of activation appears to disagree with other reports indicating that c-fos expression is greater in the caudal vs. the rostral DRN following stressful procedures (Grahn et al., 1999). The reasons for these discrepancies are unclear but may stem from arbitrary differential separation of the DRN into rostral and caudal components or methodological or species differences (ours is the first study of Syrian hamsters; others have used rats and mice).
Inhibition of Arousals-Induced Phase Shifting by Stress

As outlined in the introduction, the critical stimulus for shifts of a non-photic nature remains to be determined. There are, however, two enticing possibilities. First, it may be that arousal with some minimal level of locomotion is necessary for non-photic resetting. Alternatively, arousal alone may be sufficient to produce resetting but, for this to be true, the stress associated with restricting locomotion, or the anxiety associated with some arousal inducing drugs, must attenuate the shifts that might otherwise be produced by these manipulations. The current results demonstrate that activation of the serotonergic rostral DRN and the noradrenergic LC, as indexed by c-fos expression, occur exclusively under stressful conditions that do not promote phase shifting. Is it possible that activation of one or more of these areas could inhibit non-photic inputs to the circadian clock thereby attenuating arousal-induced phase shifts? Anatomical and electrophysiological evidence suggest that this may be the case.

First, activation of the IGL appears both necessary and sufficient for non-photic clock resetting. Electrical stimulation of this area produces non-photic like shifts (Rusak et al., 1989) while lesioning this structure attenuates or blocks wheel confinement induced phase advances (Biello et al., 1991; Janik and Mrosovsky, 1994). As well, many non-photic procedures increase the expression of c-fos in IGL neurons, particularly in those immunoreactive for NPY (Janik and Mrosovsky, 1992; Janik et al., 1995; Mikkelsen et al., 1998; Antle and Mistlberger, 2000). The DRN is the exclusive source of 5-HT input to the hamster IGL (Meyer-Bernstein & Morin, 1996) and electrophysiological studies have shown that 5-HT actions in the IGL are inhibitory. For example, serotonergic agonists inhibit the spontaneous and light-induced activity of the
majority of IGL cells (Ying et al., 1993), and electrical stimulation or lesions of the DRN decrease or increase the firing rate of IGL neurons, respectively (Blasiak and Lewandowski, 2003). Given the inhibitory action of serotonin in the IGL and the pivotal role of this region in non-photic shifting, it is likely that serotonin release in this area, induced by stressful or anxiogenic procedures, inhibits NPY and/or other neurochemical output to the SCN and may, therefore, inhibit arousal-induced clock resetting. Activation of the LC may ultimately produce the same result, as NE appears to mediate the DRN response to inescapable stress (Grahn et al., 2002). The LC also projects directly to the IGL (Vrang et al., 2003) and, therefore, may also exert its own effects in this region. We are unaware, however, of any electrophysiological investigations of the actions of NE in the IGL, however. See Fig. 27 for an illustration of this model.

Further support for this preliminary model stems from the observation that anxiogenic drugs that increase arousal but do not perturb circadian phase also tend to activate the DRN and the LC. Caffeine and yohimbine have been reported to increase c-fos expression in these areas (Singewald and Sharp, 2000; Abrams et al., 2005; Deurveilher et al., 2006).

Although a stress attenuation hypothesis is conditionally supported by the data cited above, this idea appears inconsistent with observations that phase shifts do occur in some animals aroused by procedures that are stressful. Resident-intruder interactions, open field exposure and brief intermittent footshock, for example, can induce phase advances in hamsters when presented during the midday (Mistlberger et al., 2003; Cain et al., 2004). However, stress procedures produce differential patterns of neural activity depending on factors such as modality, intensity and escapability (Maywood et al., 1998;
Grahn et al., 1999; Lowry, 2002; Amat et al., 2005), and it may be that particular patterns of activation promote clock resetting while others may oppose it. As well, it should be noted that the stressful procedures capable of inducing shifts all allow for ambulation and, as locomotor activity attenuates stress correlates (Greenwood et al., 2003; Greenwood et al., 2005), it is possible to conceive of ways in which stress, mitigated by locomotion, could gate the sensitivity of the clock to neural inputs encoding non-specific arousal. This idea is supported by the observation that the magnitude of phase shifts in response to resident-intruder interactions or open field exposure are positively correlated with one or more indices of forward locomotion (Mistlberger et al., 2003).

The assertion that stress-induced 5-HT release in the IGL inhibits non-photic outputs to the clock also appears to be incompatible with the observation that serotonin is released in the IGL in association with effective non-photic procedures (Grossman et al., 2004). Novel wheel exposure and electrical stimulation of the DRN, for example, have been reported to increase serotonin release in this area (Grossman et al., 2004) and to induce phase advances (Meyer-Bernstein and Morin, 1999; Glass et al., 2000). How is it possible that procedures with disparate effects on circadian phase can both increase IGL 5-HT? One possibility is that serotonergic afferents to the IGL are topographically organized and that the different arousal procedures induce disparate patterns of 5-HT release in the IGL. It is important to point out that microdialysis studies lack the spatial resolution needed to precisely determine where serotonin is being released in the IGL, and can only determine if release has occurred. There is evidence to suggest that different areas of the DRN send projections to specific areas of the IGL (Meyer-Bernstein and Morin, 1996) but the extent to which the arousal procedures differentially affect 5-
HT release in these areas is unknown. The current results, however, suggest that activation of specific areas of the DRN (e.g., the interfascicular region), may promote clock resetting while activation of other afferents may inhibit shifts to arousal. Functional tract tracing studies (in progress) will be required to test these hypotheses.

**Methodological Issues**

Examination of \textit{c-fos} expression in the nervous system is a powerful tool for the identification of functional circuitry mediating various behavioural and physiological processes and has provided a wealth of information since its introduction. However, the usefulness of this technique is limited by the interpretational ambiguity associated with negative results. This uncertainty stems from the fact that \textit{c-fos} expression is not necessarily synonymous with membrane depolarization. There are disconnects, for example, between \textit{in vivo} single unit recordings and \textit{c-fos} expression, and this is clearly evident in our own data. The firing rate of the majority of MnR, DRN, and LC neurons, for example, is positively correlated with increased arousal and locomotor activity (Trulson and Jacobs, 1979; Rasmussen et al., 1984; Rueter et al., 1997), yet, in the current study, these neurons did not express \textit{c-fos} following wheel confinement or sleep deprivation by gentle handling. Findings of this nature are not unprecedented in the sleep literature, however (Cirelli and Tononi, 2000), and other studies have reported that, in other species, spontaneous wake or short-term forced wakefulness is not associated with increased \textit{c-fos} expression in the DRN or the LC (Yamuy et al., 1995; Janusonis and Fite, 2001; Lu et al., 2006). As well, many of the brain areas known to be involved in wakefulness do not appear to increase \textit{c-fos} expression in response to stimulant
administration (Lin et al., 1996; Scammell et al., 2000; Deurveilher et al., 2006). The reasons for these differences are unclear but may relate to insufficient stimulation or failure to activate the specific signal transduction pathways necessary for c-fos induction.

Conclusions

In summary, the current results show that arousal procedures with disparate effects upon circadian phase differentially activate central monoaminergic systems and add further to the evidence for function specificity in the serotonergic system. c-fos expression in the rostral DRN or in the LC is associated with physical restraint, an arousal procedure that does not perturb circadian phase (Mistlberger et al., 2003; See Chpt. 5 and Appendix A), but not with wheel confinement or sleep deprivation by gentle handling, effective non-photic procedures. Thus, activation of the rostral DRN or the LC, as indexed by c-fos expression, is neither necessary nor sufficient for shifts of a non-photic nature. Interestingly, the effective non-photic procedures slightly increased c-fos expression in the caudal interfascicular DRN region. Thus, further study is warranted to delineate the role of this area, if any, in non-photic phenomena. Activation of serotonergic MnR, however, was not associated with any of the arousal procedures. Exclusive activation of the rostral DRN and the LC by physical restraint also suggests that these areas may be involved in a stress-induced attenuation of phase shifts to arousal. This contention is supported by the observation that afferents from the DRN are the exclusive source of 5-HT in the IGL, activation of the IGL appears both necessary and sufficient for shifts of a non-photic nature, and 5-HT has an inhibitory action in this region. Proposal of this model is preliminary, however, and it must be emphasized that
further work is needed to support this conjecture. Nonetheless, the current results suggest that activation of specific areas of the DRN may promote clock resetting while others may inhibit phase shifts to arousal.
Figures

Figure 8. Photographs of three of the behavioral arousal procedures used in the current study. A. sleep deprivation by gentle handling. B. wheel confinement. C. Stress-loaded restraint.
Figure 9. Representative photomicrographs of coronal brain sections stained for TrpOH and c-Fos at each of the rostral-caudal levels (relative to bregma) examined. The lines delineate each of the subregions of the rostral raphe nuclei according to Paxinos and Watson (1996).
Figure 10. Representative photomicrographs illustrating the effects of the behavioural manipulations on c-fos expression in TrpOH-ir neurons in the DRN. (A) The DRN at approximately −8.0mm relative to bregma. Also shown is double labelling of c-Fos-ir/TrpOH-ir following (B) DD (C) WC (D) SD and (E) SLR. Nuclear c-Fos-ir appears black and TrpOH-ir appears light brown. The black arrows indicate c-Fos-ir and the blue arrows TrpOH-ir.
Figure 11. The effects of behavioural manipulations on c-fos expression in specific subpopulations of TrpOH-ir neurons in the DRN. The shaded bars represent the number of double-labelled c-Fos-ir/TrpOH-ir neurons and the open bars represent the number of single labelled TrpOH-ir neurons. The percentages indicate the proportion of TrpOH-ir that also showed c-Fos-ir following the behavioural manipulations. Data are shown as means ± SEM. *** = p < .05 vs. all other conditions, ### = p < .01 vs. all other conditions, * = p < .05 vs. DD.
Figure 12. Effects of the behavioural manipulations on \( c-fos \) expression in TrpOH-immunonegative neurons in specific subregions of the DRN. Data are shown as means ± SEM. * = \( p < .05 \); ** = \( p < .01 \); *** = \( p < .001 \).
Figure 13. The effects of behavioural manipulations on c-fos expression in TrpOH-ir neurons in the MnR and PMnR. The shaded bars represent the number of double-labelled c-Fos-ir/TrpOH-ir neurons and the open bars represent the number of single labelled TrpOH-ir neurons. The percentages indicate the proportion of TrpOH-ir that also showed Fos-ir following the behavioural manipulations. Data are shown as means ± SEM. * = p < .05 vs. DD and SD.
Figure 14. The effects of behavioural manipulations on c-fos expression in TrpOH-immunonegative neurons in the MnR and PMnR. Data are shown as means ± SEM. * = p = .05; ** = p < .01.
Figure 15. Representative photomicrographs illustrating c-fos expression in the LC at approximately -10.04mm relative to bregma following (A) DD (B) WC (C) SD and (D) SLR. Nuclear c-Fos-ir appears black and TrpOH-ir appears light brown.
Figure 16. The effects of behavioural manipulations on c-fos expression in the LC. Data are shown as means ± SEM. *** = p < .001 vs. all other conditions.

-9.68mm

-9.80mm

-10.04mm
CHAPTER 4: DIFFERENTIAL EFFECTS OF AROUSAL PROCEDURES ON CIRCADIAN RYTHMS IN SYRIAN HAMSTERS: NEURAL CORRELATES IN THE HYPOCRETIN SYSTEM AND THE INTERGENICULATE LEAFLET

Abstract

In Syrian hamsters, some procedures for stimulating behavioural arousal (e.g., running in a novel wheel and sleep deprivation by gentle handling with minimal activity; Reebs & Mrosovsky, 1989a, Antle & Mistlberger, 2000), when applied during the mid rest period, can markedly phase advance circadian rhythms, while other arousal procedures do not (e.g., physical restraint, caffeine, modafinil; Antle et al., 2001; Mistlberger et al., 2003; Chpt. 2). The neural basis for this differential effect of arousal procedures on clock resetting is unknown. We used c-fos expression as a marker for neuronal activation to determine whether these arousal procedures differentially activate two non-photic inputs to the circadian system, the thalamic intergeniculate leaflet (IGL, thought to be critical for arousal-induced phase shifting; Morin & Allen, 2006) and the hypothalamic hypocretin system (which depolarizes arousal related cell groups throughout the brain and innervates both the IGL and the suprachiasmatic nucleus circadian clock; (Mintz et al., 2001; Saper et al., 2001). c-fos expression in hypocretin neurons, hypothalamic non-hypocretin neurons, and in the IGL was increased by novel wheel running, sleep deprivation, and physical restraint, but not by systemic injections of modafinil (300mg/kg) or caffeine (75 mg/kg), at doses that are strongly alerting. Spatial analysis
revealed few differences in the percentage of Hcrt-1 cells expressing c-fos following each treatment. Taken together, these results suggest that activation of hypocretin neurons (e.g., as in the restraint condition) is not sufficient to induce phase shifts, and that gating of arousal effects on circadian clock phase may be downstream from the hypocretin system and from IGL neurons activated by the restraint procedure.

**Introduction**

Convergent evidence indicates that mammalian circadian rhythms are regulated by a master pacemaker localized to the hypothalamic suprachiasmatic nucleus (SCN; Moore & Eichler, 1972; Stephan & Zucker, 1972; Welsh et al., 1995; Ralph et al., 1990). Under constant conditions, this centralized oscillator free runs with a periodicity close to, but not necessarily equal to, 24h. Photic stimulation, transduced by photoreceptive retinal ganglion cells (Gooley et al., 2001; Hattar et al., 2002; Hannibal et al., 2002) and transmitted to the SCN via the retinohypothalamic tract (Moore and Lenn, 1972), results in discrete phase advance or delay shifts that offset the intrinsic period of the SCN such that it approaches 24 h and thus entrains to the external light-dark cycle (Pittendrigh and Daan, 1976). Although light is considered the primary zeitgeber for circadian rhythmicity, varied non-photic stimuli also can perturb circadian phase (Mrosovsky, 1996; Mistlberger et al., 2000). In Syrian hamsters, for example, novel wheel confinement (Reebs & Mrosovky, 1989a; Wickland & Turek, 1994), dark pulses (Ellis et al., 1982; Mistlberger et al., 2002), or triazolam injection (Turek & Losee-Olson, 1986; Turek & Van Reeth, 1988), procedures that increase ambulation, can produce large phase
advance shifts in locomotor activity rhythms when applied during the mid-to-late light period.

