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The role of serotonin in photic phase shifting: location of action and molecular underpinnings

by

Victoria M. Smith

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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Abstract

The serotonergic system modulates the effects of light on the mammalian circadian system. Some serotonergic drugs can greatly enhance the magnitude of the effect light has on shifting the circadian clock. BMY7378, a 5-HT_{1A} mixed agonist/antagonist, enhances photic phase shifts when administered in the late subjective night in hamsters, but not mice. In hamsters, BMY7378 binding directly to receptors in the median raphe nucleus (MRN) appears to be necessary for this effect, but binding in the suprachiasmatic nuclei (SCN) does not appear to be necessary, nor are the serotonergic fibers connecting the MRN directly to the SCN necessary. In order to produce its photic phase shift enhancement effect, BMY7378 alters the activation of some biochemical cascade components, as well as the expression of some genes that are activated or expressed following light exposure in the late subjective night. Buspirone, a 5-HT_{1A} partial agonist, normally attenuates photic phase shifts when administered during the late subjective night, but instead enhances phase shifts in mice lacking the 5-HT_{1A} receptor. The activation of some biochemical cascade components and genes expressed following light exposure are differentially altered following Buspirone administration, depending on whether there was an attenuation or potentiation of photic phase shifts, with or without the 5-HT_{1A} receptors respectively. Together, these results exemplify the complexity of the ability of the serotonergic system to modulate the effects that light stimuli have on the expression of mammalian circadian rhythms.

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List of Abbreviations

Measurement Terms:

°C	degrees Celsius
cm	centimeter
kg	kilogram
lux	unit of illuminance
µg	microgram
µm	micrometer
mg	milligram
mL	milliliter
mM	millimolar
M	molar
nm	nanometer

Other Terms:

3V	3rd ventricle
5-HT	serotonin, 5-hydroxytryptamine
5,7-DHT	5,7-dihydroxytryptamine
8-OH-DPAT	(±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide
ANOVA	analysis of variance
bp	base pairs
BMY7378	8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride
CalB	calbindin
cAMP	cyclic adenosine monophospate
CRE	Ca^{2+}/cAMP response element
CREB	cAMP response-element binding protein
CREM	cAMP response-element modulator
Cry	*cryptochrome* gene
Cry	cryptochrome protein
CT	circadian time
DD	constant darkness
DAB	diaminobenzidine
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DRN	dorsal raphe nucleus
ENK	enkephalin
ERK	extracellular signal-regulated kinase
GABA	gamma aminobutyric acid
GHT	geniculohypothalamic tract
GRP	gastrin-releasing peptide
haPer1	hamster *Period1* gene
haPer2	hamster *Period2* gene
ICER	inducible cAMP early repressor

IEG	immediate-early gene
IGL	intergeniculate leaflet
IHC	immunohistochemistry
i.p.	intraperitoneal
-IR	-immunoreactivity, -immunoreactive
KO	knockout
LD	light/dark cycle
LL	constant light
LP	light pulse
MAPK	mitogen-activated protein kinase
mPer1	mouse *Period1* gene
mPer2	mouse *Period2* gene
MRN	median raphe nucleus
mRNA	messenger ribonucleic acid
NAN-190	1-(2-methoxyphenyl)4-[4-(phthlaimido)butyl]piperazine hydrobromide
NMDA	*N*-methyl-D-aspartic acid
NPY	neuropeptide Y
OX	optic chiasm
PACAP	pituitary adenylate cyclase-activated polypeptide
PBS	phosphate buffered saline
PBSx	phosphate buffered saline with 0.1% Triton X-100
P-CREB	phosphorylated cAMP response-element binding protein
Per	*Period* gene
Per	Period protein
P-ERK	phosphorylated extracellular signal-regulated kinase
PRC	phase response curve
RHT	retinohypothalamic tract
ROD	relative optical density
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SERT	serotonin transporter
SP	substance P
tRNA	transfer ribonucleic acid
VIP	vasoactive intestinal polypeptide
VP	vasopressin
WT	wildtype
ZT	zeitgeber time

1. General introduction

Chronic desynchronization between the human endogenous circadian pacemaker and the rhythmic external environment often leads to numerous health consequences. Unfortunately, this desynchronization is inherent to the 24-hour nature of society in the 21st century. Shift work and the phenomenon known as "jet lag," which results from rapid travel across multiple time zones, have become a normal part of everyday life. Currently, there are no practical medications or therapies commercially available that aid in the acceleration of the resynchronization of the circadian system. Although light has the ability to reset the mammalian circadian pacemaker, its effects are minimal in magnitude. Thus the development of a chronobiotic, which would potentiate the effects of light would be extremely beneficial to thousands of individuals in all walks of life. Serotonin (5-HT) is known to play a regulatory role in the circadian system, and has been shown to modulate the effects of photic input to the circadian pacemaker. Thus, compounds such as BMY7378, with both serotonergic agonistic and antagonistic properties, that are capable of greatly potentiating the effects of light on the circadian system, could be a huge asset in today's fast-paced world. This dissertation examined the location of action and molecular underpinnings for the 5-HT_{1A} mixed agonist/antagonist BMY7378 to greatly potentiate the effects of light on the rodent circadian system, as well as the ability of the 5-HT_{1A} partial agonist buspirone to alter the effects of light both in terms of receptor contribution and molecular underpinnings.

1.1 Circadian rhythms: Overview

1.1.1 Evolutionary perspective

All organisms have basic needs that must be met in order to survive. They require food, shelter, and access to a mate, in order to ensure their own survival as well as the survival of the species. However, all organisms on Earth evolved in a rhythmic environment, resulting from the geophysical properties of the solar system. The Earth rotates around the sun, the moon rotates around the Earth, and the Earth rotates about its own axis. Thus, temporal factors such as the changing of the seasons as well as the day-night cycle play a vital role in an organism's ability to be successful in its particular niche. An organism's ability to tailor its daily activities to the generally predictable rhythms in its surrounding environment is adaptive for maximizing its chances of survival and reproduction (Dunlap *et al.*, 2004).

Every species has two options for the organization of its behaviour in a temporal manner – either an individual can follow exogenous timing cues, and directly respond to environmental changes in determining when to perform certain activities, or it can rely on an endogenous pacemaker which can be modulated by environmental stimuli to ensure it remains properly synchronized to the external cycles. The anticipation of repetitive temporal events allows an organism to optimally organize its behaviour and physiology, such that everything occurs at the most advantageous time of day, and thus increasing genetic fitness (Dunlap *et al.*, 2004). However, it is also necessary to be able to adapt to slight fluctuations in the precise timing of these repetitive temporal events, not only in

terms of the changes in day length as the seasons change, but also as a result of travel between time zones.

Several types of rhythms can be observed in biological systems, including: circannual rhythms (period of about one year), circalunar (period of about one lunar cycle), infradian (period of more than a day), circadian (period of about one day) and ultradian (period of less than a day; Refinetti, 2006). Circadian rhythms (*circa* = approximately; *dies* = a day) can be found in species ranging from prokaryotes to mammals, and constrain behaviour and physiology to species-specific times of the day (Sharma, 2003). Although it is not explicitly known, it has been speculated that an endogenous circadian rhythm may have developed as an evolutionary solution to the problem of cellular processes sensitive to UV radiation. Thus, some processes could have been necessarily restricted to nighttime when UV radiation was lowest, leading to rhythmicity that was circadian in nature (Pittendrigh, 1993).

For centuries, it was believed that biological rhythms were solely the result of the periodic nature of the external environment (Moore-Ede *et al.*, 1982). Not until the middle of the 20th century was it generally accepted by the research community that this rhythmicity was in fact the product of an internal timekeeping mechanism, within the organism itself (Edery, 2000). This fundamental paradigm shift led to an explosion of scientific research investigating the biological rhythms of numerous species. Since that time, extensive research has been done on the marked rhythmicity of many biological processes in both plants and animals. The first experiment which tested this belief was conducted by Jean Jacques d'Ortous de Mairan in 1729, where he observed that the

leaves of a specific plant open and close at consistent intervals in conjunction with the light/dark cycle, which persisted regardless of the presence of those external time cues (Moore-Ede *et al.*, 1982). It seemed that this leaf behaviour was endogenously generated. Subsequently, Augustine de Candolle showed that the rhythmic opening and closing of leaves in the *Mimosa pudica* plant was not only endogenously generated, but also had a period that was slightly shorter than the external light/dark cycle (LD) when in constant conditions (Moore-Ede *et al.*, 1982). This suggested to de Candolle that the plant was able to 'freerun' with a unique endogenous period when no longer synchronized to the light cycle of the external environment (Moore-Ede *et al.*, 1982).

1.1.2 Entrainment

Photic information received by the suprachiasmatic nuclei (SCN) from the retinas is the primary cue for entraining, or synchronizing, an animal's endogenous circadian rhythms to the external light/dark cycle (Hirota & Fukada, 2004). The day-night cycle serves to adapt mammals to their external environment such that the animal's behaviour remains tied to the rhythms in its environment, and the chances of survival are optimized with the ability to anticipate environmental changes (Cermakian & Sassone-Corsi, 2002; Gachon *et al.*, 2004; Panda & Hogenesch, 2004). The circadian system is normally synchronized to the environmental light/dark cycle, and therefore has a period of approximately 24 hours (Moore-Ede *et al.*, 1982; Reppert & Weaver, 2001). This synchronization allows organisms to optimally coordinate their physiological processes with the external world, and to predict daily changes in the environment (Holzberg & Albrecht, 2003; Antle & Silver, 2005). Yet, in the absence of external time cues, these

endogenous rhythms continue to oscillate with a period of about a day, although the exact period length varies slightly, and is species-specific (Pittendrigh & Daan, 1976a; Moore-Ede *et al.*, 1982; Roenneberg *et al.*, 2003).

Since the period of mammalian circadian rhythms are only approximately 24 hours, a correction must be made on a daily basis to keep the rhythms in synchrony with the length of the day. Gachon *et al.* (2004) used the example of a nocturnal animal being able to anticipate approximately when it is dusk. If the animal wakes up and the sun has not yet set, the subsequent evening it will wake up a little later in an attempt to prevent being seen by a diurnal predator when it emerges from its burrow on future evenings. Similarly, the animal anticipates approximately when it will be dawn, such that if the animal stays up too long and risks exposing itself to diurnal predators it will attempt to wake up a little earlier on subsequent evenings to ensure it has returned to the burrow before the sun rises on subsequent days.

Only when the oscillation of a zeitgeber ("time-giver") falls within a fairly narrow range of period lengths that is sufficiently close to 24 hours, called the limits of entrainment, can entrainment result (Aschoff & Pohl, 1978). It is possible to experimentally entrain an animal to a light/dark cycle with a period within the species-specific circadian range, generally between 21 and 27 hours (Pittendrigh & Daan, 1976b). It does not matter what the actual time of 'sunrise' is in an animal's environment, it is merely necessary that the length of the 'day' and 'night' be appropriate for the species.

Mammals continue to exhibit circadian rhythms under constant lighting conditions, either constant light (LL) or constant dark (DD). When an animal is not

entrained to any light-dark cycle, it is said to be freerunning. In this situation, statements of ‘day’ and ‘night’ are changed to ‘subjective day’ and ‘subjective night.’ These terms simply make reference to the portion of the normal activity cycle to which the animal is behaving most similarly. Thus, in constant lighting conditions, ‘subjective day’ refers to when a nocturnal animal is behaving as though it is daytime (generally sleeping/inactive) and ‘subjective night’ refers to when the animals is behaving as though it is nighttime (generally active), regardless of what the actual external time-of-day is. The period of the freerunning rhythm is no longer tied to the 24-hour light/dark cycle, and thus varies somewhat from 24 hours. It is dependent upon the particular species as to whether an animal will tend to freerun with a period slightly less than 24 hours or slightly greater than 24 hours, and there is also a degree of inter-individual variability within a species such that all individuals will not freerun with exactly the same period. Constant darkness is frequently used to determine the freerunning period of the activity cycle in nocturnal rodents. Since it would be difficult to see any change in the circadian rhythm for an animal entrained to a light/dark cycle, the majority of research involving adjustments to the circadian rhythm is done under constant lighting conditions in order to maximize the effects that can be observed. Light stimuli play an integral role in both the entrainment of the circadian system, and in adjustments that can be made to these endogenous rhythms.

1.2 Rhythmic desynchronization

Numerous health, safety, and social problems arise when endogenous circadian rhythms become desynchronized from the external environmental cycle. There are several causes of desynchronization, including jet lag, shift work, and circadian rhythm

disorders, but regardless of the cause the results can be disastrous. Although the percentage of individuals who are considered to be shift-workers (i.e. either working the night shift, working on a rotating shift, or working extended shifts) is fairly small, some of the most important positions in society are subject to these types of work schedules (Dunlap *et al.*, 2004). Doctors, nurses, emergency service workers, pilots, and nuclear power plant operators, are just a few examples of the occupations in which individuals are often subjected to shift work schedules. The circadian disruptions inherent in these sorts of positions not only affect the circadian rhythms of the individual workers, but can also have delaterious effects on those people under their care.