The similar characteristics of the non-photic phase response curve across a wide range of behavioural manipulations suggest that some non-specific aspect(s) of these procedures mediates clock resetting. To date, several convergent lines of evidence indicate a critical role for locomotion. For many non-photic procedures, there is a positive relationship between the amount of ambulation observed during a manipulation and the size of the resultant phase shift (Mrosovsky & Salmon, 1990; Mistlberger et al., 1996; Bobrzynska and Mrosovsky, 1998; Mistlberger et al., 2003). As well, the common feature of all effective non-photic manipulations is the ability to produce arousal in association with some level of locomotor activity. Manipulations that prevent locomotor activity but sustain arousal (e.g., physical restraint, confinement to a pedestal over water) do not induce phase shifts (Mistlberger et al., 2003). Similarly, restricting locomotor activity attenuates phase shifts to triazolam and to dark pulses during the mid-subjective day (e.g., Van Reeth & Turek, 1989; Reebs et al., 1989; Dwyer & Rosenwasser, 2000). Moreover, systemic administration of caffeine or modafinil at doses that promote arousal but do not have large effects upon locomotor activity also do not perturb circadian phase (See Chpt. 2, Antle et al., 2001). Thus, it appears that some minimal amount of locomotion is necessary for non-photic clock resetting.

Alternatively, it may be that arousal alone is sufficient for clock perturbation. For this latter supposition to be true, however, it must be the case the “stress” somehow blocks phase shifts to arousal. Behavioural procedures that sustain arousal but restrict locomotor activity are classic stressors and, in hamsters, have been shown to increase
indices of stress such as cortisol (Mistlberger et al., 2003). Further evidence for this supposition includes the observation that a cortisol synthesis inhibitor increases phase shifts to a 3h sleep deprivation procedure, a manipulation that increases cortisol in its third hour (Mistlberger et al., 2003). As well, there is a negative correlation between the number of interventions needed to maintain wakefulness during SD, and the size of the resulting phase advance (Antle & Mistlberger, 2000). Furthermore, arousal-inducing anxiogenic drugs (e.g., caffeine, yohimbine) do not shift circadian phase (Antle et al., 2001; See Appendix B).

Despite the remaining questions surrounding the behavioural underpinnings of non-photic phase shifting, significant progress has been made in discerning the underlying physiology. The thalamic intergeniculate leaflet (IGL) and the midbrain serotonergic median and dorsal raphe nuclei, structures that project directly or indirectly to the SCN (Meyer-Bernstein and Morin, 1996; Moga and Moore, 1997; Hay-Schmidt et al., 2003), have been implicated in clock resetting by non-photic stimuli (Harrington et al., 1985; Meyer-Bernstein and Morin, 1996). Activation of the IGL appears both necessary and sufficient for shifts of a non-photic nature (e.g., Rusak et al., 1989; Biello et al., 1991; Wickland and Turek, 1994) and, in hamsters, several of the neurotransmitters associated with the geniculohypothalamic tract (neuropeptide Y, GABA, and enkephalin) contribute to clock resetting (Morin & Allen, 2006). The body of evidence implicating serotonergic inputs in non-photic shifting is less internally consistent, however (Morin, 1999; Mistlberger et al., 2000). Serotonergic stimulation is sufficient to induce non-photic like phase advances under certain conditions (Cutrer et al., 1994a; Bobrzynska et
al., 1996b; Ehlen et al., 2001) and, in some cases, does not appear necessary for non-photic clock resetting (Bobrzynska et al., 1996b).

Although putative SCN non-photic afferents have been identified, the pathways upstream require further elucidation. As well, the locus for the disparate phase shifting effects of the varied arousal procedures, which presumably activate many neural systems in common, remains to be identified. Given the body of evidence implicating arousal and locomotion in non-photic clock resetting, systems involved in the control of these behaviors are of particular interest. Assessing activation of these systems by the arousal procedures with divergent effects phase resetting effects may be especially instructive.

The hypocretin (hcrt; also known as orexin) system is comprised of a population of hypothalamic cells that synthesize the neuropeptides hcrt-1 and hcrt-2 and project to the circadian system and virtually all arousal-related cell groups in the brain (Peyron et al., 1998; Date et al., 1999; Mintz et al., 2001; Nixon and Smale, 2005; Vidal et al., 2005). These peptides have been functionally linked to arousal and locomotion (e.g., Chemelli et al., 1999; Torterolo et al., 2003; Mieda et al., 2004). Hcrtergic activity follows a circadian pattern with a zenith near the end of the active period and a nadir during the period of inactivity (Estabrooke et al., 2001; Martinez et al., 2002). As well, systemic or icv delivery of the hcrt-s produces arousal, likely due to the excitation of wake-active brain areas (Hagan et al., 1999; Espana et al., 2001; Yamanaka et al., 2002), and increases locomotor activity (Hagan et al., 1999; Ida et al., 1999; Jones et al., 2001; Kotz et al., 2002). Furthermore, the sleep disorder narcolepsy, characterised by excessive daytime sleepiness and cataplexy (Siegel, 1999; Dauvilliers et al., 2003; Scammell, 2003), is
associated with deficiencies of the hcrt system (Lin et al., 1999; Peyron et al., 2000; Thannickal et al., 2000; Mignot et al., 2002).

Given the close associations between arousal, locomotor activity, and hcrt release, and the physiological substrate for hcrticergic input to the circadian system, it may be that these neuropeptides contribute to non-photic clock resetting by behavioural means. As a first step in evaluating this hypothesis, hamsters were exposed to behavioural and pharmacological arousal procedures and examined, using immunohistochemical techniques, for the expression of c-fos (Morgan and Curran, 1991) in Hcrt-1 expressing neurons. As the hcrt system may not be a homogenous population of cells and are likely functionally organized (e.g., Espana et al., 2005), we examined topographical differences in c-fos expression across the population of hcrticergic neurons. Given the evidence for the IGL as the final common pathway for non-photic stimuli to the SCN, we also examined c-fos expression in this area.

Methods

Animals and Housing

Young male Syrian hamsters (Charles River, Montreal, PQ, Canada; 80-140g) were housed under a 14:10 LD cycle in standard polypropylene cages (45 X 25 X 20cm) with wire mesh bottoms. The cages were equipped with 17cm diameter stainless steel running wheels and wheel revolutions were measured via mechanical switches interfaced with a computer. A PVC pipe was provided in the home cage for added comfort and light avoidance, and food and water were provided ad libitum.
Drugs

Modafinil (2[(diphenylmethyl)sulfinyl]acetamide; Cephalon, Inc., West Chester, PA.) was suspended in a sterile solution of 0.25% methylcellulose (Sigma-Aldrich Canada Ltd.) immediately prior to i.p. injection and administered at a dose of 300mg/kg. Caffeine (Sigma-Aldrich Canada Ltd) was dissolved in sterile saline and administered i.p. at a dose of 75mg/kg. These dosages have been previously reported to induce significant arousal in hamsters over several hours following administration (See Chpt. 2, Antle et al., 2001).

General Procedures

Once stable entrainment was attained, the animals (n = 24) were randomly divided into four groups (n = 6 each) and exposed to various arousal procedures with distinct effects upon circadian phase. These three-hour procedures began at zeitgeber time 6 (i.e., ZT6, by convention six hours before dark onset) and the overhead room lights were turned out at the start of each manipulation.

For the DD control condition, a treatment that does not shift the clock, the hamsters were simply left undisturbed in their home cage. The wheel confinement (WC) procedure consisted of confinement to a novel 33 cm diameter Wahmann running wheel, a procedure that stimulates running in most hamsters, and induces large (2 h or more) phase advance shifts in those animals that run more or less continuously (Mrosovsky, 1996a). Hamsters in the sleep deprivation by gentle handling condition (SD) were continuously observed in the home cage and stimulated (tactile or auditory) upon adoption of a sleep posture. This procedure induces phase advance shifts equivalent to
those seen following WC (Antle & Mistlberger, 2000). For the stress-loaded restraint (SLR) condition, hamsters were placed into PVC tubes (15 cm long, 4 cm internal diameter) sealed on either side with rubber mesh and stimulated with compressed air to prevent behavioural sleep (See Mistlberger et al., 2003). Despite the sustained arousal produced by this latter manipulation, the SLR procedure has little effect upon circadian phase (Mistlberger et al., 2003). The SD and SLR manipulations were carried out under dim red light (~ 1 lx) and running wheels were locked during the DD and SD conditions.

A second group of hamsters were administered vehicle \((n = 2, \text{saline}; n = 2, 0.25\% \text{methylcellulose})\), the atypical arousing compound modafinil \((n = 6)\), or caffeine \((n = 6)\). Neither of these agents shift circadian phase despite their ability to promote arousal during the midday (See Chpt. 2, Antle et al., 2001). To ensure arousal over the period from ZT6-9, caffeine was administered at ZT5.5 and modafinil at ZT4 (See Chpt. 2, Antle et al., 2001).

**Immunocytochemistry**

At ZT9, immediately following the behavioural manipulations, the animals were euthanized with sodium pentobarbital and perfused transcardially with 50 ml of 0.1M phosphate-buffered saline (PBS, \(\text{ph} \sim 7.3\)) followed by 50 ml of 4% paraformaldehyde in PBS (PFA, \(\text{ph} \sim 7.3\)). Brains were removed, postfixed for 2h in PFA, and cyroprotected in 20% sucrose in PBS overnight. The tissue was sectioned (50\(\mu\)m thickness) using a cryostat and stored in de Olmos cyroprotectant (Watson et al., 1986) at -20°C until immunocytochemical processing. The sections were processed as described below and
were washed in PBSx (0.3% Triton X-100 [Sigma] in PBS, 3 X 5 min rinses) between each step and incubated at room temperature (RT) unless otherwise indicated.

To visualize c-fos expressing cells the sections were first washed in PBSx then incubated with 10% normal horse serum (NHS, Vector Laboratories, Ltd.) in PBSx for 1h. Next, the tissue was incubated with goat anti-Fos IgG (Santa Cruz Biotechnology, Inc., cat. # SC-52-G, 1:2000 in 1% NHS in PBSx) for 36-48h at 4°C followed by exposure to a biotinylated horse anti-goat antibody (Vector Laboratories, Ltd., 1:250 in 1% NHS in PBSx) for 1h. The sections were subsequently exposed to 0.3% H₂O₂ in PBSx for 30 min to inactivate endogenous peroxidase and then incubated with an avidin-biotin-horseradish peroxidase complex (ABC, Standard Vectastain Elite Kit, Vector Laboratories, Ltd.) for 1h. Last, the stain was developed with diaminobenzidine (DAB, 0.04% in 0.2M Tris buffer containing 0.1% H₂O₂ and 0.03% NiCl₂) to produce a blue/black reaction product.

To label Hcrt-1 synthesizing cells, the sections were subsequently incubated in 10% normal donkey serum (NDS, Jackson Immunoresearch Laboratories, Inc.) in PBSx for 1h followed by incubation with a rabbit anti hcr-1 IgG (CalBiochem, cat. # PC345, 1:333 in 1% NDS in PBSx) for 36-48h at 4°C. The tissue was then exposed to a biotinylated donkey anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc., 1:500 in 1% NDS in PBSx) for 2h followed by a 1h incubation with ABC. Finally, the stain was developed with DAB (0.04% in Tris buffer containing 0.1% H₂O₂) to produce a brown cytoplasmic product. The tissue was then mounted on gelatin-coated microslides, allowed to dry, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped with Permount (Fisher Scientific).
Cell Counting

Three sections per animal (correspondingly approximately to 1.8 mm, 2.0 mm, and 2.3 mm posterior to bregma, Morin & Wood, 2001) were digitised using a standard light microscope (Nikon Eclipse 80i) connected to a digital camera (Retiga 2000R CCD, QImaging). Pictures comprising individual sections were stitched together with Photoshop Elements (Adobe) to produce high-resolution images. Next the relevant sections were counterstained with cresyl violet and digitally photographed to identify the landmarks necessary for the consistent placement of counting boxes. Digital pictures of the counterstained sections were temporarily overlaid the initial images to bilaterally anchor three mediolaterally contiguous counting boxes (each measuring 600 μm X 800 μm) to the third ventricle and the dorsal border of the ventromedial hypothalamic nucleus (Fig. 17). All Hcrt-1-ir cells, c-Fos-ir nuclei, and double-labeled cells falling within these boxes were quantified with the aid of ImageJ (NIH) by two observers blind to treatment conditions. Counts by the two raters for two entire slices were highly correlated (r = 0.98, p < .01).

Also, where possible, one rater also counted the number of c-Fos positive nuclei in one slice spanning the mid region of the IGL (from –2.0mm to –2.9 mm relative to bregma, (Morin and Wood, 2001). For each slice, the numbers of c-Fos positive nuclei falling within the borders of the IGL Hcrt-1-ir fibers were recorded. (See Fig. 22). This region included the strip of tissue falling directly between the dorsal and ventral lateral geniculate nuclei and excluded the ventral aspect of the area as defined by Morin and Wood (2001).
Statistical Analysis

Overall multifactorial mixed design ANOVAs were used to assess the general effects with the behavioral and pharmacological manipulations as a between subjects factor and brain area as a within subjects factor. The Greenhouse-Geisser correction was used to correct for differences in sphericity across repeated measures and, where appropriate, oneway between ANOVAs followed by the Bonferroni or Games-Howell comparisons were used to assess treatment effects within each brain area. All analyses were carried out with SPSS 11.5 (SPSS Inc., Chicago, IL, USA) and the significance level was set at 0.05. The data are expressed as mean cell count ± SEM.

One WC animal only ran 2581 revolutions (as compared to a mean of 4224 revolutions for the whole WC group) and, as previous investigations indicate that this subject was unlikely to have shifted (Reebs & Mrosovsky, 1989b; Wickland & Turek, 1991), he was dropped from the analysis. Due to histological problems, two animals from the DD condition and one animal each from the MOD and CAFF conditions were also excluded. As the data from the DD and vehicle control groups did not differ significantly (comparisons not shown), it was combined for the overall analysis.

Results

Characteristics of Hcrt-1 and c-Fos Immunoreactivity

The distribution of Hcrt-1-immunoreactivity (ir) appeared consistent with that described previously for the hamster (Mintz et al., 2001; Vidal et al., 2005; Nixon & Smale, 2007). Brown Hcrt-1-ir was observed throughout the cytoplasm of many lateral hypothalamic neurons, and was present in fibers throughout the brain. c-Fos positive cells
appeared as dark-stained nuclei over a wide range of areas (Fig. 18). Elimination of one or both of the primary antibodies resulted in no specific staining.