The rapid desynchronization inherent to phenomena such as rotating shift work schedules and jet lag can lead to numerous negative consequences. A wide range of health problems, including increased risk of cardiovascular disease and cancer, are the more serious of the potential medical conditions which can result from chronic desynchronization between one's internal circadian rhythms and the external environment (Rajaratnam & Arendt, 2001; Knutsson, 2003; Dunlap *et al.*, 2004; van Mark *et al.*, 2006). However, reduced concentration and increased reaction times are just a few of the adverse effects which can have disastrous effects on the innocent people around those experiencing this form of chronic desynchronization, potentially leading to accidents as small as traffic collisions, and as major as the Exxon Valdez oil spill and the Chernobyl nuclear disaster (Dunlap *et al.*, 2004). In order to minimize the effects of such desynchrony and develop potential treatments to accelerate resynchronization, it is

necessary to fully understand the mechanisms responsible for the synchronization of our endogenous circadian system to the rhythmic world.

1.3 The master pacemaker

Numerous behavioural and physiological processes in mammals, including activity cycles and the expression of certain genes, are governed by the daily oscillation of endogenously generated circadian rhythms (Reppert & Weaver, 2001). In 1972, two independent research groups made a ground-breaking discovery – the location of the endogenous mammalian circadian pacemaker (Moore & Eichler, 1972; Stephan & Zucker, 1972). In the 41 years since that discovery, an enormous body of evidence has been accumulated confirming that the suprachiasmatic nucleus is indeed the location of the master circadian pacemaker in the mammalian brain (Weaver, 1998; Lee *et al.*, 2003). This circadian clock is seated in the anterior hypothalamus, and consists of a group of approximately 20,000 neurons in a pair of nuclei directly dorsal to the optic chiasm and ventral to the third ventricle (Reppert & Weaver, 2001). This small bundle of neurons is responsible for managing and coordinating the timing of both physiology and behaviour. The exact mechanism of how these cells perform this immensely complex task of coordinating all biological rhythms in the body remains elusive to researchers.

Lesioning the SCN was shown to completely ablate the adrenal corticosterone rhythm, as well as the circadian drinking and locomotor rhythms of rats (Moore & Eichler, 1972; Stephan & Zucker, 1972). However, only complete SCN lesions have the ability to cause an animal to become fully arrhythmic, and in fact it appears that only a small amount of SCN cells need to remain intact, approximately 20%, in order for

rhythmicity to be preserved (Rusak, 1977; Davis & Gorski, 1988). Specifically, the preservation of caudal pathways to the SCN appears to be more critical in the sparing of entrainment and rhythmicity than those pathways that are rostral to the SCN (Harrington *et al.*, 1993). Yet, transplants of fetal SCN tissue are able to restore rhythmicity to SCN-ablated animals (Sawaki *et al.*, 1984; Lehman *et al.*, 1987). Importantly, the arrhythmic host receiving a fetal SCN transplant not only becomes rhythmic once again, but instead of simply restoring the original period of the host, it actually takes on the specific circadian period of the donor (Ralph *et al.*, 1990).

1.4 The endogenous circadian system

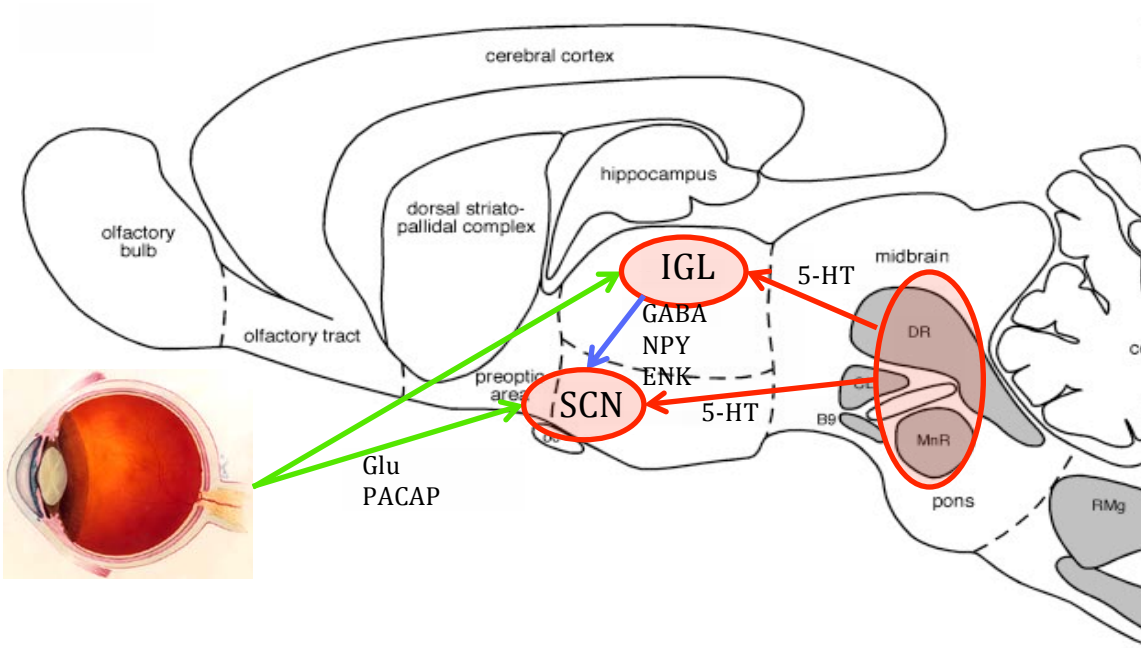
1.4.1 Afferent pathways to the SCN

Normal vision is not necessary for entrainment to a specific light cycle or to shift an animal's circadian phase (Hirota & Fukada, 2004). The photoreceptor that serves to reset the circadian clock does not require functional rods or cones in the retina (Freedman *et al.*, 1999). Instead, the light information hitting a subset of intrinsically photosensitive retinal ganglion cells, containing melanopsin, project to the SCN through the retinohypothalamic tract (RHT; Berson, 2003; Hirota & Fukada, 2004). These projections release glutamate and pituitary adenylate cyclase-activated polypeptide (PACAP) onto retinorecipient cells in the SCN. This process leads to the remodeling of chromatin and the subsequent expression of clock genes (Pittendrigh & Daan, 1976b; Morse & Sassone-Corsi, 2002). Light information is also received by the SCN indirectly from the geniculohypothalamic tract (GHT) via the intergeniculate leaflet (IGL; Morin, 1994; Harrington, 1997; Meijer & Schwartz, 2003). A third major afferent connection to the

SCN is a dense serotonergic projection originating in the raphe nuclei, which predominately terminates in the ventromedial region of the SCN (Meyer-Bernstein & Morin, 1996). In the hamster, neurons from the median raphe project directly to the SCN, however projections from the dorsal raphe connect to the SCN mainly indirectly through the IGL (Morin, 1999; Morin & Meyer-Bernstein, 1999). A summary of the major afferent pathways to the SCN can be found in Figure 1.1.

Figure 1.1 Major afferent pathways to SCN.

A schematic representation of the major afferent pathways leading into the SCN. The retinohypothalamic tract (RHT) brings photic information from the retinas to both the suprachiasmatic nucleus (SCN) and the intergeniculate leaflet (IGL), releasing glutamate (Glu) and pituitary adenylate cyclase-activated polypeptide (PACAP). The IGL then sends projections to the SCN via the geniculohypothalamic tract (GHT), releasing GABA, neuropeptide Y (NPY) and enkephalin (ENK). The raphe nuclei also send serotonin (5-HT) projections to both the SCN and the IGL.


cerebral cortex
hippocampus
dorsal striato-
pallidal complex
olfactory
bulb
olfactory tract
IGL
GABA
NPY
ENK
5-HT
midbrain
DR
SCN
5-HT
B9
MnR
pons
RMg
Glu
PACAP

1.4.2 The suprachiasmatic nucleus

1.4.2.1 Regions of the SCN

Although the SCN is endogenously rhythmic as a whole, not all cells in the SCN exhibit their own endogenous circadian rhythm (Aton & Herzog, 2005). The cells that do not express an overt rhythm receive input directly from the retinas, and then relay that light information to the rhythmic cells (Hirota & Fukada, 2004; Antle & Silver, 2005). Those neurons in the SCN which are intrinsically rhythmic in their gene and protein expression exhibit a multitude of different periods, and a daily signal from a subset of SCN cells appears to synchronize the phases of those individual oscillators to the mean period of the group of neurons (Antle & Silver, 2005). These rhythmic and non-rhythmic cells are located in distinct regions of the SCN – the light-sensitive retinorecipient cells are said to be located in the ventrolateral 'core' of the SCN, whereas the rhythmic cells are located in what is called the dorsomedial 'shell' (Moore *et al.*, 2002; Lee *et al.*, 2003; Antle & Silver, 2005).

In the hamster, the SCN 'core' cells that receive direct retinal innervation are thought to be of four different phenotypes: gastrin-releasing peptide (GRP), vasoactive intestinal polypeptide (VIP), calbindin (CalB), and so-called 'cap' cells that are responsive to GRP and contain phosphorylated extracellular signal-regulated kinase (P-ERK) at night (Antle & Silver, 2005). However, in the mouse, the cells of this 'core' region only express GRP, with no further subdivision in cell sub-populations. This is also the region of the SCN that predominantly receives extensive serotonergic projection from the midbrain raphe nuclei (Rea *et al.*, 1994). In contrast, the SCN 'shell' cells are

intrinsically rhythmic in their expression of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes, and mainly express vasopressin (VP; Antle & Silver, 2005).

1.4.2.2 Gene expression in the SCN

Although only cells in the SCN shell are endogenously rhythmic in their expression of clock genes, cells in the SCN core which receive retinal input express clock genes immediately following exposure to light (Hamada *et al.*, 2004). Core cells of the SCN express immediate-early genes such as *c-fos*, *Fra-2* and *JunB*, as well as the clock genes *Per1* and *Per2,* in response to a light pulse during the subjective night, but do not rhythmically express these genes (Guido *et al.*, 1999b; Schwartz *et al.*, 2000; Antle & Silver, 2005). In contrast, cells in the SCN shell rhythmically express the clock genes *Per* and *Cry* through an autoregulatory transcription-translation feedback loop (Holzberg & Albrecht, 2003). Yet, these rhythmic shell cells do not all express *Per* genes in a common phase, and some may actually be slave oscillators that are under the control of other pacemaker cells (Lee *et al.*, 2003; Hamada *et al.*, 2004).

The mechanism of the negative autoregulatory transcription-translation feedback loop of clock genes in the SCN shell has been extensively described (for review see Reppert & Weaver, 2001; Okamura, 2004; Antle & Silver, 2005). Briefly, the loop begins with the dimerization of CLOCK and BMAL and the subsequent binding of this complex to the E-Box promoter regions of the *Per* and *Cry* genes, which activates the transcription process. As PER and CRY proteins accumulate in the cytoplasm, peaking approximately 6 to 9 hours after the peak expression levels of their respective mRNA (Lee *et al.*, 2001), they dimerize and move into the nucleus where they inhibit the activity of the CLOCK-

BMAL dimer (Figure 1.1). Thus, the PER-CRY dimer inhibits the further transcription of the *Per* and *Cry* genes from which they are made. Once enough of the PER-CRY dimer has been degraded and removed from the nucleus, the process of gene transcription resumes (Hastings & Herzog, 2004). This whole cycle takes approximately 24 hours to complete.

Conversely, in the non-rhythmic retinorecipient core of the SCN, *Per* genes are expressed following a light signal relayed to the neurons by the RHT (Antle & Silver, 2005). The light signal leads to the phosphorylation of cyclic adenosine monophosphate (cAMP) response-element binding protein (CREB), which then binds to the Ca^{2+}/cAMP response element (CRE) in the promoter region of several immediate-early genes as well as the *Period* genes, thus inducing their transcription (Antle & Silver, 2005). The protein products of immediate-early genes such as cFos, JunB and Fra-2 can also dimerize, enter the nucleus, and bind to the AP1 promoter region of numerous genes to induce their transcription, possibly including those genes which are relevant to clock function (Takeuchi *et al.*, 1993; Kornhauser *et al.*, 1996b; Francois-Bellan *et al.*, 1999; Caputto & Guido, 2000; Schwartz *et al.*, 2000).

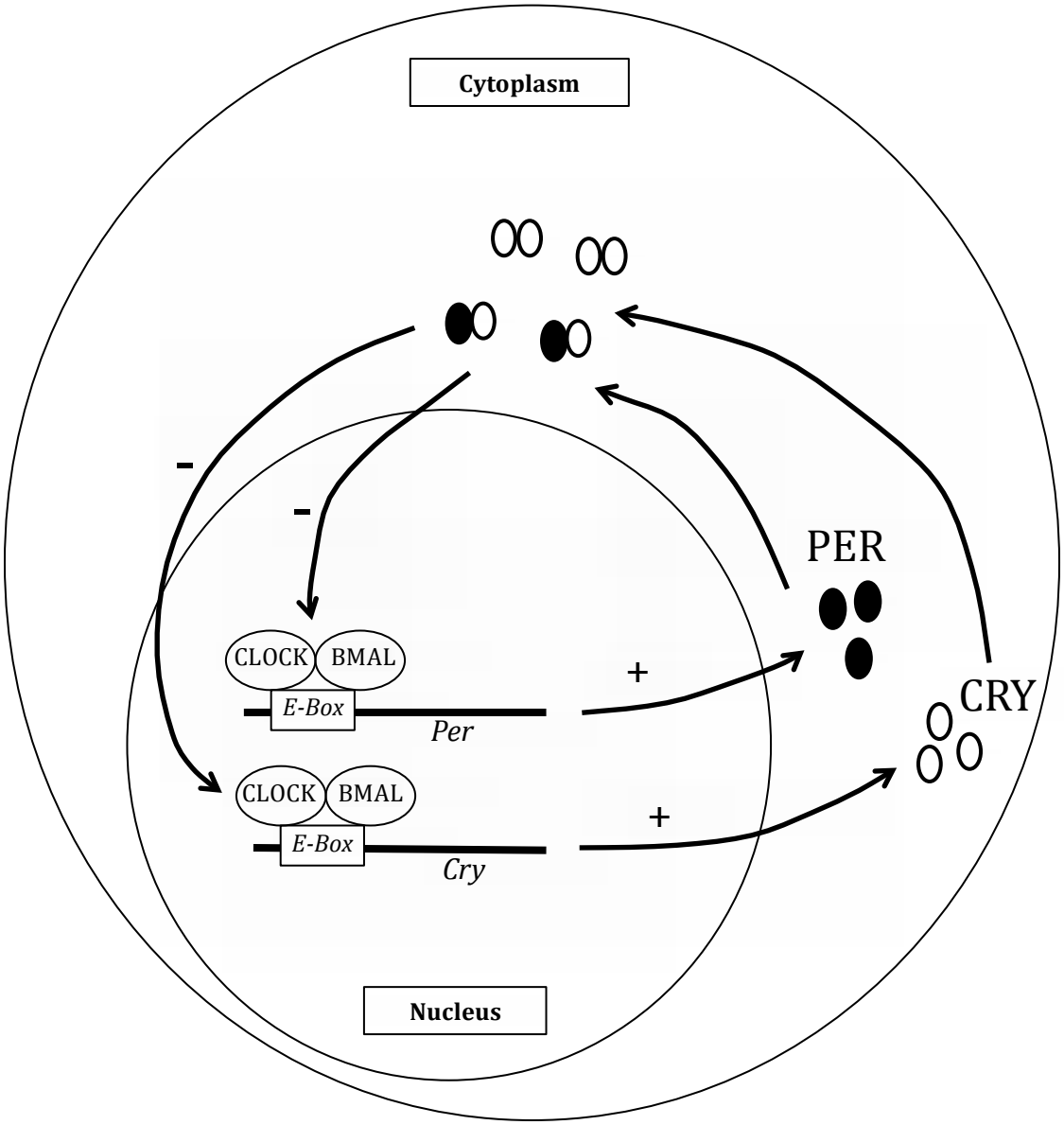
In order for it to alter the output of the circadian clock, through entrainment or photic phase shifts in response to light signals, light information must be relayed from the retinorecipient core region to the endogenously rhythmic shell (Antle & Silver, 2005). This communication between the cells of the core and the cells of the shell appears to be accomplished by multiple neurotransmitters and neuromodulators, including VIP, GRP, substance P (SP), and gamma aminobutyric acid (GABA; Hamada *et al.*, 2001; Antle &

Silver, 2005). The photic information relayed from the core cells then affects the rhythmic gene expression in the shell cells of the SCN, however the exact mechanism by which this transfer of information from core to shell is accomplished has yet to be fully understood.

The endogenously rhythmic expression of clock genes within the SCN has been shown to have a very specific temporal and spatial organization (Hamada *et al.*, 2004). It has been demonstrated that *Per1* gene expression first increases in the dorsomedial SCN, spreading ventrolaterally over the next several hours, subsequently receding rapidly, first medially and then dorsally (Hamada *et al.*, 2004). The expression of *Per1* and *Per2* extend across almost the entire SCN, spanning both the core and shell, except for a small area of GRP cells in the core in which *Per* expression is low and arrhythmic (Hastings & Herzog, 2004). Based on real-time imaging of *Per1*-driven expression of a bioluminescent or fluorescent reporter, it was shown that the majority of SCN cells express *Per1* during the day, with peak expression occurring earlier in the dorsal and lateral regions, and later in the ventral and medial regions (Hastings & Herzog, 2004). This temporally and spatially organized gene expression within the SCN can also be altered through the use of aptly timed light exposure.

Figure 1.2 Autoregulatory transcription/translation feedback loop.

A schematic representation of the negative autoregulatory transcription-translation feedback loop of clock genes in the intrinsically rhythmic cells of the SCN. The loop begins with the dimerization of CLOCK and BMAL and the subsequent binding of this complex to the E-Box promoter regions of the *Per* and *Cry* genes, which activates the transcription process. As PER and CRY proteins accumulate in the cytoplasm, they dimerize and move into the nucleus where they inhibit the activity of the CLOCK-BMAL dimer. The PER-CRY dimer inhibits the further transcription of the *Per* and *Cry* genes from which they are made and once enough of the PER-CRY dimer has been degraded and removed from the nucleus, the process of gene transcription resumes as the CLOCK-BMAL dimer interacts with the E-Box promotor regions once again.


Cytoplasm
PER
CRY
CLOCK
BMAL
E-Box
Per
CLOCK
BMAL
E-Box
Cry
+
+
-
-
Nucleus

1.4.3 Efferent pathways out of the SCN

The SCN sends various efferent projections to targets located both within the hypothalamus as well as other brain regions, with a projection pattern which appears to be predominantly ipsilateral (Swanson & Cowan, 1975; Berk & Finkelstein, 1981; Watts & Swanson, 1987; Watts *et al.*, 1987; Klein, 1991). One of the most pertinent efferent pathways consists of the SCN projections to the preoptic area, the most rostral portion of the hypothalamus, which is the region that controls many of the aspects of hypothalamic function which have circadian components, including fluid balance, reproduction, sleep, and thermoregulation (Klein, 1991). Another major efferent pathway involving many dorsocaudally directed fibers includes projections sent to the subparaventricular zone and the paraventricular nucleus of the hypothalamus. The paraventricular nucleus is considered to be a major integrative center, concerned with numerous processes relating to neuroendocrine, autonomic and behavioural functions (Klein, 1991).

In terms of projections within the hypothalamus, the SCN has also been shown to send projections to the dorsomedial and ventromedial nuclei of the hypothalamus, which in turn have extensive connections to the other areas of the hypothalamus, septum, amygdala, and brainstem. These areas have been implicated in several physiological functions including ingestive behaviour, rage, and female sexual behaviour (Klein, 1991). It also appears that there are a small number of fibers that may project to the posterior hypothalamic area and the periaquaductal gray, as well as some efferent fibers that target the contralateral SCN. Although, there is little evidence as to whether these fibers actually synapse with cells in the contralateral SCN or if they instead pass right through

and continue on to more remote targets outside the SCN (Klein, 1991). Finally, the SCN sends projections through the retrochiasmatic area, which may serve as an alternate route by which circadian information can be relayed to neuroendocrine and autonomic circuits (Klein, 1991).

There are also efferent projections from the SCN that make their way to brain areas outside the hypothalamic area. Two of the most important of these targets of SCN efferents include the rostral parts of the paraventricular and paratenial nuclei of the thalamus (Berk & Finkelstein, 1981; Watts & Swanson, 1987; Watts *et al.*, 1987; Klein, 1991). A much more minor projection of fibers from the SCN can be found targeting the ventral lateral septal nucleus, however this structure does receive a much larger indirect input through the subparaventricular zone. Finally, a laterally directed pathway has been observed which connects the SCN to the intergeniculate leaflet of the lateral geniculate nucleus (Watts & Swanson, 1987; Watts *et al.*, 1987). This pathway may be involved in reciprocal connections regulating the activity of the geniculohypothalmic tract via the intergeniculate leaflet, a major source of neuropeptide Y (NPY) innervation to the SCN which has been shown to play a role in photic modulation of circadian phase (Harrington *et al.*, 1985).

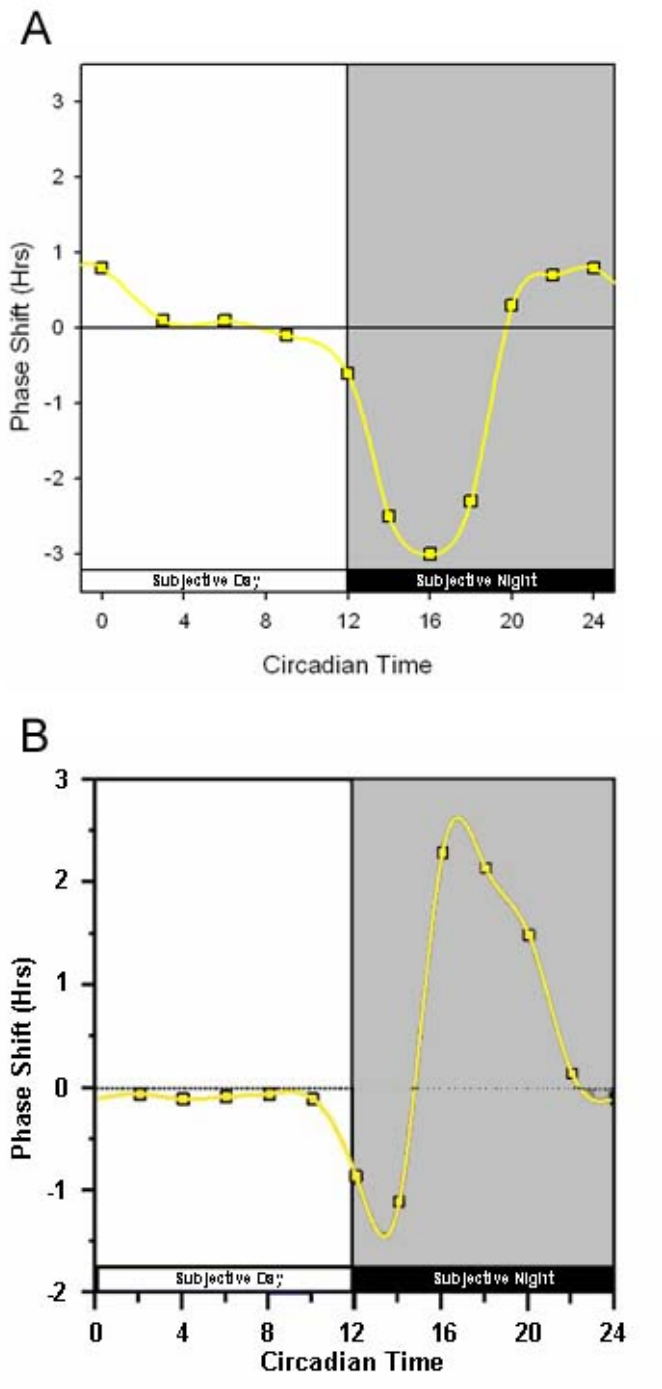
1.5 Photic phase shifting

Light exposure around dawn and dusk, as well as inappropriate light during the night, can produce a shift in the time of an animal's daily activity onset on subsequent days, leading to the animal either becoming active earlier or later than on previous days. Such a shift in the phase of a circadian rhythm is termed a phase shift, and follows a very

specific pattern called a photic phase response curve (PRC; Figure 1.2). Although there are subtle differences in exact timing between species, in nocturnal rodents this phase resetting by light is possible during the animal's subjective night (when they are active), but rarely during the subjective day (when they are inactive; Gillette & Mitchell, 2002). During the early subjective night, brief exposure to light functions to delay behavioural rhythms and causes the animal to have an activity onset that is later than expected based on previous onsets (i.e., a phase delay; Gillette & Mitchell, 2002). In contrast, a light stimulus during the late subjective night serves to phase advance an animal's behavioural rhythms, such that activity onset on subsequent days will occur at an earlier time than expected (i.e., a phase advance; Gillette & Mitchell, 2002).

Figure 1.3 Example photic phase-response curves.