**Spatial Pattern of c-Fos, Hcrt-1, and Double-Labeled Cells Following Each Arousal Procedure**

The percentage of Hcrt-1 cells expressing \( c-fos \) differed across both condition (\( F[5,29] = 10.77, p < 0.001 \)) and brain area (\( F[4.48,130.16] = 10.08, p < 0.001 \); See Fig. 19). There also was a significant condition by brain area interaction (\( F[22.44,130.16] = 3.043, p < 0.001 \)). Further examination of treatment effects revealed that \( c-fos \) expression was increased in the SLR (36.8 %\( +/-\) 9.6), SD (38.3% \( +/-\) 6.4), and WC (57.3% \( +/-\) 4.2) groups relative to the DD (9.1% \( +/-\) 2.5; See Fig. 20A) condition. The percentage of \( c-fos \) positive Hcrt-1 expressing neurons was also increased in the WC condition as compared to the MOD (15.8% \( +/-\) 3.7) and CAFF (20.8% \( +/-\) 3.1) treated animals. Visual inspection of Fig. 19 shows that, relative to DD, WC tended to increase \( c-fos \) expression in Hcrt-1-ir neurons across all areas (counting boxes) examined and, in the medial and mid levels, was also elevated relative to MOD and CAFF. No other general patterns were evident with SD, SLR, and CAFF increasing \( c-fos \) expression relative to controls in some areas. Spatial differences were particularly lacking in the anterior-posterior plane. However, the percentage of double-labeled cells tended to decrease across the medial-lateral plane, particularly for WC, and less consistently for SD.

The number of Hcrt-1 expressing cells also differed by condition (\( F[5,29] = 3.67, p < 0.05 \)) and brain area (\( F[3.42,99.27] = 59.11, p < 0.001 \)), but there was no significant condition brain by area interaction (\( F[17.12, 99.27] = 1.26, \) n.s.; See Fig. 19). Increased
numbers of single labeled Hcrt-1 cells were observed in the CAFF (1381 +/- 73) condition relative to the SLR (863 +/- 80) and SD (863 +/- 77) treatments. Further examination showed that increased numbers of Hcrt-1-ir neurons in the CAFF condition were only observed in the most posterior slice and in the most lateral counting box.

As well, the number of non-hcrtergic c-fos expressing cells differed by treatment (F [5,29] = 11.10, p < 0.001) and by brain area (F [3.15, 91.32] = 30.99, p < 0.001). There was also a significant condition by brain area interaction (F [15.74, 91.32] = 5.91, p <0.001 See Fig. 21). Overall, the results for this measure were essentially the same as that reported above for double labelling, with the exception that the SLR procedure induced more c-fos expression as compared to modafinil injection (See Fig. 20B). The general spatial pattern of results also was generally the same as that reported above for double-labeled cells. There appeared to be little difference in the pattern of results in the anterior-posterior dimension but expression did appear to decrease in the more lateral counting boxes. This appeared to particularly be the case for the SLR condition.

**c-Fos Expression in the IGL Following Each Arousal Procedure**

A oneway ANOVA revealed a significant effect of condition (F[5,31] = 13.01, p < .001) and subsequent comparisons indicated that the SLR (15.8 +/- 2.1), SD (15.7 +/- 3.3), and WC (17.3 +/- 4.8) conditions increased c-fos expression relative to the DD (0.7 +/- 0.5), MOD (0.2 +/- 0.2) and CAFF (0.0 +/- 0.0) conditions (See Fig. 22).

**Discussion**

The current results demonstrate that three behavioural arousal procedures with divergent effects upon circadian phase can elevate c-fos expression both in the hcrt
system and in the IGL. This dissociation between phase shifting and c-fos expression effects indicates that hcrt activation is not sufficient for circadian clock resetting, but leaves open the possibility that it is necessary. By contrast, two drugs, caffeine and modafinil, that increase arousal in the hamster but have little effect upon locomotor activity or circadian phase (See Chpt. 2, Antle et al., 2001), do not elevate c-fos expression in these cells. This general pattern of results also was observed for c-fos expression in hypothalamic non-hcrtergic cells and in the IGL. The latter result is in agreement with previous studies utilizing WC or SD (Janik & Mrosovsky, 1992; Mikkelsen et al., 1998; Antle & Mistlberger, 2000) but shows for the first time that an arousal procedure that does not shift circadian phase can elevate c-fos expression in the hamster IGL. This finding indicates that the effects of arousal upon IGL gene expression are more complex than originally thought and suggest that this area either is not the final common non-photic pathway to the circadian clock, or that output from this area can be gated downstream, and blocked by correlates of some arousal procedures.

Several lines of research have implicated the hcrt system in the regulation of arousal (Chemelli et al., 1999; Hagan et al., 1999; Peyron et al., 2000; Gerashchenko et al., 2003; Willie et al., 2003). Recent discoveries, however, suggest that, in rodents, this system may be more involved in the regulation of locomotor activity rather than arousal, per se. Activation of this system, as indexed by c-fos expression and CSF peptide levels, is positively correlated with the level of locomotor activity in various species under a variety of conditions (Estabrooke et al., 2001; Espana et al., 2003; Nixon and Smale, 2004). Further, increased c-fos expression in hcrtergic cells and increased Hcrt-1 release are observed following arousal associated with locomotor activity as compared to quiet
waking without movement (Kiyashchenko et al., 2002; Torterolo et al., 2003; Martins et al., 2004). Subsequent in vivo single cell recordings have confirmed relative quiescence during quiet waking and increased firing rates during ambulation (Mileykovsky et al., 2005; Lee et al., 2005).

Our results add further to the evidence indicating a strong association between hypocretic activation and locomotor activity. Differential c-fos expression was observed across the five arousal procedures with the highest levels associated with WC, presumably the manipulation with the greatest amount of locomotor activity. The levels of expression following WC were also increased relative to CAFF and MOD, agents administered at dosages that promote arousal but do not increase locomotor activity (See Chpt. 2, Antle et al., 2001; Murphy et al., 2003). In agreement with previous studies (Estabrooke et al., 2001; Modirrousta et al., 2005), increased c-fos expression was also observed following the SD procedure, a manipulation with slightly higher levels of locomotor activity as compared to MOD (~80m vs. ~20m, respectively, See Chpt. 2, Antle & Mistlberger, 2000). Thus, the increased c-Fos-ir following the SD manipulation is likely at least partially due to the increased ambulation associated with this procedure.

Activation of the hypocretin system, however, is also associated with the SLR manipulation, a procedure that prevents locomotor activity. The immobilized animals are restricted in their ability to move forward or backward but remain aroused and tend to show high muscle tone throughout the manipulation. Although the extent to which activation of the hypocretin system is associated with muscle tone as opposed to forward locomotion is unknown, it is clear that this system is also involved in regulation of the neuroendocrine response to stress. In many instances, the presentation of stressful stimuli
appears to be associated with activation of the hcrt system. Foot shock, open field exposure, cold exposure, and immobilization, for example, have been shown to increase \textit{c-fos} expression in hcrt-containing neurons (Zhu et al., 2002; Espana et al., 2003; Sakamoto et al., 2004). As well, all major divisions of the HPA axis receive hcrtergic innervation or express hcrtergic receptors (Chen et al., 1999; Date et al., 2000; Malendowicz et al., 2001; Mazzocchi et al., 2001) and central or systemic administration of the hcrt can increase plasma levels of ACTH or corticosterone (Hagan et al., 1999; Russell et al., 2001; Samson et al., 2002) and elicit HPA-mediated stress-related behaviours (Hagan et al., 1999; Matsuzaki et al., 2002; Kiwaki et al., 2004). Furthermore, prepro-hcrt knockout mice show decreased cardiovascular and behavioural responses to the resident-intruder test (Kayaba et al., 2003).

Our results add further to the data implicating the hcrt system in the response to stressors. Increased \textit{c-fos} expression was observed following physical restraint and with SD, a manipulation that also elevates plasma cortisol levels in hamsters (Mistlberger et al., 2003). Treatment with caffeine, a known anxiogenic drug, marginally increased \textit{c-fos} expression in some areas of the hcrt system. However, this was not a robust response and was non-significant overall.

The lack of \textit{c-fos} expression in hcrtergic neurons following caffeine or modafinil injection appears inconsistent with previous reports in rats indicating that systemic administration of these agents activates this system (Scammell et al., 2000; Murphy et al., 2003). However, in one study, modafinil was reported to induce \textit{c-fos} expression in only 28 hcrtergic cells counted over 3 sections (Scammell et al., 2000). Thus, our results, although non-significant, show relatively increased levels of modafinil induced \textit{c-fos}.
expression as compared to earlier investigations. With regard to caffeine, 40% of
cortergic cells in one section have been reported to express c-fos within 90 min of
administration (Deurveilher et al., 2006). The relatively decreased level of expression in
the current study (~20%) likely relates to differences in the timing of administration
across studies. In the current experiment, caffeine was administered 3.5 h prior to
sacrifice, as opposed to 90 min in the previous investigations (Murphy et al., 2003;
deurveilher et al., 2006). Therefore, in the current study, it may be that any c-fos
expression induced by caffeine had largely decayed by the time of sacrifice. If so, this
implies that the c-fos response to caffeine is transient relative to the arousal response,
which is sustained for at least 3h, comparable to the arousal induced by behavioural
procedures. Alternatively, the failure of this drug to significantly induce c-fos expression
may relate to species-specific differences.

It may be argued that the increased number of single-labeled Hcrt-1 cells
observed in the CAFF condition may have artificially lowered the percentage of double-
labeled cells observed in this treatment group. However, this seems unlikely for several
reasons. First, there is no reason to believe that an observed increase in the number of
Hcrt-1-ir cells would be limited to those that are single labeled. Thus, an increase in the
number of observed single-labelled cells would presumably also be accompanied by a
concomitant increase in the number of double-labeled cells. If anything, the bias would
lean toward detecting a statistical increase in activation. In any case, the percentage of
double-labelled cells or single-labeled Hcrt-1-ir cells observed in the CAFF condition did
not significantly differ from the DD treatment, thus suggesting that the increased number
of Hcrt-1 cells is irrelevant to the overall findings. Although the interrater reliability was
exceedingly high in the current study, the increased number of cells may be related to differences in Hcrt-1 cell counting between observers. The spatial analysis, however, revealed the significant result to be largely due to an increased number of Hcrt-1-ir cells in the most posterior slice and in the most lateral counting box.

The medial-lateral, rostral-caudal spatial analysis employed in the current study suggests little in the way of functional topography in the hypocretin system. Overall, upon visual inspection, there appear to be no large differences in the pattern of c-fos expression in either Hcrt-1-ir or non-hcretic neurons in the anterior-posterior dimension. In the medial-lateral direction, however, c-fos expression in Hcrt-1-ir cells appears to decrease following the WC treatment and a similar pattern was noted for single-labeled c-Fos positive cells, particularly for the SLR condition. At least, one previous study has also reported a similar pattern of c-fos expression in hcretic cells following wheel running (Nixon and Smale, 2004). Closer inspection at each level of analysis shows that WC tends to increase c-fos expression in Hcrt-1-ir cells relative to DD across all areas examined. No other general patterns were evident, however, with SD, SLR, and CAFF increasing c-fos expression relative to control only in some areas. Given the low power and the propensity for Type I and II statistical errors, it is difficult to ascribe any meaning to these differences. The general lack of spatial effects is probably not surprising given the arbitrary division of the hcret cell population. It must be noted however, that the current results in no way imply functional homogeneity. In fact, it is quite likely that subpopulations of hcretic cells modulate arousal, locomotor activity, neuroendocrine activation and other physiological or behavioural variables.
It also remains a possibility that the hypocretin system mediates non-photic clock resetting and that our blunt spatial analysis is unable to detect this cellular activation. For example, it has been reported that only a small proportion (1%) of hcrt neurons project to the IGL and that these neurons do not appear to be spatially organized (Vidal et al., 2005). To our knowledge, retrograde SCN tracing experiments have not been carried out but the sparse hcrtergic projections to this area (Nixon and Smale, 2007) suggest that only a small number of these neurons project to circadian clock. Therefore, the spatial analysis of a large number of hcrt neurons, as carried out in the current study, is unlikely to identify spatially separated neurons that are exclusively active during effective non-photic procedures. Functional neural tract tracing studies, utilizing retrograde tracer application in combination with c-Fos immunostaining, will be necessary to identify any functional subsets of hcrt neurons involved in the control of circadian phase.

Given the direct hcrtergic projection to the SCN (e.g., Date et al., 1999) and to the IGL (McGranaghan and Piggins, 2001; Mintz et al., 2001; Novak and Albers, 2002; Nixon and Smale, 2004; Vidal et al., 2005) and the localization of hcrt receptors in the SCN (Backberg et al., 2002), it may seem surprising that this system does not appear to perturb circadian phase. *In vitro* application of hcrt-1 or -2 have been shown to excite rat SCN neurons at low doses and to inhibit them at high doses (Farkas et al., 2002; Piggins et al., 2002). However, the effects of the hcrts on circadian phase either, *in vitro* and *in vivo*, have not been reported. Central injections of both peptides appear to induce *c-fos* expression in the SCN (Date et al., 1999) but the time of administration in these studies was not reported. As SCN *c-fos* induction is typically associated with photic stimulation, it remains a possibility that hcrt release can affect circadian parameters during the dark
period. Clearly, the generation of an *in vivo* PRC is necessary to fully elucidate the role of these peptides, if any, in the control of circadian timing.

The evidence to date suggests that activation of the IGL is both necessary and sufficient for shifts of a non-photic nature (e.g., Rusak et al., 1989; Biello et al., 1991; Wickland and Turek, 1994) with NPY, GABA, and/or enkephalin potentially transferring non-photic information to the SCN (Morin & Allen, 2006). The current results are concordant with this idea in that WC and SD, effective non-photic manipulations, elevate IGL c-Fos-ir. These observations are also consistent with results reported previously for these procedures (Janik & Mrosovsky, 1992; Janik et al., 1995; Mikkelsen et al., 1998; Antle & Mistlberger, 2000). As well, systemic administration of modafinil or caffeine, arousal-inducing pharmaceuticals that do not perturb circadian phase, did not increase the number of c-Fos positive nuclei in this structure.

However, we found that the SLR procedure, a stressful arousing procedure that does not perturb circadian phase, also elevates *c-fos* expression in the IGL. In rats, Edelstein and Amir (1995) have also observed increased c-Fos-ir following 1h of conventional restraint during the subjective day, although rats are not known to exhibit non-photic shifts to daytime arousal of any kind. These observations raise several critical issues. First, if it is assumed that the SLR-activated cells are involved in phase control, this finding challenges the status of the IGL as the final common non-photic pathway to the SCN. However, as noted above, the IGL is neurochemically diverse and it may be that the cells that are active following restraint are separate from those that mediate phase shifts (e.g., NPY, GABA, enkephalins). Without positive neurochemical identification it
is impossible to come to any firm conclusions. Thus, it will be a priority to characterize the neurochemical nature of these stress-activated cells.