Examples of a photic phase-response curve (PRC) for A) mice and B) hamsters. Indicates typical phase shift magnitudes when dim light is briefly presented at various circadian times in constant darkness.


A
Phase Shift (Hrs)
3
2
1
0
-1
-2
-3
Subjective Day
Subjective Night
0
4
8
12
16
20
24
Circadian Time
B
Phase Shift (Hrs)
3
2
1
0
-1
-2
Subjective Day
Subjective Night
0
4
8
12
16
20
24
Circadian Time

1.5.1 Effects of light intensity and duration

Although timing of light exposure is of great importance, the specific properties of the light are also pertinent, especially the intensity and duration of the light. Although there has not been a large amount of data collected regarding the effects of intensity and duration on the magnitude of phase shift elicited, there is some research looking at the effects of these two aspects of light stimuli. For example, phase shifts induced by green light (λ = 503 nm) at circadian time (CT) 19 at various intensities and durations were examined by Nelson and Takahashi (1991). Regardless of stimulus duration, it was observed that no phase shifts could be elicited in hamsters at intensities below 0.2 lux, indicating that 0.2 lux is the threshold intensity for evoking phase shifts (Nelson & Takahashi, 1991). Similar intensity thresholds have been found in hamsters, rats and mice (Foster *et al.*, 1991; Bauer, 1992; Meijer *et al.*, 1992; Sharma *et al.*, 1999). These results also demonstrate the phenomenon of temporal summation, such that stimuli can be summed over time allowing for a trade-off between intensity and duration. For example, there are two ways to elicit a 60-minute phase shift – either by a short bright light pulse or by a long dim light pulse. Thus, in general, longer duration light exposure of a specific intensity induces larger phase shifts, and larger intensity light stimuli of a given duration also induce larger phase shifts.

Originally there appeared to be a ceiling effect to this temporal summation, such that the peak phase shift appeared to be elicited following a light pulse somewhere between 30 and 60 minutes in duration (Nelson & Takahashi, 1991; Daymude & Refinetti, 1999). However, two independent studies have demonstrated that much longer

duration high intensity light exposure, of up to 12 hours in duration at ~1500 lux, can in fact increase the maximal phase shift to approximately 5 hours (Comas *et al.*, 2006; Antle *et al.*, 2007). Even so, there may be a non-photic, activity-related, component to these phase shifts since blocking running-wheel activity following the transition back to dark at the end of the light pulse reduces the phase shift substantially (Antle *et al.*, 2007). Though for simplicity and efficiency, most studies examining the phase shifting effects of brief light stimuli tend to use light exposure of 5 to 30 minutes in duration at an intensity of 30-150 lux, which is generally considered to result in a sufficient activation to essentially saturate the circadian system in terms of its response to the effects of light (Foster *et al.*, 1991; Bauer, 1992; Meijer *et al.*, 1992).

1.5.2 Mimicking and altering the effects of light

Exogenous application of some neurotransmitters or neuromodulators, which are involved in the pathway responsible for the transduction of light information to the SCN, are also able to induce phase shifts similar, both in magnitude and direction, to brief light exposure (Piggins *et al.*, 1995; McArthur *et al.*, 2000; Sterniczuk *et al.*, 2008). GRP, *N*-methyl-D-aspartic acid (NMDA), and VIP are just a few examples of such photic-like chemicals (Antle & Silver, 2005; Sterniczuk *et al.*, 2008). There are also many non-photic stimuli which are able to modulate the effect that light has on the circadian phase. For example, sleep deprivation for 8 hours during the subjective day has been shown to reduce the magnitude of light-induced phase delays (Challet *et al.*, 2001). Sleep deprivation, by a slow rate of forced activity, for as little as 6 hours has even been shown to significantly attenuate the phase delay produced by an early night light pulse

(Mistlberger *et al.*, 1997). Confinement to a novel running wheel late in the night cannot phase shift the circadian activity rhythm alone, but is capable of significantly attenuating the normal phase advance observed with a late night light pulse (Ralph & Mrosovsky, 1992; Mistlberger & Antle, 1998; Edelstein *et al.*, 2003).

It has previously been demonstrated that there is a dramatic increase in serotonin release in the SCN during wheel-running activity (Dudley *et al.*, 1998). Similarly, several studies have shown that the modulation of photic input to the circadian system induces an elevation of serotonin levels within the SCN (Asikainen *et al.*, 1997; Grossman *et al.*, 2000). Thus, it appears that increased serotonin release in the SCN is able to inhibit light-induced phase shifts of the circadian activity rhythm (Pickard & Rea, 1997a). Interestingly, pretreatment with the serotonergic antagonist metergoline is able to block the attenuation of late night photic phase advances, despite the inability of metergoline to significantly alter the photic phase shift itself (Mistlberger & Antle, 1998).

1.5.3 Light-induced protein and gene expression

Along with looking at the behavioural manifestations resulting from the modulation of circadian rhythms, it is also pertinent to investigate the changes in the expression of specific genes within the SCN that coincide with these changes in behaviour. Although cells in the SCN shell are endogenously rhythmic in their expression of several genes related to the functioning of the circadian clock, cells in the SCN core which receive retinal input express several of these same genes immediately following exposure to light (Hamada *et al.*, 2004). Core cells of the SCN express immediate-early genes such as *c-fos*, *Fra-2* and *JunB*, as well as the clock genes *Per1* and *Per2,* in

response to a light pulse during the subjective night, but do not rhythmically express these genes (Guido *et al.*, 1999a; Schwartz *et al.*, 2000; Antle & Silver, 2005). In contrast, cells in the SCN shell rhythmically express the clock genes *Period* and *Cryptochrome* (Antle & Silver, 2005).

1.5.3.1 Immediate-early genes

Upwards of 20 genes in the mammalian system are rapidly and transiently induced following treatment with several different agents, including those which trigger mitogenesis, differentiation, or membrane depolarization (Curran & Morgan, 1987). They are considered to be a part of the signal-transcription coupling cascade which functions to link extracellular stimuli with long-term adaptive responses (Curran & Morgan, 1987). They are proposed to function as third messengers, in that they mediate further changes in gene expression which convert short-term extracellular signals into long-lasting cellular changes (Guido *et al.*, 1999a). Included in this unique class of genes termed immediate-early genes (IEGs) are the family of *fos*-like genes, such as the proto-oncogene *c-fos* and a Fos-related antigen termed *fra-2*, as well as the family of *jun* genes, such as *JunB* and *JunD* (Kornhauser *et al.*, 1996b). The two types of patterning in gene expression observed in the cells of the SCN, one dependent upon photic input during the subjective night and the other being spontaneously rhythmic during the subjective day, can both be observed in the expression of the *c-fos* and *JunB* genes within the SCN (Guido *et al.*, 1996b; Guido *et al.*, 1999b; Edelstein *et al.*, 2003).

These two expression patterns are generally observed in different cell sub-populations of the SCN. The ventrolateral region of the SCN shows very little rhythmic

expression of *c-fos*, with only tiny peaks of expression in the early night and during the day (Sumova & Illnerova, 2005). Instead, these ventral SCN cells demonstrate light-induced expression of *c-fos* and *JunB* during the subjective night (Guido *et al.*, 1996b; Guido *et al.*, 1999b). The light-induced expression of the protein product of the *c-fos* and *JunB* genes, cFos and JunB, appear relatively quickly (within one hour after light exposure) and last for approximately 4 and 8 hours respectively (Morgan & Curran, 1989). In contrast, the dorsomedial SCN cells are spontaneously rhythmic in their *c-fos* expression, with a peak level of expression during the early day and the lowest levels of expression in the early night. Whereas, *JunB* expression peaks in the transition from subjective night to subjective day and lasts for at least 4 hours (Guido *et al.*, 1996b; Guido *et al.*, 1999b; Sumova & Illnerova, 2005). The IEG *fra-2* is found both in the dorsomedial and ventrolateral regions of the SCN, but demonstrates a light-induced increase in expression only in the ventrolateral region, with an expression distribution pattern matching that of *c-fos*, particularly in the ventrolateral region following light exposure (Francois-Bellan *et al.*, 1999). Fra-2, the protein product of the *fra-2* gene, shows a peak level of expression somewhere between 5 and 8 hours following the light exposure, thus allowing for a possible continued impact on circadian rhythmicity up to 8 hours after the presentation of a light stimulus (Morgan & Curran, 1989).

Circadian activity rhythms can be adjusted by aptly timed nocturnal light exposure, as previously discussed. Changes in the expression of several IEGs have been shown to coincide with such subjective night light exposure (Guido *et al.*, 1999a; Guido *et al.*, 1999b). It is thought that the light-induced expression of IEGs could be an early

event in the response cascade which ultimately leads to light-induced phase alterations of the circadian pacemaker (Rea, 1992). However, the induction of the IEG *c-fos* alone is not sufficient, or even required, for inducing a shift in the circadian activity rhythm. This was discovered because mice with a null mutation at the *c-fos* gene locus are still able to respond to a phase-shifting light pulse (Honrado *et al.*, 1996). Further, sensitivity to light exposure, in terms of induced gene expression, diminishes in the early subjective day despite robust spontaneous expression of *c-fos* and *JunB* (Guido *et al.*, 1996b; Guido *et al.*, 1999a). Yet, blocking the expression of both c-Fos and JunB proteins, using antisense oligonucleotides directed against *c-fos* and *JunB* mRNA, inhibited light-induced phase shifts of the circadian rhythm (Wollnik *et al.*, 1995). This indicates that although the IEG *c-fos* may not be necessary, the expression of the AP-1 complex in some form (a heterodimer with Fos and Jun or a homodimer between two members of the Jun family) is necessary for the elicitation of photic phase shifts.

Light-induced *c-fos* and *JunB* expression appears to only be seen when the light exposure occurs at a time when behavioural phase shifts are generated (Rusak *et al.*, 1990; Rusak *et al.*, 1992; Amir *et al.*, 1998; Guido *et al.*, 1999a). This temporal specificity has been demonstrated by exposing animals to light throughout the day and night, and observing that a significant increase in the number of cells expressing the protein products of these genes is observed only at night, with the greatest concentration of each being found in the retinorecipient ventrolateral cells of the SCN (Amir *et al.*, 1998; Guido *et al.*, 1999a). It has also been demonstrated that there is a unique spatial expression of *c-fos* and *JunB*, depending on whether the light is presented during the

early or late subjective night (Rea, 1992; Guido *et al.*, 1999a). In the early subjective night, when light exposure results in a phase delay, their expression is mainly confined to the ventral region of the SCN. In contrast, when light is presented late in the subjective night, when a phase advance of the rhythm is generally observed, expression is found in both the ventral as well as the dorsal cell sub-populations of the SCN (Rea, 1992; Guido *et al.*, 1999a).

1.5.3.2 Period genes

The temporal and spatial expression of clock genes, discussed previously, is highly influenced by exposure to light during the subjective night (Yan & Silver, 2004). Substantial light-induced expression of *mPer1* in the core region of the SCN has been observed during the entire subjective night, whereas *mPer2* expression is mostly seen in the SCN core after a light pulse in the early subjective night (Yan & Silver, 2002). Quite a different pattern emerges in the shell of the SCN, where light-induced phase delays in the early subjective night cause *mPer2* expression but very little *mPer1* expression. The opposite is true for phase-advancing light pulses in the late subjective night in which *mPer1* is highly expressed and not *mPer2* (Yan & Silver, 2002). After a light pulse in the early subjective night, *Per1* expression is mainly seen in the ventrolateral GRP cells of the SCN, whereas *Per2* expression is much broader and is seen also in the VP cells (Dardente *et al.*, 2002). Hamada *et al.* (2004) found *Per1* induction in the core region of the SCN (in the CalB-expressing cells) approximately one hour following a light pulse, and in the dorsomedial shell approximately 30 minutes later. Thus, it appears that phase shifts occur only when light-induced *Period* gene expression spreads from the core to the

shell of the SCN (Yan & Silver, 2002). More specifically, light-induced *Per1* and *Per2* gene expression in the SCN shell are associated with phase advances and phase delays respectively (Yan & Silver, 2002).

Further evidence of the spatial and temporal organization of *Per* gene expression in the SCN is seen when a light-pulse is given during the late subjective night, when endogenous levels of mPer1 and mPer2 proteins are rising, and the resultant expression of *mPer1* is further increased while *mPer2* is unaffected (Yan & Silver, 2004). This increased *Per1* gene expression subsequently leads to significantly increased Per1 protein expression starting between 4 and 9 hours following the light exposure (Field *et al.*, 2000). In contrast, when a light pulse falls during the early subjective night, when endogenous levels of the two Per proteins are falling, expression of both *mPer1* and *mPer2* are increased (Yan & Silver, 2004). This increased expression of both *Period* genes results in significantly increased Per1 protein expression starting approximately 4 hours after the light exposure (Field *et al.*, 2000). These results imply that there are complex integrations between different cell types behind the differential expression of the *Per* genes when induced by light, which appears to be a requirement for phase adjustments to the circadian rhythm (Dardente *et al.*, 2002).

During the subjective day, a very different pattern of expression emerges. Both *Per1* and *Per2* are expressed similarly, restricted mainly to the region defined by the VP cells of the SCN (Dardente *et al.*, 2002). Although there does not appear to be a role for light in the induction of *Per* gene expression, with no phase-shifting effects, during the day, there is a general up-regulation of *Per1* and *Per2* expression during the subjective

day following a 30 minute light pulse (Dardente *et al.*, 2002; Challet *et al.*, 2003). This effect was more pronounced when the light pulse was administered in the early subjective day, and *Per2* expression was markedly increased with extended light exposure, of up to 3 hours, whereas *Per1* was not increased with such an extended light exposure (Challet *et al.*, 2003).

It is important, however, to note that mice genetically engineered to lack either mPer1 or mPer2 are able to normally entrain to a light-dark cycle and generally remain rhythmic in constant darkness, although *Per2*-deficient mice can gradually become arrhythmic (Oster *et al.*, 2002; Bae & Weaver, 2003; Spoelstra *et al.*, 2004). Thus, it appears that although *Per2* may be a necessity for proper functioning of the circadian clock in constant darkness, the clock genes *Per1* and *Per2* may not be necessary for light-induced phase shifts of the circadian activity rhythm. In fact, mice deficient for any one of the *Per1*, *Per2*, *Cry1*, or *Cry2* genes are able to phase shift relatively normally, to both advancing and delaying light exposure (Spoelstra *et al.*, 2004). However, *Per2*-deficient mice also lacking a functional *Cry2* gene remain fully rhythmic in constant darkness, whereas *Per2*/*Cry1*-deficient mice are similar to mice lacking only the *Per2* gene and gradually become arrhythmic in constant darkness (Oster *et al.*, 2002). Conversely, mice deficient of both *mCry1* and *mCry2*, which are arrhythmic both behaviourally as well as in their *mPer1* and *mPer2* expression in the SCN, are still able to demonstrate *mPer1* and *mPer2* expression in response to a brief light stimulus known to phase shift the circadian clock (Okamura *et al.*, 1999). These studies appear to indicate that although the *Period* genes may be sufficient, they are not strictly necessary for photic phase shifting of the

circadian activity rhythm. *Per* and *Cry* genes appear to interact in the production of photic phase shifts, with the possibility that having one may compensate for the loss of another.

1.5.3.3 Biochemical pathways

Although it is essential to understand the light-induced expression of numerous IEGs and clock genes in terms of their effects on circadian rhythms, light-induced changes in the circadian system depend upon the photic information from the retina being relayed to the SCN cells. Then the subsequent initiation of transcription of those pertinent genes mentioned above. In order to accomplish this, SCN neurons contain second-messenger signaling pathways that couple the incoming photic stimuli with the transcription of immediate-early genes as well as *Period* genes within SCN cells (Butcher *et al.*, 2003). An important example of a second-messenger pathway is the mitogen-activated protein kinase (MAPK) pathway, which consists of three kinases, one of which is extracellular signal-regulated kinase (ERK; Butcher *et al.*, 2003). This MAPK cascade has been shown to be activated by light in a phase dependent manner in the SCN, such that it parallels the effects of light on the expression of immediate-early genes (Obrietan *et al.*, 1998). However, MAPK pathway activation occurs very rapidly, within minutes of photic stimulation, and before an increase in immediate-early gene mRNA expression is seen (Dziema *et al.*, 2003).

The disruption of this MAPK cascade has been shown to attenuate both the phase shifting effects of light and photic-induced immediate-early gene expression (Butcher *et al.*, 2002; Coogan & Piggins, 2003; Dziema *et al.*, 2003). The activation of the MAPK

pathway by a light stimulus leads to the rapid activation of ERK, through its phosphorylation, which then dimerizes and moves into the nucleus of the cell (Butcher *et al.*, 2003; Dziema *et al.*, 2003). This translocation of phosphorylated ERK into the nucleus is considered to be a key connection between the MAPK pathway and the activation of transcription of genes in the nucleus (Butcher *et al.*, 2003). Specifically, P-ERK has been shown to interact directly with clock gene products of the transcription/translation feedback loop (Sanada *et al.*, 2002).

It has also been determined that light-induced phase shifts are consistently accompanied by the phosphorylation of the transcription factor CREB within the SCN (von Gall *et al.*, 1998). Elevation of both Ca^{2+} and cAMP following light leads to the phosphorylation of CREB, which then binds to the CRE of the promoter region several base pairs before the transcription start site of multiple circadian-related genes, including *c-fos* and *Per1*, thus leading to their transcription into mRNA (Dunlap *et al.*, 2004). Within minutes following light exposure, the phosphorylated form of CREB (P-CREB) can be found in SCN cells, however this phosphorylation is phase-dependent, only occurring when light can induce phase shifts and immediate-early gene expression (Ginty *et al.*, 1993; Ding *et al.*, 1997). Light has also been shown to trigger the colocalized expression of the activated forms of ERK 1/2 and the activated form of CREB in cells of the SCN (Dziema *et al.*, 2003). In addition to the effects of photic stimulation only being observed during the subjective night and not during the subjective day, levels of P-CREB have also been shown to be phase-dependent, with peak levels occurring during the mid- to late-subjective night (Obrietan *et al.*, 1999). The importance of P-CREB to eliciting

light-induced phase shifts and *Per1* gene expression are highlighted when glutamate-induced as well as GRP-induced photic-like phase shifts are blocked following the application of an antisense oligonucleotide against *Per1* and an antisense oligonucleotide decoy for the CRE of the promoter region of the *Per1* gene, thus preventing transcription of the gene (Tischkau *et al.*, 2003; Gamble *et al.*, 2007). This indicates that the activation of the CREB second-messenger pathway, including its binding to the CRE promoter region of the *Per1* is likely necessary in order to induce photic phase shifts.

Another set of CRE-binding proteins, encoded by the cAMP response element modulator (CREM) gene, can either act as activators or repressors following phosphorylation, depending on the specific isoform generated (Lalli *et al.*, 1996; Sassone-Corsi, 1998; De Cesare *et al.*, 1999). This family of transcription factors is able to heterodimerize with each other in order to activate genes with CRE promoter regions, but only in certain combinations. However, some of these transcription factors can also heterodimerize with members of the Fos and Jun families of immediate-early genes (Lalli *et al.*, 1996), which could alter how Fos and Jun protein dimers interact with their own AP1 promoter region. The inducible cAMP early repressor (ICER) is a truncated product, which is transcribed along with the C-terminal segment of CREM, containing essentially only the DNA-binding domain of CREM (Figure 1.3). ICER is the only CREM isoform that is inducible in this manner, and is the only CRE-binding protein whose function is physiologically regulated by altering its cellular concentration and not its degree of phosphorylation, involving a negative autoregulatory control mechanism resulting from ICER binding to its own promoter region, and appears to include a refractory inducibility

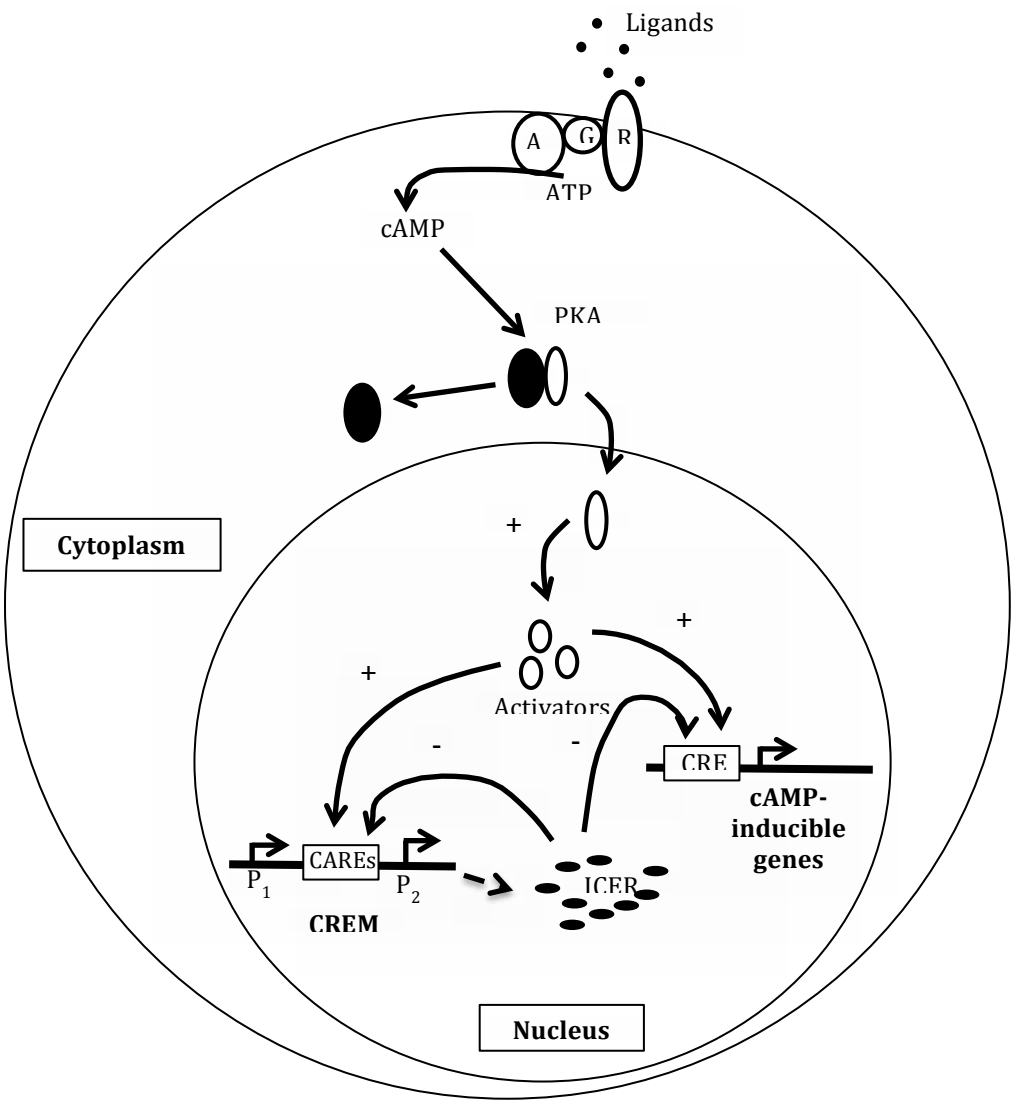
period (Molina *et al.*, 1993; Lalli *et al.*, 1996). This inducible repressor functions as a powerful repressor of cAMP-induced transcription. ICER mediates repression at substoichiometic concentrations, by heterodimerizing with all other CREM proteins as well as with CREB, generating non-activating dimers (Molina *et al.*, 1993; Lalli *et al.*, 1996; Lamas *et al.*, 1996). Following the phosphorylation of activators such as CREB, CRE-related gene expression is attenuated by two mechanisms – the dephosphorylation of the activators by specific phosphatases, as well as negative autoregulation by ICER (Lamas *et al.*, 1996).

ICER has been shown to increase in the rat SCN following light exposure in the late subjective night, at CT19 (Molina *et al.*, 1993; Schwartz *et al.*, 2005). In contrast to ERK and CREB which are phosphorylated very quickly following a phase- shifting light pulse, ICER levels begin to rise 60 minutes following the light exposure and continue to rise until at least 240 minutes (Stehle *et al.*, 1996; Schwartz *et al.*, 2005), and its expression drops 4-6 hours after induction (Molina *et al.*, 1993). However, this pattern of light-induced expression takes even longer to be evident when light exposure occurs at CT17, such that significant increases in expression are not observed until 3 hours after light exposure (Stehle *et al.*, 1996). Importantly though, Kell *et al.* (2004) observed what appeared to be constitutive expression of ICER throughout the day in the SCN of rats as well as C3H and C57BL mice, with comparable expression levels found at zeitgeber times (ZT)6, ZT11.5, ZT18 and ZT23.5. Plus, much higher levels were generally observed in mice than in rats. Yet, P-CREB and ICER have also been shown to have rhythmic expression, with declining P-CREB levels coinciding with rising levels of ICER

such that peak ICER expression is delayed by approximately 6 hours compared to the peak of P-CREB expression (Maronde *et al.*, 1999). It has been proposed that the decay of light-induced CRE-dependent mRNAs is not only the result of autorepression and mRNA instability, but may also be due in part to transcriptional inhibition by ICER (Schwartz *et al.*, 2005). This may be particularly true for those photoinducible genes which begin to decline only after ICER levels have begun to rise, including *fra-2* and *per2* (Schwartz *et al.*, 2005), or the protein products of photoinducible genes such as PER1 which also peak after ICER levels begin to rise. Thus, ICER may be a possible target for adjusting the effects that light can have on the circadian system, by adjusting the rate or timing of the repression of CRE-induced gene expression following the activation of intracellular biochemical cascades by light.