These findings also raise several questions regarding the potential sites and mechanisms for the hypothesized interaction between stress and phase shifts to arousal. As noted in the introduction, stressful arousal procedures that restrict locomotor activity and anxiogenic drugs do not phase shift circadian rhythms (Antle et al., 2001; Mistlberger et al., 2003; See Chpt. 5 and Appendix A and B). Therefore, it is possible that stress may inhibit phase shifts to arousal. The potential biological bases of any stress-induced attenuation remain to be elucidated but the mechanisms have been postulated to involve a serotonergic inhibition of non-photic IGL output (See Chpt. 3). If one assumes that the same population of cells are activated by both effective non-photic procedures and by physical restraint, then the observation that the SLR procedure induces IGL c-fos expression suggests that the site(s) of arousal/stress interactions must lay outside of the IGL, at the level of the SCN. On the other hand, assuming that some or all of the IGL neurons activated by stress are separate from those involved in phase control, it may be that some of the cells expressing c-fos following the SLR procedure inhibit IGL output to the SCN. For example, the stress activated IGL cells may be inhibitory interneurons that can decrease arousal-induced IGL activity. Alternatively, these stress-activated IGL neurons may separately project to the SCN and directly or indirectly influence arousal-induced neurotransmitter release or signal transduction at the level of the clock. Whatever the case, neurochemical phenotyping and functional tract tracing studies will be needed to distinguish between these competing hypotheses and other potential explanations.
Technical Considerations

Prepro-hcrt is proteolytically processed to produce one molecule each of mature hcrt-1 and hcrt-2 (Sakurai et al., 1999) and the distribution of these two peptides appear to overlap (Peyron et al., 1998). However, apparent differences in the fiber staining patterns of both peptides have been reported and, following wheel running, divergent patterns of c-fos expression across Hcrt-1 and -2 expressing cells have been noted (Cutler et al., 1999b; Nixon and Smale, 2004). Therefore, it is possible that immunostaining for prepro-hcrt or hcrt-2 may have yielded divergent results. However, a recent comprehensive examination of hcrt-1 and -2 immunoreactivity across several rodent species concluded that, for both peptides, there are few differences in the distribution of fibers across structures (Nixon & Smale, 2007). This latter result indicates that the Hcrt-1 immunostaining likely identified the entire population of hcrt expressing cells.

Perhaps a more daunting issue, are the problems associated with using c-fos expression as a measure of neuronal activity. Examination of c-Fos-ir is a powerful tool for the identification of neural circuitry mediating various behavioural and physiological processes. However, as discussed earlier (See Chpt. 3), the usefulness of this technique is limited by the interpretational ambiguity associated with negative results. This uncertainty stems from the fact that c-fos expression is not necessarily synonymous with membrane depolarization. There are disconnects, for example, between in vivo single unit recordings and c-fos expression. The reasons for these differences are unclear but may relate to insufficient stimulation or failure to activate the specific signal transduction pathways necessary for c-fos induction.
Another issue to consider is the fact that it is impossible to determine if a sacrificed animal would have actually phase advanced in response to the behavioral manipulations. This is less of a concern with WC, given the clear relationship between wheel revolutions and resulting shifts. There have been cases, however, where hamsters do not phase shift despite running over 10,000 wheel revolutions (Mrosovsky & Biello, 1994). With SD deprivation it is difficult to predetermine which animals may shift in response to the procedure. We have considered utilizing light-induced SCN c-fos expression to confirm shifting, an approach used previously in this laboratory (Antle & Mistlberger, 2000), but, following WC, delaying sacrifice until ZT11.5 results in a significant decrease in the percentage of Hcrt-1-ir neurons expressing c-fos (Webb et al., unpublished results).

Conclusions

In sum, the current results indicate that arousal procedures differentially activate the hcr system and the IGL. Wheel confinement and sleep deprivation, effective non-photic procedures increased c-fos expression in the hcr system while caffeine and modafinil are without effect. Physical restraint, however, an ineffective non-photic stimulus, also increases c-fos expression in these cells. Thus, the hypocretin system is not sufficient to induce clock resetting. A similar pattern of c-Fos immunostaining also was observed in the IGL, raising questions regarding the status of this structure as the final common pathway to the clock and its potential as a site for the hypothesized interaction between stress and phase shifts to arousal.
Figures

Figure 17. Illustration of the counting boxes used for spatial analysis. Each box measured 600 μm X 800 μm. The dots represent Hert-1 immunoreactive neurons. V = 3rd ventricle; VMH = ventromedial hypothalamic nucleus, OT = optic tract.
Figure 18. A representative photomicrograph illustrating c-Fos (black arrow) and Hcrt-1 (red arrow) immunoreactivity. Double-labeled cells are indicated by the presence of two arrows.
Figure 19. Effects of behavioural and pharmacological manipulations on the number of single labelled c-Fos cells, single labelled Hcrt-1 cells, and double-labelled cells by brain area. The shaded bars represent the number of double-labelled c-Fos-ir/Hcrt-1-ir neurons and the open bars represent the number of single labelled Hcrt-1-ir neurons. The percentages indicate the proportion of Hcrt-ir cells that also showed c-Fos-ir following the manipulations. Data are shown as means ± SEM. * = significantly different from DD; A = significantly different from MOD; B = significantly different from CAFF; C = significantly different from SLR; D = significantly different from SD; E = significantly different from WC. One symbol represents p < .05, two symbols represent p < .01, three symbols represent p < .001. Uppercase letters indicate differences in the percentage of double-labelled cells while lower case letters indicate differences in the number of single-labelled Hcrt-1 cells.
Figure 20. Effects of the behavioural and pharmacological manipulations on c-fos expression in Hcrt-1-ir cells and Hcrt-1 immunonegative neurons. A. Effects of behavioural and pharmacological manipulations on the number of single labelled Hcrt-1 cells, and the percentage of double-labelled cells. The shaded bars represent the number of double-labelled c-Fos-ir/Hcrt-1-ir neurons and the open bars represent the number of single labelled Hcrt-1-ir neurons. The percentages indicate the proportion of Hcrt-ir cells that also showed Fos-ir following the manipulations. B. Effects of the behavioural and pharmacological manipulations on c-fos expression in Hcrt-1-immunonegative neurons.* = significantly different from DD; A = significantly different from MOD; B = significantly different from CAFF; C = significantly different from SLR; D = significantly different from SD; E = significantly different from WC. One symbol represents p < .05, two symbols represent p < .01, three symbols represent p < .001. Uppercase letters in panel A indicate differences in the percentage of double-labelled cells whilst lower case letters indicate differences in the number of single labelled hcr-1 cells. Data are shown as means ± SEM.
Figure 21. Effects of the behavioural and pharmacological manipulations on c-fos expression in Hcrt-1-immunonegative neurons by brain area. Data are shown as means ± SEM. The shaded bars represent the number of double-labelled c-Fos-ir/Hcrt-1-ir neurons and the open bars represent the number of single labelled Hcrt-1-ir neurons. The percentages indicate the proportion of hcrt-ir that also showed c-Fos-ir following the manipulations. Data are shown as means ± SEM. * = significantly different from DD; A = significantly different from MOD; B = significantly different from CAFF; C = significantly different from SLR; D = significantly different from SD; E = significantly different from WC. One symbol represents p < .05, two symbols represent p < .01, three symbols represent p < .001.
Figure 22. c-Fos expression in the intergeniculate leaflet (IGL) following the various arousal procedures. A. Photomicrograph illustrating c-Fos and Hcrt-1 immunoreactivity in the IGL of a control and a stress-loaded restraint treated animal. B the number of c-Fos positive cells observed in the IGL following each treatment. The asterisks indicate a significant difference from the DD, MOD, and CAFF groups with \(*\)\(= p < .01\) and \(*\*\)\(= p < .001\).
CHAPTER 5: STRESS, GLUCOCORTICOIDS, AND NON-PHOTIC PHASE SHIFTING IN SYRIAN HAMSTERS

Abstract

In Syrian hamsters, circadian rhythms can be phase shifted by arousal procedures that permit locomotion (e.g., wheel confinement, sleep deprivation, Reebs & Mrosovsky, 1989; Antle & Mistlberger, 2000) but not by stressful arousal procedures that restrict ambulation (e.g., physical restraint, confinement to a platform over water; Mistlberger et al., 2003). Therefore, it may be that some minimal amount of locomotor activity is necessary for non-photic clock resetting or, alternatively, under some circumstances, stress may block phase shifts to arousal. To explore the effects of stress applied following a phase shifting procedure, hamsters were subjected to physical restraint following wheel confinement. As well, to investigate whether blocking glucocorticoid receptors might reveal a phase shifting effect to a stressful arousal procedure, hamsters were treated with the glucocorticoid antagonist mifepristone or vehicle prior to immobilization stress. Phase shifts to wheel confinement were not affected by subsequent restraint stress and no phase shifts were evident following restraint with vehicle or mifepristone. As well, immobilization stress transiently decreased locomotor activity only in one experiment, suggesting that stress-induced suppression of wheel running is not a robust effect. These results confirm that physical restraint, applied during the mid-to-late light period, does not perturb circadian phase and suggest that, if stress attenuates phase shifts to arousal, it
likely does so at the input stage and through a mechanism separate from glucocorticoid receptor activation.

**Introduction**

The phylogenetically ancient hypothalamus is the seat of mammalian homeostasis and located within this structure are several systems designed to deal with environmental challenges. One, the circadian system, is designed to anticipate the predictable daily physiological changes associated with life on a rotating planet. The master circadian pacemaker, localized to the suprachiasmatic nucleus (SCN), is at the heart of this system and drives the daily rhythms in physiology and behaviour that have evolved to deal with these predictable changes (Moore & Eichler, 1972; Stephan & Zucker, 1972; Ralph et al., 1990). Another hypothalamic system, the stress system, is designed to counter those challenges that are unpredictable. In the face of an imminent threat, this system initiates activation of the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic nervous system, ultimately resulting in the mobilization of energy resources and increased cardiovascular tone (Sapolsky, 2002).

In the absence of environmental time cues, the SCN free runs with a periodicity close to, but not necessarily equal to, 24h and, without resetting, this endogenous oscillator would eventually drift out of phase with the external light/dark cycle, thus losing its adaptive utility. However, on a daily basis, light, transduced by photoreceptive retinal ganglion cells (Gooley et al., 2001; Hattar et al., 2002; Hannibal et al., 2002) that innervate the SCN (Moore and Lenn, 1972), resets the phase of the clock such that its
average period approaches 24h and it remains in synchrony with the external environment (DeCoursey, 1960; Pittendrigh & Daan, 1976).

Although photic stimulation is considered the primary zeitgeber for mammalian circadian timing, in Syrian hamsters, non-photic manipulations that increase locomotor activity or arousal also can perturb circadian phase (Mrosovsky, 1996a; Mistlberger et al., 2000). Running stimulated by confinement to a novel wheel or sleep deprivation with minimal activity, for example, can phase advance locomotor rhythms when presented during the mid-light period (i.e., the normal rest period in nocturnal species; Reebs & Mrosovsky, 1989; Wickland & Turek, 1991; Antle & Mistlberger, 2000). Given that the generalized stress response is necessarily associated with increased arousal, it seems only reasonable to assume that stressors presented during the light period may also perturb circadian phase. The circadian response to stressors is complex, however, and phase shifts occur only under limited circumstances.

Inescapable foot shock, resident-intruder interactions, and open field exposure, all classical stressors, have been reported to phase advance hamster locomotor activity rhythms when presented during the mid-subjective day (Cain et al., 2004; Mistlberger et al., 2003), although the magnitude of shifts and the percentage of animals exhibiting shifts in response to these stimuli are lower by comparison to wheel running or sleep deprivation by gentle handling. As well, many of the behavioural manipulations capable of inducing non-photic like phase shifts activate the stress system at one or more levels. Saline injections, cages changes, or exposure to a novel environment, for example, have been reported to increase plasma glucocorticoid levels in hamsters or in rats (Mead et al., 1992; Sumova et al., 1994; Buijs et al., 1997; Dishman et al., 1998). However, when
locomotor activity is restricted, phase shifts do not occur despite the presence of sustained arousal. Physical restraint via confinement to a tube or to a small platform over water, for example, do not produce phase shifts in hamsters (Mistlberger et al., 2003). The current body of evidence, therefore, suggests two possibilities regarding the behavioural antecedents of non-photic clock resetting. First, it may be that shifts in response to arousal procedures are induced by the neural correlates of locomotor activity, and that even low levels of activity are sufficient if sustained over 3 h (e.g., as in the sleep deprivation by handling procedure, which is associated with ~80 m distance in 3 h, or 0.3 m/min, Antle & Mistlberger, 2000). Phase shifts induced by some stress procedures (e.g., footshock, resident/intruder interactions, and open field exposure) may be caused by the locomotion stimulated by these manipulations, and the variability of shifts to these stimuli may be because the amount (intensity or duration) of locomotion is near some lower threshold for eliciting shifts. Alternatively, it may be that arousal alone is sufficient to shift the clock and that phase shifts to stress procedures are absent, smaller, or less likely because the neural or endocrine correlates of stress block phase shifts to arousal. Phase shifts may occur to some stressful procedures because of concurrent expression of locomotor activity, which in other paradigms has been shown to attenuate or prevent behavioural or physiological effects of stress (Soares et al., 1999; Dishman et al., 2000; Greenwood et al., 2005). When locomotor activity is entirely prevented, this mitigating influence is absent, and any clock resetting effects of being awake in the usual sleep period would be entirely blocked by stress correlates. This latter hypothesis while less parsimonious, is testable.
This chapter describes two experiments designed to further probe the stress attenuation hypothesis. In Experiment 1, we determined if non-photic phase shifts could be blocked by subsequent exposure to a stressor. This was assessed by subjecting hamsters to physical restraint immediately following novel wheel confinement. This experiment was suggested by other work demonstrating mutual antagonism of photic and non-photic stimuli (inhibition of phase shifts to activity, and the reverse; Challet & Pevet, 2003; Yanielli & Harrington, 2004). If locomotor activity and some correlates of stress are similarly antagonistic, then a stress stimulus applied after a bout of wheel running may attenuate or block shifts to this procedure. In Experiment 2, we investigated whether glucocorticoids, a major neurohormonal correlate of the stress response, might be responsible for the lack of phase shifts to some stress procedures. Mifepristone, a glucocorticoid receptor antagonist, was administered prior to physical restraint and the effects upon circadian phase were assessed. If glucocorticoid receptor activation inhibits phase shifts to arousal, then blocking these receptors may reveal phase shifts to restraint stress.