Figure 1.4 Intracellular cAMP biochemical cascade.

A schematic representation of the cAMP signal transduction pathway whereby ligands interact with metabotropic receptors (R) and result in altered gene expression. Ligand binding activates G-proteins (G), which stimulates associated adenylyl cyclase (AC), leading to the conversion of ATP into cAMP and then the dissociation of the inactive tetrameric protein kinase A (PKA) complex into its active catalytic subunits and regulatory subunits. The catalytic subunits migrate into the nucleus, where they phosphorylate and activate transcriptional activators. Activators interact with the cAMP response element (CRE) of the promotor region of cAMP-inducible genes to activate transcription. Activators also lead to the transcription of CREM P_2 promotor via the CARE elements, rapidly increasing ICER protein levels, which then repress cAMP-induced transcription, including its own promotor. ICER levels eventually fall, which releases the repression and permits a new cycle of transcription activation.


Ligands
A
G
R
ATP
cAMP
PKA
Cytoplasm
+
+
+
Activators
-
-
CRE
cAMP-
inducible
genes
CAREs
P1
P2
ICER
CREM
Nucleus

1.6 Serotonergic modulation of circadian rhythmicity

1.6.1 The role of serotonin in the circadian system

The neurotransmitter serotonin has been extensively researched in regards to its ability to modulate the activity of the SCN. The SCN receives dense serotonergic innervation arising from projections mainly from the median raphe nucleus (MRN; as in the hamster), but also partially from the dorsal raphe nucleus (DRN) in some species (particularly in the rat; Pickard & Rea, 1997a; Yamakawa & Antle, 2010). Both retrograde and anterograde tracing techniques have demonstrated that serotonergic neurons from the median raphe project to the SCN (Meyer-Bernstein & Morin, 1996). Specifically, anterograde tracing of median raphe efferent neurons projecting to the SCN revealed synapses in a pattern similar to that of 5-HT neuron fibers in the SCN, and serotonin-labeled fibers in the SCN demonstrate a retrograde tracing mainly to the median raphe (Jiang *et al.*, 2000). These efferent projections from the MRN form both axodendritic and axosomatic synapses with SCN neurons receiving retinal input, and hence serotonergic innervation mainly resides in the core region of the SCN (Pickard & Rea, 1997a).

Serotonin generally has an inhibitory effect on the central nervous system, and appears to modulate the effects of light on the SCN by dampening the excitatory effects light has on retinorecipient cells (Meyer-Bernstein & Morin, 1996; Mistlberger, 2000). The daily profile of 5-HT release in the region of the SCN, in a light/dark cycle or constant darkness, indicates an abrupt extracellular increase in 5-HT which peaks at the light/dark transition or onset of subjective night, with a subsequent decrease during the

night and into the following day (Dudley *et al.*, 1998). This rise in 5-HT levels corresponding to activity onset, at the beginning of the subjective night, lends support to the idea that the 5-HT output rhythm is not driven by light input. Serotonin has been shown to have numerous physiological effects on the SCN. A few of these actions include an inhibitory outward current at the postsynaptic membrane of some neurons, presynaptic inhibition of glutamate release from the RHT, reduced release of GABA from presynaptic terminals, as well as an excitatory inward current in some neurons (Jiang *et al.*, 2000). Also, electrical stimulation of the median raphe and the application of 5-HT to the SCN have been shown to depress the firing rate of most SCN neurons (Pickard & Rea, 1997a).

Disruption of the serotonergic projections to the SCN, as well as pharmacological manipulation of the synthesis or degradation of serotonin, has been extensively shown to alter various aspects of circadian rhythms in rodents (Rea *et al.*, 1994). The removal of the afferent projections from the MRN to the SCN through the application of a neurotoxin has been shown to produce three main effects on the circadian system (Pickard & Rea, 1997a). Re-entrainment to shifted light/dark cycles is accelerated, larger light-induced phase shifts result from the administration of a 30-minute light pulse, and there is a significant increase of the freerunning period when in constant light. These three findings suggest that these 5-HT fibers play a role in the modulation, specifically inhibition, of light input from the retina to the SCN (Rea *et al.*, 1994). Thus, it is not surprising that increased endogenous serotonin in the SCN, stimulated by exogenous

application of tryptophan, was able to attenuate the phase shifting effects of light (Glass *et al.*, 1995).

Currently, there are a total of 18 serotonin receptor subtypes recognized in the brain, 6 of which are believed to be present in the SCN: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{5A}, and 5-HT_{7} (Glennon, 1995; Smith *et al.*, 2001; Niesler *et al.*, 2007). However, these receptors are coupled to very different G-protein-coupled second-messenger systems within a cell, and are located in very different parts of the cell (i.e. some are found post-synaptically while others are found as autoreceptors), leading to very different effects when activated (Hannon & Hoyer, 2008). Thus, the specific contributions of each of these receptors in the functioning of the SCN are still not well understood, and thus it is difficult to determine which serotonin receptors are responsible for the mediation of photic effects on the circadian system. Although a plethora of serotonin agonists and antagonists are available which can pharmacologically manipulate serotonin activity by differentially activating receptors in the SCN, each individual agonist or antagonist has a unique receptor specificity and efficacy, which result in varying effects on the circadian system following their administration.

Much research has been done with serotonergic agonists and antagonists in order to elucidate the role of serotonin in the circadian system. However, there are seemingly conflicting results surrounding the effects of such pharmacological manipulations. The agonistic or antagonistic effect of a substance on the circadian system is dependent upon the mode of application, the species under consideration, and most importantly the

location of the receptor at which the substance is an agonist or antagonist – e.g. whether it has its action at a post-synaptic receptor or a pre-synaptic autoreceptor.

1.6.1.1 Affinity for serotonergic receptor subtypes

One of the difficulties in determining exactly which receptor subtypes contribute to, and mediate, the serotonergic effects observed on the mammalian circadian system, as well as exactly how they accomplish these effects, lies in the lack of selective 5-HT receptor ligands (Smith *et al.*, 2001). The majority of serotonin receptor agonists and antagonists have an affinity for more than one receptor subtype, but to varying degrees (Ying & Rusak, 1997). Some chronobiotics previously assumed to be highly selective, to the point of being the prototypical choice for studies of a specific receptor subtype, have since been shown to bind to multiple receptor subtypes (Sprouse *et al.*, 2004b).

An example of this is 8-OH-DPAT, which was originally thought to be a highly selective 5-HT_{1A} receptor agonists, but is now known to be active at 5-HT_7 receptors as well (Sprouse *et al.*, 2004b; Gardani & Biello, 2008; Smith *et al.*, 2008). Other examples include the non-selective 5-$HT_{1,2}$ antagonist metergoline, the mixed 5-$HT_{1A/1B}$ agonist TFMPP, the mixed 5-$HT_{2/7}$ receptor antagonist ritanserin, and the receptor antagonist clozapine which is primarily a dopamine antagonist but also has an affinity for 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_6 and 5-HT_7 (Glass *et al.*, 1995; Smith *et al.*, 2001; Saifullah & Tomioka, 2003). Similarly, the serotonin mixed agonists/antagonists NAN-190, BMY7378, S 15535, and MDL 73005 EF are proposed to have their action mainly at the 5-HT_{1A} receptors, but also have affinities for other receptors as well (Claustre *et al.*, 1991; Gannon, 2003). NAN-190 is generally considered to be a high affinity competitive

antagonist at postsynaptic 5-HT_{1A} receptors, but it has also been shown to be a partial agonist at somatodendritic 5-HT_{1A} autoreceptors, as well as having an affinity for 5-HT_2 and 5-HT_7, dopamine-D_2 and α_1-adrenergic receptors (Glennon *et al.*, 1988; Rydelek-Fitzgerald *et al.*, 1990; Claustre *et al.*, 1991; Millan *et al.*, 1994; Glass *et al.*, 1995; Portas *et al.*, 2000; Wesolowska *et al.*, 2002; Saifullah & Tomioka, 2003). Similarly, BMY7378 has been shown to be a 'partial' agonist of 5-HT_{1A} receptors and an agonist at 5-HT_{1A} autoreceptors, along with demonstrating marked dopamine-D_2 antagonistic abilities (Millan *et al.*, 1994; Hjorth *et al.*, 1995). Therefore, it is obvious that much more research needs to be performed in order to fully understand the effects that these different drugs are able to produce in the mammalian circadian system.

1.6.2 Non-photic phase shifts mediated by the serotonin system

1.6.2.1 Serotonergic mediation of activity and arousal

Serotonin is thought to couple activity and arousal to the circadian system, and thus is also thought to participate in non-photic phase shifts of the circadian system, as well as activity-induced modulation of responses to light. Through the use of microdialysis to measure endogenous 5-HT, it was demonstrated that activity induced by novel running wheel access during the night suppressed 5-HT release, whereas the same induced activity during the day led to a stimulation of 5-HT release (Dudley *et al.*, 1998). Such novelty-induced running for 3h during the day has also been shown to cause phase shifts, of approximately 3.5h, in the activity rhythm in hamsters (Bobrzynska *et al.*, 1996b). Although, this high amplitude activity does not seem to be strictly necessary, as 3h of daytime arousal without induced activity has been shown to also stimulate 5-HT

release and induce large magnitude phase shifts (Antle & Mistlberger, 2000; Grossman *et al.*, 2000).

High amplitude activity induced during the early or late night does not cause phase shifts in the activity rhythm (Mistlberger & Antle, 1998). Yet, novel wheel running-induced activity beginning before, and continuing through, a brief light pulse during the late subjective night significantly attenuates the normal phase shift induced by light at that circadian phase (Mistlberger & Antle, 1998). Induced activity combined with light exposure in the early subjective night, however, did not have any effect on the magnitude of phase shifts normally observed with an early subjective night light pulse (Mistlberger & Antle, 1998). This attenuation of the photic response by activity was found to be serotonin-dependent, such that pretreatment with the serotonin antagonist metergoline prevented the attenuation of the photic phase shifts by activity, but without affecting the levels of activity elicited by access to the novel running wheel (Mistlberger & Antle, 1998).

1.6.2.2 Chronobiotic mediation of non-photic phase shifts

There is also a large body of evidence indicating a role for the serotonin system in modulating the circadian system and producing non-photic phase shifts through the use of pharmacological agents targeting serotonin receptors. For example, it appears that serotonin and its agonists can produce phase shifts during the subjective day, both *in vivo* and *in vitro* (Smith *et al.*, 2001). The systemic injection of 8-OH-DPAT or buspirone have been shown to result in phase advances during the mid-subjective day (Tominaga *et al.*, 1992; Bobrzynska *et al.*, 1996a; Challet *et al.*, 1998; Gardani & Biello, 2008; Smith

et al., 2008). This non-photic phase shift induction by 8-OH-DPAT has even been observed in multiple types of lighting conditions, including a light/dark cycle (LD), constant light, and constant darkness which are similar to those induced by wheel-running (Shibata *et al.*, 1992; Tominaga *et al.*, 1992; Edgar *et al.*, 1993; Cutrera *et al.*, 1994; Bobrzynska *et al.*, 1996a; Cutrera *et al.*, 1996). Either systemic, or intra-raphe injection of 5-HT agonists have been shown to result in phase advances during the mid-subjective day (Mintz *et al.*, 1997; Challet *et al.*, 1998; Antle *et al.*, 2003).

However, this non-photic effect appears to be species-specific. For example, in both mice and hamsters, the 5-$HT_{1,2}$ agonist quipazine was unable to induce phase shifts at a range of circadian phases. In contrast, 8-OH-DPAT induced phase shifts in hamsters when delivered intraperitoneally or directly into the raphe nuclei, but not when injected into the SCN (Bobrzynska *et al.*, 1996a; Mintz *et al.*, 1997; Antle *et al.*, 2003). Also, 8-OH-DPAT suppressed rhythmic daytime c-Fos in hamsters, whereas there was no effect in mice at the circadian times tested (Antle *et al.*, 2003). Yet, 8-OH-DPAT has since been shown to induce significant phase advances when administered 6 hours prior to activity onset in mice (Gardani & Biello, 2008; Smith *et al.*, 2008). Similarly, the specific effect can also depend on the exact agonist used along with which species is tested, with quipazine administered during the middle of the day increasing rhythmic Fos in mice but instead decreasing Fos in hamsters (Antle *et al.*, 2003).

In terms of the mechanism by which these chronobiotics produce their effects on the circadian system, 5-HT_{1A} agonists may produce phase advances during the subjective day by reducing the expression of *Per1* and *Per2* in the nocturnal rodent SCN (Horikawa

et al., 2000). It was originally presumed that the non-photic effects of 8-OH-DPAT were strictly the result of binding to 5-HT_{1A} receptors (Sprouse *et al.*, 2004b). A 5-HT_{1A} antagonist was shown to block 8-OH-DPAT-induced phase shifts, further supporting the proposed role of the 5-HT_{1A} receptor (Sprouse *et al.*, 2005). This is quite contrary to what this same research group had found previously, where it appeared the primary action of 8-OH-DPAT was achieved through binding to 5-HT_7 receptors in hamsters (Sprouse *et al.*, 2004b). Yet another study by the same research group, still regarding the action of 8-OH-DPAT, showed that it was actually the activation of 5-HT_{5A} receptors which produced a shift in the circadian rhythm (Sprouse *et al.*, 2004a). Obviously, the effect of 8-OH-DPAT on the circadian system is highly complex, and the precise mechanism of action has yet to be fully elucidated.

Thus, taking advantage of the generation of mice genetically lacking particular 5-HT receptors has allowed for more precise research into how the 5-HT agonist 8-OH-DPAT actually operates on the circadian rhythm (Sprouse *et al.*, 2005; Gardani & Biello, 2008; Smith *et al.*, 2008). It was observed that the *in vitro* application of 8-OH-DPAT to SCN slices at mid-day induced phase advances in both normal mice and the 5-HT_7 receptor knockout mice. This indicated that the lack of 5-HT_7 receptors did not hinder the effect of 8-OH-DPAT, and thus the effect was surmised to be the result of activity at the 5-HT_{1A} receptors. In support of this, systemic administration of 8-OH-DPAT in the middle of the subjective day to mice lacking 5-HT_{1A} receptors induced no significant phase advances, indicating the necessity of this receptor subtype for this effect (Smith *et al.*, 2008). However, the administration of 8-OH-DPAT

systemically to 5-HT_7 receptor knockout mice also produced no significant phase shifts in the mid-subjective day, where phase shifts were induced in the wild type mice (Gardani & Biello, 2008). Therefore, it appears that there is an essential role for both the 5-HT_{1A} and 5-HT_7 receptors in the non-photic phase shifting abilities of 8-OH-DPAT when administered in the middle of the day.

Not only does it appear that multiple serotonin receptors may be necessary for the non-photic effects observed with 8-OH-DPAT, but it has also been shown that serotonergic fibers which connect the raphe nuclei to the SCN are essential for the action of 8-OH-DPAT (Schuhler *et al.*, 1998). These projections from both the median and dorsal raphe nuclei appear to be quite important to the ability of 5-HT and its agonists to induce behavioural phase shifts. In fact, 8-OH-DPAT appears to act to alter the circadian system not by activity in the SCN itself, but instead via action directly in the raphe nuclei (Mintz *et al.*, 1997; Antle *et al.*, 2003). Further, stimulation of the dorsal and median raphe nuclei in hamsters has been found to elicit phase advances on its own (Meyer-Bernstein & Morin, 1999). Thus, the serotonergic projections from the raphe nuclei to the SCN play some sort of role in eliciting phase shifts in the circadian system, and perhaps the involvement of 5-HT_{1A} and 5-HT_7 receptors in such phase shifts may be partly through these raphe nuclei projections.

Along with the non-photic effects of serotonin agonists and the role of the raphe nuclei on the circadian system, the effects that antagonists may have on their own as well as on the effects induced by agonists must also be considered. As such, several serotonin antagonists have been shown to block agonist-induced effects on the circadian system,

while having no effect on photic responsiveness when administered alone (Ying & Rusak, 1997; Gannon, 2003). Pretreatment with three different 5-HT_{1A} receptor antagonists (WAY-100635, NAN-190, and p-MPPF) were shown to block the effects of 8-OH-DPAT, while the partial agonist/antagonist BMY7378 had no blocking effect (Chen *et al.*, 2002). Yet, due to its action as a mixed autoreceptor agonist, systemic administration of BMY7378 is able to suppress the effects of 5-HT on the SCN (Mistlberger, 2000). The serotonin antagonist WAY-100635 was unable to induce any phase shifts when administered in isolation, but did induce significant release of 5-HT in the SCN during the subjective day (Antle *et al.*, 2000).

1.6.3 Serotonergic mediation of photic phase shifts

1.6.3.1 Serotonin agonists and antagonists

In general, it has been shown that 5-HT agonists have the ability to modify the response of the SCN to light exposure, and it has been reported that serotonin receptor agonists may be able to mimic photic-like input to the SCN and independently induce phase shifts of the circadian rhythm (Kohler *et al.*, 1999; Kennaway *et al.*, 2001; Smith *et al.*, 2001; Graff *et al.*, 2005). The assertion has been made that 5-HT and its agonists always inhibit photic responsiveness of the circadian system (Gannon, 2003), however there is an increasing body of evidence disagreeing with this statement.

For example, the serotonin$_{1,2}$ agonist quipazine was shown to decrease the number of cells expressing Fos-like immunoreactivity induced by a light pulse (Selim *et al.*, 1993). Similarly, the 5-HT_{1A} agonist 8-OH-DPAT has been shown to inhibit both behavioural phase shifts as well as *c-fos* expression in the SCN stimulated by light

exposure during the latter part of the subjective night (Rea *et al.*, 1994; Rea & Pickard, 2000). Although 8-OH-DPAT was not able to significantly alter the circadian phase when administered alone in the late subjective night, it dose-dependently inhibited light-induced phase advances (Rea *et al.*, 1994). It has also been shown to attenuate photic phase shifts when administered systemically or directly to the SCN (Rea & Pickard, 2000; Antle *et al.*, 2003). However, the effect of 8-OH-DPAT appears to be somewhat species-dependent, with the attenuation of light-induced phase shifts observed in hamsters being completely absent in mice (Antle *et al.*, 2003). Yet, in rats, 8-OH-DPAT is able to mimic the immediate and long-term effects of light on the melatonin rhythm, and reduced light-induced Fos expression to a much smaller degree than seen in the hamster (Glass *et al.*, 1994; Kennaway *et al.*, 1996; Recio *et al.*, 1996).

Alternatively, and contrary to the assertion that serotonergic agonists always inhibit light-responsiveness, the highly selective 5-HT_{1A} agonist MKC-242 injected systemically has been shown to potentiate phase advances and delays induced by a light pulse (Moriya *et al.*, 1998; Takahashi *et al.*, 2002), exemplifying the necessity of looking at exactly what type of receptor is being utilized by a drug. MKC-242 administered 30 minutes prior to a light pulse in the early subjective night was shown by Takahashi *et al.* (2002) to prolong the light-induced expression of *mPer1* and *mPer2* in the mouse SCN. This prolonged gene expression directly corresponded to the potentiation of photic phase delays. MKC-242 was also noted to have no effect on *mPer1* or *mPer2* expression in the SCN without exposure to light. Further, *mPer1* and *mPer2* expression in the ventral portion of the SCN (likely the core area) was inducible by light, whereas their expression

was not induced by light in the medial SCN (likely corresponding to the shell area) and instead demonstrated a strong rhythmic expression of the genes and proteins (Takahashi *et al.*, 2002).

Although it has been reported that 5-HT_{1A} antagonists can reverse the action of agonists or produce the opposite effect (Ying & Rusak, 1997), it has also been shown that these compounds are able to potentiate phase advances in the late subjective night (Rea *et al.*, 1995). For example, pre-treatment with the specific 5-HT_{1A} antagonist WAY-100635 has been shown to significantly potentiate a light-induced phase delay during the early subjective night. However, it was ineffective at adjusting the magnitude of phase shifts during the late subjective night (Smart & Biello, 2001). Similarly, Rea *et al.* (1995) found that the serotonin antagonist NAN-190, originally thought to be strictly an antagonist, is also able to substantially potentiate photic phase shifts, when administered systemically in both the early and late subjective night. There are also several other substances which were originally thought to be strictly serotonergic antagonists, but have since been shown to also have agonistic properties as well.

1.6.3.2 Serotonin mixed agonists/antagonists

There are several substances that act as serotonergic antagonists, but also appear to bind to some receptors agonistically, and are thus considered to be a class of drugs which is distinct from those strictly labeled as being either an agonist or antagonist. Thus, there is a unique third class of drugs that are termed mixed agonists/antagonists due to their action as antagonists at presynaptic axoaxonic serotonin heteroreceptors on the RHT, as well as agonists at postsynaptic 5-HT somatodendritic autoreceptors. Four such

drugs are BMY7378, S 15535, MDL 73005 EF, and NAN-190 (Portas *et al.*, 2000; Wesolowska *et al.*, 2002; Gannon, 2003)

Administration of NAN-190, BMY7378 or S15535 during the late subjective night can greatly potentiate photic phase shifts; inducing shifts approximately 250-300% compared to control treatments (Rea *et al.*, 1995; Byku & Gannon, 2000; Wesolowska *et al.*, 2002; Gannon, 2003; Gannon & Millan, 2006). However, BMY7378 is not able to significantly potentiate light-induced phase delays in the early subjective night (Gannon, 2003). The administration of NAN-190 without any other treatment also results in significantly larger phase advances, along with a trend towards larger phase delays (Sterniczuk *et al.*, 2008). More recently, S15535 was shown to potentiate the effect of light nearly three-fold in the late subjective night (Gannon & Millan, 2006). It was also discovered that when BMY7378 is administered during the mid- to late-subjective day it produced a phase advance in the circadian rhythm without any light exposure (Gannon, 2003). As an added complication in their mechanism of action, NAN-190 and BMY7378 have both been shown to potentiate photic phase shifts when administered up to 6 hours after the onset of the light exposure (Kessler *et al.*, 2008; Lungwitz & Gannon, 2009).

It is likely that NAN-190 and BMY7378 regulate part of the circadian system through their action to block the inhibitory effects of 5-HT release (Rea *et al.*, 1995; Dudley *et al.*, 1998). Plus, the agonistic action of NAN-190 at presynaptic somatodendritic 5-HT_{1A} autoreceptors leads to decreased release of 5-HT at the SCN (Dudley *et al.*, 1998). This decreased 5-HT release coincides well with the suspected mechanism of action of this class of mixed serotonin agonist/antagonists, such that it

could represent the outcome of an agonistic action at the median raphe to prevent serotonin release onto the SCN along with an antagonistic action at the RHT or SCN cells to block serotonergic inhibition at these location. Thus, this two-facetted decrease of inhibition to the SCN would result in an overall potentiated photic phase shifting effect of pre-treatment with this class of drugs.

1.6.3.3 Special case of BMY7378 and NAN-190

A relatively large body of research has been dedicated to determining the mechanism of action of these serotonin mixed agonists/antagonists, especially for NAN-190. A major complication regarding the mechanism by which NAN-190 and BMY7378 potentiate photic phase shifts stems from their marked lack of receptor specificity. Specifically, NAN-190 has been shown to be a potent antagonist at postsynaptic 5-HT_{1A} receptors, but it also has potent α_1-adrenoceptor blocking properties (Claustre *et al.*, 1991). NAN-190 is also a partial agonist of presynaptic 5-HT_{1A} receptors, and has been considered by some to be at least a partial agonist at somatodendritic 5-HT_{1A} autoreceptors (Portas *et al.*, 2000; Wesolowska *et al.*, 2002). Recently, NAN-190 has been shown to significantly attenuate photic phase shifts in mice lacking the 5-HT_{1A} receptor, in contrast to its normal potentiation effect observed in normal mice (Smith *et al.*, 2010). In fact, NAN-190 is also believed to bind to 5-HT_7 receptors (Lovenberg *et al.*, 1993). Thus, although it is apparent that part of the ability of NAN-190 to greatly potentiate photic phase shifts is indeed through binding at the 5-HT_{1A} receptor, it must also be binding to some other receptor which produces an opposing effect. Therefore, it appears that NAN-190 is a mixed agonist/antagonist at 5-HT_{1A} receptors, which also has

some affinity for numerous other receptors, and it is still largely unclear exactly how (at the cellular and molecular level) and where drugs such as NAN-190 function to induce such large magnitude phase shifts.

Considered to be highly efficacious, when it is administered in advance of a light pulse, BMY7378 was found to greatly potentiate late subjective night photic phase advances of the hamster circadian activity rhythm (Rea *et al.*, 1995; Gannon, 2003). Byku and Gannon (2000) found BMY7378 to potentiate phase advances by approximately 250-300% compared to control treatments. However, it is not able to significantly potentiate light-induced phase delays in the early subjective night (Gannon, 2003). It was also discovered that when BMY7378 is administered during the mid- to late-subjective night it produced a phase advance in the circadian rhythm without any light exposure (Gannon, 2003).