Methods

Animals and Housing

Young male Syrian hamsters (Charles River, Montreal, PQ, Canada; 80-140g) were housed under a 14:10 LD cycle in opaque polypropylene cages with stainless steel mesh bottoms. The cages were equipped with 17.5cm running wheels and PVC pipes for light avoidance. Wheel revolutions were recorded via a microswitch interfaced with a computer and displayed with Circadia. Food and water were provided ad libitum.
Drugs

Mifepristone (Sigma), a glucocorticoid receptor antagonist, was dissolved in corn oil and administered subcutaneously at a dose of 80 mg/kg. This dosage is well above that reported to increase prepro-enkephalin mRNA levels in this species (Jimenez et al., 1999; Franklin and Jimenez, 2006).

Experiment 1: Does subsequent exposure to physical restraint block phase shifts induced by novel wheel confinement?

Once stable entrainment was achieved, hamsters (n = 12) were subjected to two separate conditions beginning at zeitgeber time 6 (i.e., ZT6, by convention six hours before dark onset). The first condition consisted of confinement to a novel 33cm Wahlmann wheel for 3h, a manipulation that produces large phase advances in hamsters that run more or less continuously (Mrosovsky, 1996a). The second condition also involved confinement to a Wahlmann wheel but was followed immediately by physical restraint for 3h. The restraint manipulation involved placement into a PVC tube (15 cm long, 4 cm internal diameter) sealed on either side with steel mesh combined with intermittent compressed air stimulation to prevent behavioural sleep (See Mistlberger et al., 2003). Despite the sustained arousal produced by this stress-loaded restraint (SLR) manipulation, it has little effect upon circadian phase when carried out during the light period (Mistlberger et al., 2003). The overhead room lights were turned out at the beginning of each procedure and remained out for three days following the manipulations. The restraint procedure was carried out under dim red light (~ 1 lx). A period of two weeks between manipulations was allotted for re-entrainment.
Experiment 2: Does pharmacological blockade of glucocorticoid receptors influence the effect of physical restraint upon circadian phase?

Upon stable entrainment, two groups of hamsters (n = 6 per group) were subjected to three conditions counter balanced for order within each group. At ZT5, the first group received vehicle (corn oil, s.c.) alone, mifepristone alone, or vehicle injection followed by the SLR procedure from ZT6-9. A second group also was administered vehicle or mifepristone at ZT5 but, following a separate mifepristone administration, was subjected to the SLR procedure from ZT6-9. Upon completion of the injections, the room lights were turned out and remained out for three days following the manipulations. The restraint procedure, however, was carried out under dim red light (~ 1 lux). There was a minimum period of 10 days between each procedure to allow for reentrainment and drug washout.

Phase Shift Assessment and Activity Analysis

Phase shifts were calculated by comparing the time of spontaneous activity onset on day 2 of constant darkness following the manipulation with the average time of activity onset during the 3 days prior to the manipulation day (the so-called Aschoff Type II procedure (Mrosovsky, 1996b). A computer algorithm was used to identify the onset of the main period of daily wheel running.

Stressful procedures have been reported previously to effect wheel-running activity when applied in the early active period (Seifritz et al., 1998; Mistlberger and Antle, 2006). Therefore, the influence of each manipulation on total daily activity over a 5-day baseline, the manipulation day, and the three days of DD was examined, and,
where significant effects were found, further analysis was undertaken to explore their time course. To this end, activity was summed across five blocks spanning the latter half of the subjective day and the entire subjective night (ZT6-9, ZT9-12, ZT12-15, ZT15-18, and ZT18-22).

Phase shift and activity measures were evaluated by repeated measures ANOVAs, and independent or paired t-tests where appropriate. The Greenhouse-Geisser correction for degrees of freedom was utilized in cases where sphericity assumptions were violated. Means are presented +/- the standard error of the mean.

Results

Experiment 1: Subsequent exposure to physical restraint does not affect phase shifts induced by wheel confinement

Wheel confinement alone and wheel confinement followed by physical restraint produced equivalent phase advance shifts of 133 +/- 22 min and 134 +/- 26 min, respectively (t [11] = -0.062, n.s., Fig. 23). The activity levels during the WC procedure also did not differ by condition (3537 +/- 456.1 vs. 3763 +/- 498.4; t[11] = -0.79, p > .05; Fig. 24B).

A multifactorial within ANOVA of the percent change in total daily activity relative to an average 5-day baseline over the days following each treatment (the manipulation day and the three days of constant darkness) revealed an SLR-induced decrease in wheel running (F[1,11] = 5.91, p = .05), but no significant effect of day (F[3,33] = 0.47, p > .05) and a non-significant interaction (F[1.69,18.56] = 2.05, p > .05). Subsequent comparisons indicated that, relative to WC alone, the SLR procedure
significantly reduced activity only on the manipulation day (80.2 +/- 5.4% vs. 48.6 +/- 6.7%; t [11] = 3.20, p < .01; Fig. 24A). Following the detection of a significant condition by time of day interaction (F[2.07, 22.73] = 8.00, p < .001), further analysis of the time course of this effect on the manipulation day revealed an SLR-induced decrease in activity at ZT9-12 (731 +/- 252 vs. 0 +/- 0; t [11] = 2.90, p < .05), ZT12-15 (2230 +/- 536 vs. 55 +/- 41; t[11] = 4.05, p < .01) and ZT15-18 (1250 +/- 433 vs. 2 +/- 1; t[11] = 2.89, p < .05; Fig. 24B).

Experiment 2: Blockade of glucocorticoid receptors does not influence the phase response to physical restraint.

Oneway within ANOVAs revealed that, in both groups, none of the treatments induced significant phase shifts (Group 1: F[2,8]= 1.378, p > .05.; Group 2: F[1.1, 4.2] = 4.268, p > .05, Fig. 25). An independent t-test revealed that the phase shifts induced by restraint were not influenced by mifepristone administration (4 +/- 7.3 min vs. 11 +/- 3.9 min, t [7.5] = -0.900, p > .05).

To further explore the inhibitory effect of restraint stress on subsequent home cage wheel running activity, a multifactorial within ANOVA examining the percent change in total daily activity relative to an average 5-day baseline by day and condition revealed that for, Group 1, mifepristone alone had no effect upon wheel running (F[2,10] = 0.93, p > .05), nor was there an effect of day (F[3,15] = 1.21, p > .05) or a significant interaction (F[6,30] = 2.27, p > .05; Fig. 26). For Group 2, there also was no significant effect of condition (F[2,8] = 4.03, p > .05) and a non-significant interaction (F[6,24] =
2.00, p > .05), There was, however, a significant effect of day (F[3, 12] = 7.21, p < .01; Fig. 26).

**Discussion**

Overall, the current results confirm earlier observations that restraint stress, applied during the mid to late subjective day, does not perturb circadian phase in hamsters despite producing sustained arousal (Mistlberger et al., 2003). We also found that physical restraint, applied immediately following the wheel confinement procedure, does not attenuate phase shifts to this manipulation. Thus, any stress-induced attenuation of non-photic clock resetting must occur at an earlier time point, potentially during the input stage. The current results also indicate that mifepristone does not influence the phase response to physical restraint and suggest that any effects of stress upon non-photic phase shifting must be mediated by glucocorticoid independent mechanisms. As well, it was observed that the SLR procedure suppressed locomotor activity during the subsequent dark period. However, as this was observed in only one experiment, it does not appear to be a robust effect.

That physical restraint does not influence phase shifts to wheel confinement is not completely unexpected. Several lines of evidence suggest that clock resetting by non-photic means is rapid and complete by the end of the arousing procedure. Suppression of SCN *per* gene expression, for example, is evident immediately following a 3h wheel confinement (Maywood et al., 1999). As well, following sleep deprivation by gentle handling, *c-fos* can be induced in the SCN by photic stimulation at ZT10, a time when the SCN is normally unresponsive to light (Antle & Mistlberger, 2000). Similar results have been observed following saline injections and indicate that resetting is complete within an
hour of stimulus presentation (Mead et al., 1992), a time frame similar to that proposed for photic resetting in hamsters (Best et al., 1999). Therefore, it is probable that the clock was already well advanced prior to the stress procedure, and that the opportunity for any stress-induced influence had passed. That is, stress may prevent non-photic stimuli from resetting the clock when applied during the arousal procedures, but cannot block a phase shift that has already occurred.

However, light applied immediately following wheel confinement has been reported to attenuate or block phase shifts in response to this procedure (Mrosovsky, 1991). Thus, there is an opportunity to inhibit non-photic clock resetting in the hours immediately following an activity pulse. Given the advanced phase of the clock at this time, however, any effects are likely mediated by changes in clock gene expression. Light, for example, presumably increases SCN Per levels at this time, thereby delaying rhythms and counteracting any non-photic effects. Although the effects of physical restraint on hamster SCN Per levels are unknown, it is quite likely that this procedure does not induce expression in this species. This is supported by the observations that forced swimming or physical restraint do not elevate per 1 or 2 expression in the murine SCN (Takahashi et al., 2001). As well, short-term immobilization, saline injection, or social conflict do not increase SCN fos expression in the hamster or rat (Mead et al., 1992; Edelstein and Amir, 1995; Kollack-Walker et al., 1999). Therefore, unlike photic/non-photic interactions, any stress-induced attenuation of arousal-induced resetting likely does not occur at the level of SCN clock gene expression and may involve an extra-SCN site or sites. It is possible, for example, that the neural correlates of stress somehow inhibit the ability of non-photic stimuli to access the circadian clock. This is
speculation, however, and further research is needed to support this idea. The application of CRF, glucocorticoid or noradrenergic agonists in combination with effective non-photic procedures may be useful in this regard.

The current results also indicate that glucocorticoids likely do not participate in any stress-induced attenuation of phase shifts to arousal. Short-term immobilization, a procedure that produces sustained arousal, did not induce phase shifts despite the systemic administration of mifepristone. This observation, however, should not be construed as a decisive blow to a stress attenuation hypothesis. The physiological response to stress is multifaceted and the mechanism of action may include several other stress-associated hormones and/or neurotransmitters. CRF receptors, for example, are distributed throughout the brain (Hemley et al., 2007) and central administration of this peptide reduces the amplitude of locomotor activity rhythms in hamsters (Seifritz et al., 1998), similar to the suppression of nighttime locomotor activity by daytime restraint observed in the current study. Therefore, it may be that activation of these receptors is critical to any attenuation effects and further studies utilizing CRF antagonists and other pharmacological compounds may be warranted.

A single dose of mifepristone was employed in the current study and it may be argued that this dosage was too small to exert any effects upon glucocorticoid binding. However, our dose is relatively high in comparison to previous studies and was selected to overcome the lowered ability of mifepristone to cross the rodent blood brain barrier (Heikinheimo and Kekkonen, 1993). At lower doses (30 -50mg/kg), mifepristone has been reported to lower prepro-enkephalin mRNA levels in hamsters and to block the elevation of corticosterone levels or changes in midbrain glutamate receptor expression in
rats when administered 15 min – 1 h prior to inescapable stress (Jimenez et al., 1999; Moldow et al., 2005). Therefore, it is likely that the current dose was sufficient to block central glucocorticoid receptors during the restraint procedure and that higher doses also would be without effect.

In one of the experiments, physical restraint, applied during the mid-to-late day, transiently decreased locomotor activity over the subsequent dark period. These observations are consistent with previous results showing that, in hamsters or rats, resident-intruder interactions or central CRF administration suppress locomotion in a similar manner (Meerlo et al., 1997; Seifritz et al., 1998). However, the stress-induced decrease in wheeling running was observed in only one of the two current experiments and thus does not appear to be a robust effect.

It also has been previously reported that physical restraint alone, without air stimulation and applied during the early dark period, produces an initial decrease in activity followed by a rebound increase in wheel running (Antle & Mistlberger, 2006). The reasons for the lack of rebound activity in the current study are unclear but likely relate to procedural differences. Physical restraint with air may further increase activation of the HPA as compared to restraint alone, and, given that the stress-induced decrease in wheel running appears to be mediated by CRF in hamsters (Seifritz et al., 1998), this modified restraint procedure may result in comparatively increased inhibition. Alternatively, the effects of stress upon wheel running may be time-of-day dependant.

In conclusion, phase shifts to wheel confinement are not affected by subsequent physical restraint, suggesting that any stress-induced attenuation of phase shifts to arousal likely occurs at the input level. As well, glucocorticoid receptor antagonism does not
influence the effect of restraint stress upon circadian phase. Thus, any effects of stress on phase shifts to arousal must be mediated by glucocorticoid independent mechanisms. In addition, the current results add to the body of evidence indicating that, in hamsters, stressful procedures transiently decrease wheel-running behaviour. However, as stress induced attenuation was observed in one experiment, this does not appear to be a robust effect.
Figures

Figure 23. Phase shifts in response to wheel confinement with or without subsequent physical restraint. (A) A representative actogram from animal hSTWC11 showing phase shifts in response to wheel confinement (WC) from ZT6-9 with and without subsequent physical restraint (SLR). Each horizontal line represents a 24 h period with wheel revolutions plotted in 10 min bins from left to right. Wheel running is indicated by vertical deflections and shading marks the dark period of the LD cycle. The ‘v’ markers designate the beginning and end of novel wheel confinement and the unfilled box represents the restraint procedure. (B) Mean phases shifts to wheel confinement with and without subsequent physical restraint. Data shown as mean +/- SEM.
Figure 24. The effects of wheel confinement (WC) with and without subsequent restraint stress (WC + SLR) on total daily wheel running activity (A) Percent change in total daily activity following WC and WC+SLR relative to an average 5-day baseline on the manipulation day and three days of constant dark. (B) Number of wheel revolutions by varied time blocks across the manipulation day. * = p < .05, ** = p < .01
Figure 25. Phase shifts in response to vehicle, mifepristone, and physical restraint with and without pre-treatment with mifepristone. A. Two representative actograms from animals C3P6 and C4P2 showing phase shifts in response to vehicle (Veh), mifepristone (Mife), and physical restraint (SLR) with and without pre-treatment with mifepristone. Vehicle injections are represented by unfilled diamonds, mifepristone injections by filled diamonds, and physical restraint by unfilled boxes. B. Mean phase shifts to vehicle, mifepristone, and physical restraint with and without pre-treatment with mifepristone. Group numbers are shown above each bar. Note that data for both the veh and mife conditions for Group 1 and 2 have been pooled for this figure. Data shown as mean +/- SEM.
Figure 26. Percent change in total daily wheel running relative to an average 5-day baseline induced by vehicle and mifepristone with and without restraint stress over the manipulation day and three days of constant darkness.
CHAPTER 6: DISCUSSION AND GENERAL CONCLUSIONS

During the subjective day, varied non-photic procedures that increase locomotor activity (e.g., confinement to a novel running wheel) can markedly phase advance circadian rhythms in Syrian hamsters (Turek & Losee-Olson, 1986; Reebs & Mrosovsky, 1989a; Van Reeth & Turek, 1989). Initial research to establish the behavioural antecedents of non-photic clock resetting has revealed vigorous physical activity to be unnecessary, as simply keeping the animals awake induces phase advance shifts comparable in size to those seen with wheel confinement (Antle and Mistlberger, 2000). Thus, the critical variable appeared to be arousal. Subsequent work, however, has indicated that behavioural arousal is not always sufficient. Stressful arousal procedures that prevent ambulation (e.g., physical restraint, confinement to a platform over water) do not effect circadian phase despite continuous, EEG-confirmed waking with high muscle tone (Mistlberger et al., 2003). The current body of data therefore supports two possible hypotheses regarding the nature of the critical non-photic stimulus. It may be that non-specific arousal with some minimal level of locomotor activity is necessary for non-photic resetting. Alternatively, behavioural arousal alone may be sufficient to phase advance the circadian pacemaker, but for this to be true, an inhibitory action of stress must be invoked.

The current series of experiments add further to the body of evidence implicating locomotor activity in non-photic phenomena. As discussed previously, the common feature of all effective non-photic manipulations is the ability to produce arousal in
association with some level of locomotor activity. Manipulations that prevent locomotor activity but sustain arousal do not induce phase shifts (Mistlberger et al., 2003) and restricting locomotor activity attenuates shifts to triazolam and to dark pulses (e.g., Reebs et al., 1989; Van Reeth and Turek, 1989; Dwyer and Rosenwasser, 2000a; Rosenwasser and Dwyer, 2002). As well, the magnitude of phase shifts induced by many non-photic manipulations are positively related to indices of forward locomotion (Mrosovsky and Salmon, 1990; Mistlberger et al., 1996; Bobrzynska and Mrosovsky, 1998; Mistlberger et al., 2003). We have now shown that modafinil, an atypical alerting compound that potently stimulates wakefulness but only marginally increases locomotor activity, does not perturb circadian phase when administered at midday. This is the case both under a normal LD schedule and following one night of constant light, a procedure known to potentiate shifts to non-photic stimuli (Mistlberger et al., 2002; Knoch et al., 2004; Landry and Mistlberger, 2005; Knoch et al., 2006). Likewise, we have confirmed and extended previous findings by showing that physical restraint, even when applied following a night of constant light, does not induce non-photic like shifts (See Chpt. 5 and Appendix A). Thus, even under optimal conditions for non-photic clock resetting, phase advances do not occur in the absence of ambulation. A working hypothesis, therefore, is that a sustained low level of locomotor activity is necessary for shifts of a non-photic nature.

If this is the case, however, what remains unclear is the amount of locomotor activity required to reset the circadian pacemaker. With a 3h wheel confinement procedure, there is a clear sigmoidal relationship between the number of wheel revolutions and the resultant shifts, with large advances evident at over 4000 revolutions
and little or no shifts with lower levels of activity (Bobrzynska and Mrosovsky, 1998). Nevertheless, vigorous sustained activity over a three-hour period is clearly not necessary for behavioural resetting as sleep deprivation, open field exposure, or social interaction, procedures with relatively low levels of ambulation (~80m for SD), are sufficient to phase advance the clock (Antle & Mistlberger, 2000; Mistlberger et al., 2003). One possible interpretation of these data is that the ambulatory threshold required for non-photic resetting varies by situation. It may be for example, that arousal (or some correlate thereof) and ambulation interact and that, under some circumstances, increased arousal lowers the amount of locomotor activity required to perturb circadian phase. This idea is partially supported by our observations that, with modafinil administration, large phase shifts to wheel confinement are observed following relatively low levels of locomotor activity. This is an intriguing possibility with the potential for therapeutic application and, therefore, warrants further investigation.

Another perspective is that the sigmoidal relationship between the number of wheel revolutions accumulated in 3 h and the magnitude of phase shifts reflects a critical role for sustained arousal or locomotion, and not for the intensity or total amount of locomotion. Hamsters that run in a novel wheel tend to run continuously, which is necessary to accumulate 4,000 or more revolutions. Those that don’t run continuously (classified as ‘sluggards’ by Mrosovsky & Biello, 1994) typically accumulate between 0 and 2,000 revolutions. When hamsters are not running when confined to a novel running wheel, they are not awake (unpublished observation), and lack of sustained waking may prevent the clock from receiving sufficient non-photic stimulation (whatever its nature) for phase resetting to occur. Modafinil may shift leftward the dose-response curve for
‘activity-induced’ shifting because it prevents hamsters from sleeping when they are not running. The fact that modafinil alone is associated with very low levels of spontaneous locomotion and is completely devoid of phase resetting action indicates that locomotion likely does play an important role. Sleep deprivation induces large shifts and on average 3-fold more locomotor activity, but the total distance travelled in 3h remains very low, much less than even ‘sluggard’ hamsters that may run 1,000 revolutions but nonetheless fail to shift, presumably due to repeated napping. Taken together, these observations suggest an important role for sustained waking with some locomotor activity; the dose response relation between wheel running and shifting obtained from novel wheel confinement experiments may be an artifact of the necessary relationship between continuity of waking and amount of running- to run a lot, the hamsters has to stay awake. If there is a dose-response relationship between amount of locomotor activity and phase shifting, it is likely to be in the very low range of activity, e.g., between the amount associated with modafinil injections and the amount associated with sleep deprivation by gentle handling.

The current results also appear to strengthen the evidence indicating that arousal per se is not sufficient to induce shifts of a non-photic nature. As outlined above, stressful arousal-inducing manipulations that restrict locomotor activity or the administration of anxiogenic drugs (Antle et al., 2001; See Chpt. 5 and Appendix B) do not induce phase shifts (Van Reeth et al., 1991; Mistlberger et al., 2003) despite the presence of wakefulness. Therefore, it may be that the stress and/or anxiety associated with these procedures somehow blocks phase shifts to arousal. As modafinil does not have anxiogenic properties (Simon et al., 1994; van Vliet et al., 2006), administration of this
atypical alerting agent offered a novel approach to separating locomotor activity and arousal while at the same time avoiding the confounding influence of stress and/or anxiety. In our hands, this compound produced sustained arousal without a concomitant increase in ambulation, but did not perturb circadian phase. Therefore, behavioural arousal alone appears to be insufficient for clock resetting even in the absence of stress and/or anxiety. We are not aware of evidence that modafinil stimulates HPA correlates of stress, but if it did, and these correlates were inhibitory to non-photic shifting, then we would have expected modafinil to have shifted the dose-response curve for ‘activity-induced’ phase shifts to the right, rather than to the left as we observed.

Previous studies have indicated that phase shifts to light exposure are inhibited by concurrent wheel running or by acute sleep deprivation (Ralph & Mrosovsky, 1992; Mistlberger & Antle, 1998; Mistlberger et al., 1997; Challet et al., 2001; Christian & Harrington, 2002). We found that modafinil does not affect photically induced advances or delays and thus show for the first time that behavioural arousal alone is not sufficient to attenuate light induced phase shifts. Given that non-photic attenuation of photic phase resetting appears to be mediated by NPY, GABA, the enkephalins and/or serotonin release (Gamble et al., 2005; Weber et al., 1998; Tierno et al., 2002; Mintz et al., 2002), it is likely that modafinil administration does not induce release of these neurotransmitters at the level of the clock, a suggestion consistent with this agent’s failure to perturb circadian phase during the midday.

Arousal, however, is a non-specific term encompassing a range of behavioural states and it is likely that the constellation of neural and endocrine correlates unique to each of these states has differential effects upon the circadian pacemaker. As outlined
above, which of these correlates is critical for phase shifting has not been fully resolved, and it is conceivable that some correlates promote shifting, while others block shifting. Under some circumstances, for example, it may be that stress and/or anxiety concurrent with wakefulness may attenuate arousal-induced clock resetting. This hypothesis is supported by several pieces of evidence. First, as noted above, stressful procedures that restrict ambulation do not induce phase advance shifts during the mid light period despite high levels of arousal (Mistlberger et al., 2003; See Chpt. 5 and Appendix A). As well, there is a negative correlation between the number of interventions needed to maintain wakefulness during sleep deprivation and the size of the resulting phase advance (Antle & Mistlberger, 2000). Furthermore, administration of the cortisol synthesis inhibitor metyrapone increases the magnitude of phase shifts induced by sleep deprivation (Mistlberger et al., 2003) and, with the exception of modafinil, the arousal inducing agents that do not perturb circadian phase (i.e., caffeine, yohimbine) also have anxiogenic effects in animal models (Pellow et al., 1985; Baldwin et al., 1989; Johnston and File, 1989; Antle et al., 2001; See Appendix B). Moreover, caffeine administration has been reported to attenuate phase shifts to wheel confinement (Antle et al., 2001). Therefore, it may be that the neural correlates of these stressful and/or anxiogenic procedures inhibit phase shifts to arousal. Our observation that restraint stress attenuates phase shifts to the midday LD transition normally seen with short-term constant light exposure (Knoch et al., 2004; Knoch et al., 2006; Landry & Mistlberger, 2005; See Chpt. 2) further supports this contention (See Appendix A).

The current series of experiments also have provided novel information regarding when stress is likely to have an influence on non-photic resetting and have ruled out some
potential mechanisms of action. We have shown that physical restraint, applied immediately following the wheel confinement procedure, does not attenuate phase shifts to this manipulation. Therefore, any stress-induced attenuation of non-photic clock resetting must occur at an earlier time point, perhaps during input stage. As well, administration of the glucocorticoid receptor agonist mifepristone does not influence the phase response to physical restraint, indicating that any effects of stress upon non-photic phase shifting must be mediated by glucocorticoid independent mechanisms. As noted previously, this observation should not be construed as a serious blow to the stress-attenuation hypothesis as the response to stress is multifaceted and numerous other molecular effecters may underlie the hypothesized inhibitory effects.

Admittedly, a stress-attenuation model is somewhat tenuous and hangs only on a few key pieces of indirect evidence. Several experiments, including those from our own laboratory, have yielded results seemingly at odds with this proposal. For instance, phase shifts are elicited by some stressful arousal procedures. Intermittent foot shock, resident-intruder interactions, and open field exposure all have been reported to produce phase advances in hamsters when presented during the light period (Cain et al., 2004; Mistlberger et al., 2003). These stressful manipulations, however, do not restrict locomotor activity and it may be that the ambulation induced by these procedures somehow mitigates the effects of stress. Alternatively, stress has differential effects on the monoaminergic and peptidergic systems that project directly or indirectly to the SCN depending on factors such as modality (e.g., visceral, sensory, cognitive/conditioned), intensity and escapability (Maywood et al., 1998; Grahn et al., 1999; Lowry, 2002; Amat
et al., 2005), and it may be that particular patterns of stress-induced neural activation permit clock resetting while others may inhibit this process.

Regardless of the nature of the non-photic stimulus and the hypothesized influence of stress, previous investigations have identified two prominent non-photic inputs to the circadian clock and the current studies have yielded novel information regarding their potential roles in pacemaker resetting. The GHT, originating in the thalamic IGL, utilizes NPY, enkephalin and GABA (Morin and Blanchard, 2001) and, in hamsters, activation of this pathway appears both necessary and sufficient for non-photic clock resetting (Rusak et al., 1989; Biello et al., 1991; Janik and Mrosovsky, 1992, 1994; Janik et al., 1995; Mikkelsen et al., 1998). The other pathway, stemming from the median raphe nucleus, utilizes serotonin (Meyer-Bernstein & Morin, 1996). However, the body of evidence implicating this transmitter in clock resetting is conflicting and inconsistent at best (Morin, 1999; Mistlberger et al., 2000).

The observations that WC and SD induce non-photic shifts and elevate IGL c-Fos-ir, while modafinil and caffeine do neither, are consistent with the idea that the IGL is the final common non-photic pathway to the clock. However, the SLR manipulation, a stressful arousing procedure that does not perturb circadian phase, also elevates c-fos expression in this structure. A similar result was reported previously for the rat, although that species does not exhibit phase shifts to midday activity or arousal (unpublished observations; Mrosovsky, personal communication). Expression of c-fos in the IGL hamsters by restraint indicates that neurons in the IGL respond to arousal without locomotor activity, and that ‘non-photic’ output from the IGL may be blocked by some correlate of stress, as we have speculated. A critical next step in this analysis is to
identify the neurochemical phenotypes and projection patterns of these stress-activated IGL neurons.

Other data from the c-fos mapping studies indicates that activation of the rostral serotonergic DRN, the MnR, or the noradrenergic LC, is not necessary for non-photic clock resetting. In general, both novel wheel confinement and sleep deprivation by gentle handling, effective non-photic procedures, failed to increase c-fos expression in these areas. By contrast, physical restraint, an arousing procedure that does not result in clock resetting, elevated c-fos expression in the DRN and the LC. Thus, activation of these areas is also not sufficient for non-photic clock resetting by behavioural means. As outlined previously, involvement of the rostral ascending serotonin system in non-photic phenomena remains contentious. 5,7-dihydroxytryptamine lesions (Bobrzynska et al., 1996a; Meyer-Bernstein and Morin, 1998) or serotonergic antagonists (Antle et al., 1998) do not attenuate phase shifts in response to some non-photic procedures. As well, 5-HT agonists do not induce phase shifts in mice (Antle et al., 2003) or hamsters (Mintz et al., 1997; Antle et al., 2000) unless 5-HT receptors are supersensitized by pre-treatment with a 5-HT synthesis inhibitor (Ehlen et al., 2001) or short-term constant light exposure (Knoch et al., 2006). Furthermore, elevation of serotonin in the SCN to levels similar to that observed during wheel confinement or electrical stimulation does not induce shifts (Antle et al., 2000). Thus, the previous findings in concert with the present observations, suggest that, in some cases, the activation of serotonergic outputs to the SCN or IGL is not necessary for shifts of a non-photic nature and, under normal physiological conditions, is not sufficient to reset the circadian pacemaker. The same also may be concluded for noradrenergic inputs to the circadian system.
Instead, as the rostral DRN and the LC are selectively activated by an arousing stress procedure, the current findings suggest that these structures may be involved in the hypothesized stress-induced attenuation of phase shifts to arousal outlined above. This contention is based upon the following observations. Given the inhibitory action of serotonin in the IGL (Ying et al., 1993; Blasiak & Lewandowsk, 2003), and the pivotal role of this region in non-photic shifting (Rusak et al., 1989; Biello et al., 1991; Janik & Mrosovsky, 1994; Janik et al., 1995; Janik & Mrosovsky, 1992; Mikkelsen et al., 1998), it is likely that serotonin release in the IGL, induced by physical restraint or other stressful or anxiogenic procedures, inhibits output to the SCN and thereby blocks arousal-induced clock resetting. Activation of the LC may ultimately produce the same result, as noradrenergic input to the DRN appears to mediate the response to inescapable stress. In rats, pharmacological blockade of DRN α1 noradrenergic receptors attenuates the behavioural consequences of exposure to an uncontrollable stressor (Grahn et al., 2002). See Fig. 27 for a schematic of the proposed model.

Indirect support for this model stems from the observation that anxiogenic drugs that increase arousal but do not perturb circadian phase (i.e., caffeine and yohimbine) also increase c-fos expression in the DRN and in the LC (Singewald and Sharp, 2000; Abrams et al., 2005; Deurveilher et al., 2006). In contrast, modafinil, a non-anxiogenic alerting compound that does not induce clock resetting (See Chpt. 2), does not increase c-fos expression in the feline DRN or in the LC (Lin et al., 1996). In rats, however, this agent, when administered at night, has been reported to slightly increase c-fos expression in the LC but not in the DRN (Scammell et al., 2000).
The stress attenuation model outlined above is preliminary in nature and therefore requires further empirical validation. First, it will be essential to determine if the cells that express c-fos in response to physical restraint actually project to the IGL. We are currently using functional neural tract tracing techniques to verify that this is the case. We are also actively examining the effects of local DRN blockade of several stress-related neurochemicals upon the circadian response to physical restraint. In animal models, the DRN is critical for the behavioural effects induced by inescapable stress (Maier et al., 1993; Amat et al., 2005) and CRF and norepinephrine release in this area appear to be necessary for these behavioural changes (Grahn et al., 2002; Hammack et al., 2003). Theoretically, if these inputs are involved in a stress-induced inhibition of shifts to arousal then the administration of CRF or noradrenergic receptor antagonists to the DRN should gate shifts to physical restraint. Clearly, the proposed model as it stands outlines numerous predictions that are experimentally tractable.

The current results also show that, following several behavioural and pharmacological arousal procedures with disparate effects upon circadian phase, c-fos is expressed differentially in the hypocretin system. This immediate early gene correlate of neural activity was increased by wheel confinement, short-term sleep deprivation, and stress-loaded restraint. Thus, activation of the hcrt system is not sufficient to induce shifts. Caffeine and modafinil administration, arousal procedures that do not phase shift the clock (Antle et al., 2001; See Chpt. 2), also slightly elevated c-fos expression but the increase was non-significant compared to controls. Reduced activation of hcrt cells by compounds that fail to induce non-photic shifts is consistent with a necessary role for
hert, but it remains possible that greater $c-fos$ expression would have been revealed by reducing survival time after the drug treatment.

In conclusion, the current series of studies have yielded novel findings regarding the behavioural antecedents and neural mechanisms of non-photic phase shifting in Syrian hamsters. Although it has now been 20 years since the first report of circadian clock phase resetting in response to activity/arousal inducing stimuli, a full neurobiological account of this phenomenon still awaits.
Figures

Figure 27. A schematic representation of the circadian system and the stress attenuation model. The plus symbols indicate putative excitatory inputs, the minus symbols inhibitory inputs, and the question marks inputs of unknown influence. According to the model, arousal during the subjective day excites the IGL leading to the release of one or more excitatory neurotransmitters in the SCN, thus resulting in a phase advance. Inescapable stress concurrent with arousal, however, leads to increased excitation of the LC and/or the DRN, ultimately resulting in the release of 5-HT in the IGL and a subsequent decrease in IGL output to the SCN.
APPENDIX A: SHORT-TERM CONSTANT LIGHT DOES NOT POTENTIATE PHASE SHIFTS TO PHYSICAL RESTRAINT IN SYRIAN HAMSTERS

Introduction

Although light is considered the primary zeitgeber for circadian rhythms, in Syrian hamsters, manipulations that increase arousal and locomotor activity can perturb circadian phase. Confinement to a novel running wheel or sleep deprivation, for example, can phase advance locomotor activity rhythms when applied during the mid-light period (Reebs & Mrosovsky, 1989a; Antle & Mistlberger, 2000). Phase shifts in response to non-photic manipulations are greatly increased by short-term exposure to constant light (LL). During the midday, shifts to novel wheel confinement, sleep deprivation, intra-SCN NPY or systemic 8-OH-DPAT injections are potentiated (~2.5 fold) by one to three days of LL (Mistlberger et al., 2002; Knoch et al., 2004; Landry & Mistlberger, 2005; Knoch et al., 2006; See Chpt. 2).

Utilizing an Aschoff Type II design, stress-loaded restraint (SLR), an immobilization procedure with concomitant compressed air stimulation to sustain arousal (Mistlberger et al., 2003), does not perturb circadian phase. This seems somewhat surprising given the body of evidence implicating arousal in non-photic clock resetting. However, it may be that arousal alone, in the absence of locomotor activity, does not provide sufficient stimulation to shift the clock and that, in this situation, optimal conditions are necessary to observe any resultant phase shifts. This has been observed for other manipulations. For example, lower doses of 8-OH-DPAT that are ineffective under
LD conditions can produce large phase shifts following a period of LL (Knoch et al., 2006). To further investigate this possibility, we examined the effect of short-term LL on the phase response to restraint stress during the light period.

The mechanisms of LL-induced potentiation of non-photic clock resetting are unclear but may involve increases in non-photic clock inputs. The LL-induced suppression of locomotor activity first focused attention upon changes in the serotonin system. LL suppresses SCN serotonin release during the subjective night (Knoch et al., 2004) and restoration of SCN 5-HT levels by reverse microdialysis moderately attenuates the potentiation of phase shifts to systemic 8-OH-DPAT (Knoch et al., 2004). Short-term LL, however, does not induce changes in the number of 5HT\textsubscript{1A}, 5HT\textsubscript{1B}, or 5HT\textsubscript{7} serotonin receptors in the SCN suggesting that the mechanism may involve a change in receptor sensitivity and/or intracellular modifications downstream of ligand binding, rather than an upregulation in receptor expression (Duncan et al., 2005; Knoch et al., 2006). The observation that NPY-mediated shifts are also potentiated by LL exposure suggest that changes occur in non-serotonergic clock inputs as well (Knoch et al., 2004).

Alternatively, chronic light exposure may modify a core clock parameter rendering it more susceptible to perturbation by entraining stimuli. Circadian clocks have been successfully modelled as limit cycle oscillators that can exhibit small or large phase shifts dependent upon the amplitude of the oscillation (Johnson et al., 2003). Constant light dampens locomotor activity, SCN serotonin release, and per 1 expression in the SCN core (Knoch et al., 2004; Duncan et al. 2005) and, therefore, it is possible that this decrease in pacemaker amplitude could render the clock more prone to large phase shifts. Theoretically, a decrease in amplitude should increase phase shifting in response to all...
entraining agents and not specifically to classic non-photic stimuli. Therefore, one way to test the amplitude suppression hypothesis is to examine phase shifts to other stimuli following exposure to LL. Physical restraint in the late subjective day or early subjective night produces small (~25 min) phase delays in hamster locomotor activity rhythms (Van Reeth et al., 1991; Dwyer & Rossenwasser, 2000; Rossenwasser & Dwyer, 2002; Antle & Mistlberger, 2006), and if the amplitude suppression hypothesis is correct, then phase shifts to this procedure should also be potentiated by short-term exposure to constant light. To test this possibility, hamsters were exposed to one night of constant light followed by restraint stress in the early dark period and examined to determine the effects of these procedures upon circadian phase.

Methods

Animals

Young male Syrian hamsters (80-140g; Charles River, Montreal, PQ, Canada) were housed under a 14:10 light-dark cycle in standard polypropylene cages (45 X 25 X 20cm) with stainless steel mesh bottoms. The cages were equipped with running wheels (17.5 cm diameter) and contained a PVC pipe for light avoidance. Wheel revolutions were recorded via mechanical switches, and displayed using Circadia (Dr. T. A. Houpt, Florida State University) or Clock Lab (Actimetrics, Wilmette, IL). Food and water were provided ad libitum.
Experiment 1: Does short-term constant light affect the phase response to physical restraint during the light period?

Once the hamsters (n = 12) were stably entrained to the LD cycle they were exposed to one night of constant light (~350 lux). The homecage pipes were removed during this period to ensure equal levels of light exposure across all animals. Following the night of LL, the hamsters were randomly divided into two groups and, at zeitgeber time 6 (ZT6, six hours before dark onset by convention), were left undisturbed in the homecage or subjected to 3 hours of SLR, a manipulation consisting of confinement to a small PVC tube (15cm long X 4cm internal diameter) in combination with intermittent compressed air stimulation to maintain arousal (Mistlberger et al., 2003). The overhead room lights were turned out at the start of each procedure and remained out for 3 days to allow for the assessment of phase shifts. However, during both procedures, the room was illuminated with dim red light (DDred, ~ 1 lux) from ZT6-9.

Experiment 2: Does short-term constant light affect the phase response to physical restraint during the dark period?

Upon stable entrainment, a second group of hamsters (n = 9) were exposed to one night of LL and, at ZT12, left undisturbed in the homecage or subjected to the SLR procedure as outlined above. During both conditions, the overhead lights were turned off and the room was placed into DDred. In this case, however, the colony room remained in DDred for 4 days following each manipulation. All animals were exposed to both manipulations with 8 days between each procedure to allow for reentrainment to the LD schedule.
Phase Shift Analysis

Phase shifts were measured by comparing the activity onsets on the second day following the manipulations to the average baseline onset for 3 days prior to the procedure. Data were evaluated with the appropriate t-tests and are displayed as the mean +/- the standard error of the mean.

Results

Experiment 1: Short-term LL does not promote phase advance shifts to SLR during the light period

Both the DD and SLR procedures induced small phase advances relative to baseline but the SLR condition induced a significant phase delay of 46 min relative to the control group (57 +/- 13 min vs. 11 +/- 12 min; t[10] = 2.63, p < .05; Fig. 1).

Experiment 2: Short-term LL does not potentiate phase shifts to SLR early in the dark period

Both the DD and SLR procedures induced phase advances relative to baseline but the SLR procedure did not induce phase shifts relative to the controls (23 +/- 9 min vs. 8 +/- 7 min; t[8] = 1.57, p > .05; Fig. 2).
Fig. 1. Phase shifts induced by DD or SLR at ZT6 following one night of LL. A. A representative actogram for a DD control animal. B. A representative actogram for a SLR treated animal. The “V” markers designate the beginning and end of the procedure. C. Mean phase shifts induced by DD or SLR on day 2 of DD relative to the average activity onset during baseline. * = p < .05 as compared to DD.

Discussion

Overall, the current results indicate that, under optimal conditions for behavioural clock resetting, stress-loaded restraint, applied during the mid light period, does not
induce phase shifts of a non-photic nature. Following exposure to short-term constant light, a LD transition in the midday results in large phase advance shifts (Knoch et al., 2004; Knoch et al., 2006; Landry & Mistlberger, 2005; See Chpt.2) and this effect is evident in the current results. The SLR manipulation, however, induces phase delay shifts relative to the LD transition, thus suggesting that restraint stress may attenuate phase shifts to the change in lighting conditions. The underlying physiology responsible for phase shifts to the LD transition is unknown but it has been reported that these shifts can be attenuated by systemic pindolol (a 5-HT₁A antagonist) injection (Glass, unpublished...
results). Therefore, stress may attenuate phase shifts to the LD transition by influencing serotonergic transmission. Note that this is consistent with our hypothesis that stress-induced release of 5-HT in the IGL may inhibit non-photic output to the SCN.

Alternatively, LL exposure may change the shape of the restraint PRC such that restraint-induced delays, normally evident only following restraint at the beginning of the night (Van Reeth et al., 1991; Dwyer & Rossenwasser, 2000; Rossenwasser & Dwyer, 2002; Antle & Mistlberger, 2006), instead occur at an earlier phase of the circadian pacemaker. Such an effect has been previously reported for 8-OH-DPAT. Following two days of constant light, systemic 8-OH-DPAT injections induce large Type-0 advances early in the subjective day and delays of a similar magnitude in the mid to late subjective night (Knoch et al., 2004; Knoch et al., 2006). Construction of a full restraint PRC (i.e., restraint tests across a range of circadian phases) following short-term LL exposure will be necessary to test this supposition.

As outlined in the introduction, there are two general mechanisms by which non-photic resetting may be potentiated by LL exposure. First, it may be that LL somehow potentiates non-photic input to the clock (by increasing neurotransmitter release, receptor upregulation, increased receptor sensitivity, changes in second messengers, etc.). Alternatively, according to limit cycle modelling, LL induced suppression of a core clock parameter may render the clock more susceptible to phase perturbation. If this is true then, theoretically, a decrease in pacemaker amplitude should increase phase shifting in response to all entraining agents and not specifically to arousal procedures or their neural correlates. The current results, however, argue against the amplitude suppression hypothesis. Phase shifts to restraint in the early dark period are not increased by short-
term constant light. In fact, the SLR procedure, applied at this time, did not perturb circadian phase. These observations are in agreement with a previous study indicating that phase delays in response to intra-SCN NMDA are not potentiated by exposure to constant light (Landry & Mistlberger, 2005). These results, taken together, suggest that LL induced potentiation is not due to amplitude suppression and may be specific to non-photic clock inputs during the mid light period.
APPENDIX B: SYSTEMIC ADMINISTRATION OF YOHIMBINE DOES NOT PERTURB CIRCADIAN PHASE IN SYRIAN HAMSTERS

Introduction

The serotonergic raphe nuclei and thalamic intergeniculate leaflet have been implicated in non-photic resetting (Morin, 1999, Mistlberger et al., 2000); however areas upstream require further elucidation. Convergent evidence suggests that locomotor activity and/or arousal are critical for clock resetting by behavioural means (Mrosovsky, 1996), thus, areas involved in the neural control of locomotor activity or arousal are of particular interest. The neurons comprising the noradrenergic locus coeruleus are active during waking, decrease their activity during slow wave sleep, and are virtually silent during REM sleep (Rasmussen et al., 1986), and, therefore, may potentially contribute to non-photic phase shifting during the midday.

Several observations suggest that the norepinephrine (NE) system can influence circadian timing. First, the neural substrate exists for noradrenergic input to the circadian clock. The locus coeruleus sends sparse projections to both the SCN and the IGL (Moga and Moore, 1997; Vrang et al., 2003; Horowitz et al., 2004) and α2 NE receptors have been detected in these areas (Rosin et al., 1996; Talley et al., 1996). As well, chronic administration of clonidine, an α2 noradrenergic receptor agonist, has been reported to shorten circadian period in rats under constant conditions (Rosenwasser, 1989, 1996; Dwyer and Rosenwasser, 2000b) and to transiently lengthen circadian period and change...
the phase angle of entrainment in hamsters (Dwyer & Rosenwasser, 2000). Acute clonidine administration in hamsters also has been reported to induce photic-like phase shifts and these effects are blocked by the noradrenergic neurotoxin DSP-4 (Rosenwasser et al., 1995). Chronic administration of clonidine, however, decreases photic phase advances in hamsters (Dwyer and Rosenwasser, 2000b).

Given the preliminary evidence for noradrenergic regulation of circadian rhythmicity, we examined the effect of systemic yohimbine, an α2 noradrenergic autoreceptor antagonist (thus functionally a noradrenergic agonist), on circadian phase in hamsters.

**Methods**

**Animals**

Young male Syrian hamsters (n = 20, Charles River, PQ) were housed under a 14:10 LD schedule in polypropylene cages with wire mesh bottoms and PVC pipes for light avoidance. The cages were equipped with 17.5cm running wheels and microswitches were utilized to record running wheel activity. Food and water were freely available.

**Drugs**

The α2 noradrenergic receptor antagonist yohimbine (Sigma) was dissolved in sterile saline and administered i.p. at doses of 1 and 2 mg/kg. At these doses, yohimbine has been reported to influence male sexual behaviour in hamsters (Arteaga et al., 2002),...
and to increase arousal in rats (Makela and Hilakivi, 1986) and cats (Leppavuori and Putkonen, 1980).

**Procedure**

Once stable entrainment was evident, at ZT6, the hamsters were administered vehicle, 1 mg/kg yohimbine, and 2 mg/kg yohimbine in counterbalanced order. To allow for the measurement of phase shifts, the room lights were turned out following the injections and remained out for three days. There was a minimum of 14 days between manipulations.

**Phase Shift Measurement**

The onset of locomotor activity on Day 2 of DD was compared with the mean activity onset for three days prior to drug administration. The data are displayed as mean +/- SEM.

**Results**

Repeated measures ANOVA revealed that yohimbine had no effect upon circadian phase ($F[2,32] = 0.445$, n.s., **Fig. 1**).

**Discussion**

The current results indicate that systemic administration of yohimbine during the mid-light period does not perturb circadian phase in Syrian hamsters and suggest that the NE system does not contribute to non-photic clock resetting. In addition to a direct
pharmacological effect, however, pharmaceutical agents may shift the clock indirectly via increased arousal and/or locomotor activity. In this context, it is somewhat surprising that yohimbine did not advance locomotor activity rhythms. Systemic or central administration of yohimbine, at the current doses, has been reported to increase wakefulness and locomotor activity in several species (Leppavuori and Putkonen, 1980; Pellejero et al., 1984; Makela and Hilakivi, 1986; De Sarro et al., 1987). However, we did not directly examine behavioural effects in the current study and it is possible that yohimbine does not potently induce arousal or locomotor activity in this species. Yohimbine also has well documented anxiogenic effects in rats and mice (Pellow et al., 1985; Johnston and File, 1989; Venault et al., 1993) and, assuming that this compound produced arousal in the current study, it is conceivable that the anxiogenic actions blocked the influence of arousal upon the clock. This interpretation is consistent with other evidence suggesting that stress may attenuate phase shifts to arousal. Caffeine, another anxiogenic agent, fails to induce phase shifts in hamsters despite increasing arousal (Antle et al., 2001) and confinement to a restraint tube or to a platform over water, stressful arousal conditions that restrict locomotor activity, do not perturb circadian phase (Mistlberger et al., 2003). Alternatively, it is conceivable that yohimbine did not perturb circadian phase, despite increased arousal, because it did not concomitantly increase locomotor activity.
Fig. 1 Phase shifts in response to systemic yohimbine injection at ZT6. Representative phase shifts to (A) saline, (B) 1mg/kg yohimbine or (C) 2mg/kg yohimbine. (D) Mean phase shifts to each of the three treatments.
APPENDIX C: A PRELIMINARY COMPARISON OF THREE IMMUNOPEROXIDASE TECHNIQUES TO IMPROVE NEUROPEPTIDE Y CELLULAR STAINING IN THE INTERGENICULATE LEAFLET OF THE SYRIAN HAMSTER

Introduction

Few neuropeptide Y (NPY) intergeniculate leaflet (IGL) cell bodies are observed in our laboratory with ABC immunoperoxidase immunocytochemistry (ICC). Use of the axonal transport inhibitor colchicine may be an effective technique to increase NPY-immunoreactivity (ir) but it is not compatible with behavioural studies. Tyramide signal amplification (TSA), however, may be an ideal way to increase NPY-ir without the negative side effects. Hence, we undertook to compare the number of IGL NPY-ir cells observed following ABC immunoperoxidase ICC in untreated animals and in those pre-treated with colchicine, and in untreated animals following ABC ICC with TSA.

Methods

Animals

Young male Syrian hamsters (n = 3, Charles River, PQ, Canada).

Surgery

Anaesthesia was induced in one animal with butorphanol (10 mg/kg, s.c.) and sodium pentobarbital (80 mg/kg, i.p.). The hamster was also administered Ringers
solution (5cc, s.c.) to prevent dehydration and Metacam (1 mg/kg, s.c.) as an analgesic.

Using a stereotaxic instrument a cannula was aimed at the lateral ventricle (0.4mm anterior, 1.9mm lateral, and 4.4mm ventral relative to bregma) and colchicine (100μg in 5μL) was injected unilaterally with the aid of a Hamilton syringe. The injection was carried out over 5 min, visually confirmed, and the cannula was left in place for an additional 2 min to allow for diffusion.

**Histology**

48 h after surgery, 3 animals were perfused transcardially with 50ml phosphate buffered saline (PBS, pH ~ 7.3) followed by 50ml 4% paraformaldehyde in PBS (PFA, pH ~ 7.3). Brains were removed, post-fixed in PFA for 2h, and cryoprotected in 20% sucrose in PBS overnight. Using a cryostat, the tissue was sectioned in a 1:4 series at 30um intervals through the entire IGL. The sections were stored in de Olmos cyroprotectant (Watson et al., 1986) at -20°C until immunocytochemical processing. Tissue surrounding the cannula tract in the surgerized animal was also Nissl stained and examined to determine cannula placement.

**Immunocytochemistry**

One series per animal was subjected to ICC processing as follows:

*ABC ICC With and Without Colchicine Treatment*

The tissue was washed in 0.01M phosphate buffered saline containing 0.03% Triton X-100 (PBSx, Sigma) between each step and all incubations were carried out at room temperature. The tissue was first incubated for 1 h in 10% normal donkey serum
(NDS, Jackson Immunoresearch) in PBSx containing a 1:5 dilution of avidin (Vector Labs) followed by overnight immersion in rabbit anti-NPY (Bachem, 1:10000 in PBSx containing 1% NDS and a 1:5 dilution of biotin [Vector Labs]). The following day the sections were incubated for 1 h with a biotinylated donkey anti-rabbit secondary (Jackson Immunoresearch, 1:400 in PBSx containing 1% NDS) and subsequently exposed to an avidin-biotin complex (ABC, Vector Labs, 1:200 in PBSx) for 1 h. Finally the stain was developed with 0.05% dianminobenzidine and 0.1% H₂O₂ in 0.05M Tris buffer. The tissue was mounted on gelatin subbed slides, allowed to dry, dehydrated, cleared and coverslipped with Permount (Fisher).

**ABC ICC with TSA**

Up to the ABC incubation, this tissue was treated as outlined above. These sections were incubated with ABC (1:800 in PBSx) for 1 h followed by exposure to biotinylated tyramine (Perkin Elmer, 1:250 in amplification solution) for 10 min. Next, the tissue was immersed in streptavidin-conjugated horseradish peroxidase (Perkin Elmer, 1:100 in PBSx) followed by development with 0.04% dianminobenzidine and 0.07% H₂O₂ in 0.05M Tris buffer. The sections were mounted as described above.

**Cell Counting**

All NPY-ir cells in the IGL were counted with a Nikon Eclipse 80i light microscope.
Results

NPY-ir was observable in all conditions with fibers and cells identifiable in the IGL and in many other brain areas (See Fig. 1 and Table. 1). An increase in fiber staining was apparent with TSA but the number of NPY-ir cell bodies did not increase. Colchicine appeared to be without effect but the injection may have been slightly misplaced (Fig. 2). The injection site appeared dorsal to the ventricle but the dorsal border of the ventricle appears breached.

Fig. 1. NPY-ir at three levels of the IGL with the three staining techniques.
Table 1. The number of IGL NPY-ir cells, slices with NPY-ir cells, and estimated total number of NPY cells for each staining condition.

### Discussion

The number of NPY-ir neurons observed with ABC immunoperoxidase ICC is lower than that reported previously (Morin & Blanchard, 1995; but see Morin & Pace, 2002). By contrast with NPY-ir neurons in many other brain regions in our tissue, NPY-ir in IGL neurons appears quite faint and is not easily identified. In our previous double labelling attempts (NPY and c-fos), we have observed very few NPY cells and this may be partially due to a slight increase in background from labelling the second antigen. However, most published images of double-labbelled IGL cells appear equally poor.

Colchicine did not appear to increase the number of NPY-ir cells or to effect fiber staining. This may have been due to a slightly misplaced injection, low dosage or unilateral injection. TSA appeared to increase fiber staining but did not increase the number of IGL NPY-ir cells. A lower number of slices with NPY-ir cells were observed in the TSA condition, however. This may partly be due to sampling issues as only one series out of four was stained. Twenty-five cells were counted in a single slice in the colchicine condition (data not shown) and it may be that the slices with the greatest
number of cells were located in one of the three remaining series. Nevertheless, in the sections that showed immunoreactivity there was not a dramatic increase in the number of cells observed. Therefore, it is unlikely that TSA

Fig. 2  Nissl stained sections showing the site of the colchicine injection. The slices are arranged from anterior to posterior.

will aid in identifying NPY cells in the IGL. NPY in these neurons may be trafficked out of the soma rather quickly and TSA cannot amplify a signal that does not exist. We have,
however, observed increased NPY-ir cells following Ni$^{2+}$ enhancement, a condition that is not ideal for c-Fos double labelling protocols (data not shown).

**Conclusions**

Neither colchicine nor TSA increased the number of IGL NPY-ir neurons and will likely be of little use. The lack of effect for colchicine may be due to a misplaced injection. It appears the NPY staining in the IGL is generally poor and may not be able to be improved through conventional amplification techniques. The implications for the reliability of published double-labelling results in the literature is a matter of speculation.
REFERENCES


immunoreactivity in the hamster suprachiasmatic nucleus and intergeniculate

Cain SW, Verwey M, Hood S, Leknickas P, Karatsoreos I, Yeomans JS, Ralph MR
Behav Neurosci 118:131-137.

Card JP, Moore RY (1982) Ventral lateral geniculate nucleus efferents to the rat
suprachiasmatic nucleus exhibit avian pancreatic polypeptide-like

Card JP, Moore RY (1989) Organization of lateral geniculate-hypothalamic connections

pancreatic polypeptide-like immunoreactivity in the rat hypothalamus. J Comp

Challet E, Pevet P (2003) Interactions between photic and nonphotic stimuli to
synchronize the master circadian clock in mammals. Front Biosci 8:s246-257.

Challet E, Takahashi JS, Turek FW (2000) Nonphotic phase-shifting in clock mutant

Challet E, Scarbrough K, Penev PD, Turek FW (1998) Roles of suprachiasmatic nuclei
and intergeniculate leaflets in mediating the phase-shifting effects of a
serotonergic agonist and their photic modulation during subjective day. J Biol


Ehlen JC, Novak CM, Karom MC, Gamble KL, Paul KN, Albers HE (2006) GABAA receptor activation suppresses Period 1 mRNA and Period 2 mRNA in the


Lall GS, Biello SM (2003) Attenuation of circadian light induced phase advances and
delays by neuropeptide Y and a neuropeptide Y Y1/Y5 receptor agonist.
Neuroscience 119:611-618.


Larsen PJ, Kristensen P (1998) Distribution of neuropeptide Y receptor expression in the

raphe nuclear complex to the suprachiasmatic nucleus and the deep pineal gland

Legan SJ, Reinhardt CR, Davis VA, Duncan MJ (2005) Phenobarbiral-induced delay of
the estrous cycle in proestrus golden hamsters is associated with phase advance of
the circadian pacemaker. Society for Neuroscience Abstracts.

Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL (1987)
Circadian rhythmicity restored by neural transplant. Immunocytochemical

Leppavuori A, Putkonen PT (1980) Alpha-adrenergic influences on the control of the


Mintz EM, van den Pol AN, Casano AA, Albers HE (2001) Distribution of hypocretin-
(orexin) immunoreactivity in the central nervous system of Syrian hamsters


with photic signaling in the suprachiasmatic nucleus to regulate circadian phase

Mistlberger RE (1991) Effects of daily schedules of forced activity on free-running

resetting is phase and serotonin dependent. Brain Res 786:31-38.


Mistlberger RE, Skene DJ (2004) Social influences on mammalian circadian rhythms:

Mistlberger RE, Antle MC (2006) The enigma of behavioral inputs to the circadian clock:
a test of function using restraint. Physiol Behav 87:948-954.

Mistlberger RE, Houpt TA, Moore-Ede MC (1991) The benzodiazepine triazolam phase-
shifts circadian activity rhythms in a diurnal primate, the squirrel monkey (Saimiri


response element-mediated gene expression in the suprachiasmatic nuclei. J Biol
Chem 274:17748-17756.

and NMDA receptor subunit expression in the suprachiasmatic nucleus and other

Oliver KR, Kinsey AM, Wainwright A, Sirinathsinghji DJ (2000) Localization of 5-

Pacak K, Palkovits M (2001) Stressor specificity of central neuroendocrine responses:

Academic Press.

Effects of methoxamine and alpha-adrenoceptor antagonists, prazosin and
yohimbine, on the sleep-wake cycle of the rat. Sleep 7:365-372.

elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149-
167.

Penev PD, Turek FW, Zee PC (1993) Monoamine depletion alters the entrainment and
the response to light of the circadian activity rhythm in hamsters. Brain Res
612:156-164.