NAN-190 has also been shown to be able to potentiate photic phase shifts by a substantial amount when administered in both the early and late subjective night (Rea *et al.*, 1995). However, NAN-190 administered to hamsters not only potentiates the effects of light, but is also able to potentiate phase shifts induced by the direct application of some of the peptides released along the pathway between the retina and different cell sub-populations in the SCN. A systemic injection of NAN-190 significantly potentiates the phase shifts induced by NMDA application directly into the SCN, both in the early and late subjective night, but did not affect the Fos protein expression induced by NMDA (Sterniczuk *et al.*, 2008). Similarly, NAN-190 significantly potentiated phase advances, but attenuated phase delays, following the administration of GRP directly to the SCN.

However, these attenuated phase delays are accompanied by an increase in Fos expression in the 'cap' region of the SCN core (Sterniczuk *et al.*, 2008).

NAN-190 is able to potentiate the effects of light in both dark-adapted and entrained hamsters and greatly decreases the number of days required to re-entrain to a 6 hour advance in the light/dark cycle (Kessler *et al.*, 2008). As an added complication in their mechanism of action, NAN-190 and BMY7378 have both been shown to even potentiate photic phase shifts when administered up to 6 hours after the onset of the light exposure (Kessler *et al.*, 2008; Lungwitz & Gannon, 2009). Although the molecular mechanisms underlying the potentiating effects of NAN-190 or BMY7378 when administered several hours after a phase-shifting light pulse have yet to be elucidated, BMY7378 has also been shown to decrease the amount of 5-HT released in the hamster SCN following a systemic injection (Dudley *et al.*, 1998). Thus, it is likely that NAN-190 and BMY7378 regulate part of the circadian system through their action to block the inhibitory effect of serotonin release (Rea *et al.*, 1995; Dudley *et al.*, 1998).

The agonistic action of BMY7378 at presynaptic somatodendritic 5-HT_{1A} receptors also leads to decreased release of serotonin at the SCN (Dudley *et al.*, 1998). This decreased 5-HT release coincides well with the suspected mechanism of action of this class of mixed serotonin agonist/antagonists, such that it could represent the outcome of an agonistic action at the median raphe to prevent serotonin release onto the SCN, along with an antagonistic action at the RHT or SCN cells to block serotonergic inhibition at these locations. Thus, this two-facetted decrease of inhibition to the SCN could result in an overall potentiated photic phase shifting effect of pretreatment with this

class of drugs. However, the precise brain regions (e.g. median raphe, SCN, or both) through which NAN-190 and BMY7378 are producing the majority of their potentiation action has yet to be determined. BMY7378 would be a suitable candidate for the determination of this aspect of the action of 5-HT_{1A} mixed agonists/antagonists due to the fact that it can be dissolved in saline, thus allowing it to be injected directly into different regions of the brain to observe its local effects, in contrast to the global effects observed thus far following systemic administration of the drugs.

1.6.4 Serotonergic mediation of light-induced gene expression

Administration of serotonin agonists, including 5-$HT_{1A/7}$ receptor agonists, which attenuate or block light-induced phase shifts of the rodent activity rhythm, have also been shown to attenuate the expression of Fos in the hamster SCN (Amir *et al.*, 1998). Both systemic and intra-SCN injections of the 5-HT agonist quipazine can reduce light-induced Fos expression in the SCN during the subjective night (Selim *et al.*, 1993). Similarly, two 5-HT_{1A} receptor agonists, 8-OH-DPAT and buspirone, have been demonstrated to inhibit light-induced Fos-like immunoreactivity when administered 30 minutes prior to a half-hour duration light pulse (Glass *et al.*, 1994). This inhibition was mainly observed in the ventrolateral core of the SCN, with little effect in the dorsal and lateral regions of the SCN. This inhibitory effect of 8-OH-DPAT could be completely abated by NAN-190 pretreatment, despite the inability of NAN-190 to induce a change in the Fos-like immunoreactivity when injected alone (Glass *et al.*, 1994). However, this study was performed prior to the discovery that NAN-190 increases the effects of light on

behavioural phase shifts, and thus they did not look at the effect of NAN-190 and light exposure without the 5-HT agonist pretreatment.

Administration of the selective 5-HT_{1A} receptor agonist MKC-242 30 minutes prior to a light pulse in the early subjective night was shown to prolong the light-induced expression of *mPer1* and *mPer2* in the mouse SCN (Takahashi *et al.*, 2002). This prolonged gene expression directly corresponded to the potentiation of photic phase delays. MKC-242 was also noted to have no effect on *mPer1* or *mPer2* expression in the SCN without exposure to light. Although the 5-HT_{1A} receptor agonist MKC-242 has also been shown to potentiate photic phase advances in the late subjective night, this action was antagonized by pre-treatment with the selective 5-HT_{1A} receptor blocker WAY100635. However, despite the phase-shift potentiation ability of MKC-242, it has been shown to have no effect on the light-induced *c-fos* expression in the SCN when administered 30 minutes prior to a light pulse (Moriya *et al.*, 1998). One suggested explanation of this result proposed by Moriya *et al.* (1998) was the MKC-242 could be acting downstream of the light-induced *c-fos* expression seen in the SCN. Further, this receptor agonist could be activating 5-HT_{1A} autoreceptors in the midbrain raphe to reduce the serotonin activity in the SCN, thus allowing for the potentiation of the effect of light (Moriya *et al.*, 1998). However, if this explanation were correct, it would be expected that there would be an increase in cFos expression in the SCN resulting from the decreased inhibition, which was not observed.

It has been previously shown that 5-HT_{1A} antagonists either reverse or block the action of agonists, or produce the opposite effect (Ying & Rusak, 1997; Horikawa *et al.*,

2000). Plus, it is known that 5-HT_{1A} antagonists are able to potentiate phase advances in the late subjective night (Rea *et al.*, 1995). Thus, it would be expected that the administration of a 5-HT_{1A} antagonist, such as NAN-190 or BMY7378, along with a light pulse in the late subjective night, could conceivably result in the increased expression of *Per1* and *Per2* in the SCN. However, NAN-190 has been shown to attenuate the inhibitory effect of serotonin release, as stimulated by administration of tryptophan, on the *c-fos* expression normally induced by a light pulse (Glass *et al.*, 1995). When tryptophan was administered 1 hour prior to light exposure, there was an approximately 77% reduction in the number of cells expressing Fos-like immunoreactivity compared to the light only condition. However, pretreatment with NAN-190 attenuated this suppressive effect of the tryptophan on the Fos-like protein expression. As previously reported, NAN-190 administered alone had no significant effect on the amount of Fos-like immunoreactivity detected in the SCN (Glass *et al.*, 1995). Yet, when NAN-190 was combined with a light stimulus, it was found to affect Fos expression by partially blocking the expression normally induced in the ventrolateral SCN by exposure to a light pulse (Recio *et al.*, 1996).

These results have been extended in a study investigating the effects of NAN-190, paired with a light pulse, on several components in the pathway relaying the information of a light signal from the surface of the cell to the expression of genes related to the functioning of the circadian clock (Smith *et al.*, 2010). It was discovered that although a behavioural potentiation effect was observed, which coincided with a significant decrease in the level of activation of CREB and expression of the Fos protein, no effect was

observed on the activation of ERK or on the expression of *mPer1* or *mPer2* (Smith *et al.*, 2010). These results indicate that further research is still necessary, looking at other components of the pathway leading from cell activation to gene expression following the administration of 5-HT_{1A} mixed agonists/antagonists, in order to fully understand the precise pharmacological mechanism underlying the ability of these unique drugs to potentiate the effects of light to such a great degree. Ultimately, understanding the precise mechanism of action of these drugs is necessary before a commercially available chronobiotic could be developed which could be tailored to elicit the desired effects upon the circadian system, to rapidly resynchronize the circadian system with the external world, without any undesirable side-effects.

1.6.5 Summary

Through various different receptors, serotonin acts on the mammalian circadian system to inhibit photic input to the SCN. Projections from the median raphe into the SCN are the major source of this serotonergic input, acting to dampen the responsiveness of the SCN to photic input. Although effects are somewhat species-specific, serotonergic agonists generally attenuate photic responses during the subjective night, but can also potentially induce phase shifts on their own when administered during the subjective day. In contrast, serotonin antagonists are able to prevent or reverse the effects of agonists, as well as potentiate the effects of light when delivered at the appropriate phases. Several serotonin agonists and antagonists are also known to affect the expression of certain genes and proteins involved in the molecular clockwork of the SCN. A unique class of drug termed mixed agonists/antagonists, which presumably possess both agonistic and

antagonistic properties in different brain regions, have been shown to greatly potentiate light-induced phase shifts of the circadian system. However, the complete pharmacological mechanisms underlying the abilities of these drugs, including BMY7378, remain to be fully elucidated. The precise mechanism of action is necessary before a chronobiotic could be developed which is tailored to elicit the desired effects upon the circadian system without any undesirable side effects.

1.7 Present study: Objectives and hypotheses

Serotonin is known to play a major role in the regulation of circadian rhythmicity and modulate the effects of photic input to the SCN. The 5-HT_{1A} mixed agonist/antagonist BMY7378 has been shown to greatly potentiate the phase-shifting response of the circadian system to photic stimuli. However, it is still unknown exactly how BMY7378 achieves this enhanced response to light, both in terms of the location of action and in terms of the effects it has on molecular components of the SCN cells. The purpose of the present study is four-fold: 1) to explore the location in the brain at which BMY737 binds to receptors in order to produce the effect on circadian activity rhythms and whether the direct connection between the raphe nuclei and the SCN are necessary for this effect; 2) to better understand the effects of BMY7378 on *Period* gene expression in the SCN; 3) to characterize which immediate-early genes, biochemical cascade components, and clock-related proteins demonstrate altered expression as a result of BMY7378, and are thus involved in the behavioural potentiation effect observed; and 4) to determine whether the 5-HT_{1A} partial agonist busprione affects photic phase shifts more like a traditional agonist or more like a mixed agonist/antagonist.

2. Location of action of the 5-HT_{1A} mixed agonist/antagonist BMY7378

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Running Head: Location of action of BMY7378

Author Contributions:

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- Experimental design
- Laboratory Research: stereotaxic surgeries, predictions for manipulation times, injections, brief light pulse manipulations, phase shift calculations, perfusions, tissue collection, immunohistochemistry, tissue image capturing, 5-HT lesion analysis
- Manuscript Preparation: Entered and organized data; ran statistical analyses, produced figures, composed manuscript

Ryan Jeffers:
- Experiment 1 (Intra-SCN): Assisted with carrying out predictions for manipulation times, injections, phase shift calculations, perfusions, tissue collection, and cannula placement checks.

Dr. Michael Antle:
- Assisted with experimental design, and trained me on stereotaxic surgery and immunohistochemistry techniques
- Provided laboratory, equipment and financial support necessary to carry out experiments
- Manuscript Preparation: Provided feedback and editing of the manuscript

Abstract

The mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus, and generates daily oscillations in physiology and behaviour. The circadian system is modulated by serotonin (5-HT), such that 5-HT is presumed to inhibit photic phase shifts by dampening the excitatory signal sent to the SCN following light exposure. The 5-HT_{1A} mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378) has been shown to greatly potentiate photic phase shifts when administered systemically. Here we examine the role of the SCN, median raphe nucleus (MRN), and the serotonergic fibers connecting these 2 structures on the potentiated photic phase shifts when pretreated with BMY7378. When BMY7378 was administered systemically, we reproduced previous findings of potentiated photic phase shifts in the late subjective night. When administered directly to the SCN, BMY7378 no longer had the ability to potentiate photic phase advances. However, when delivered directly to the MRN, the potentiation effect of BMY7378 remained, although to a slightly smaller degree than with systemic treatment. Finally, the serotonergic fibers projecting from the MRN to the SCN are not strictly necessary for the potentiation effect observed with BMY7378 pretreatment. These findings suggest that BMY7378 potentiates photic phase shifts by binding to receptors in the median raphe nucleus, not in the SCN, and its effect is not solely the result of reduced serotonin output from the axon terminals of cells projecting from the raphe nuclei to the SCN.

Key Words: Circadian, Suprachiasmatic Nucleus, Raphe Nuclei, BMY7378, Photic Phase Shift, 5,7-DHT Lesion

2.1 Introduction

Endogenously generated circadian oscillations in physiology and behavior are controlled by the circadian clock, which is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972). The circadian system is entrained to the daily environmental light/dark cycle by light information relayed from the retina to the SCN via the retinohypothalamic tract (RHT; Daan & Pittendrigh, 1976; Rusak & Zucker, 1979). Light exposure at specific times during the night can induce a shift in the onset of the circadian activity rhythm (Daan & Pittendrigh, 1976). Dense serotonergic input to the SCN, originating in the raphe nuclei, modulate the effects of light by dampening the excitatory effects of light on the SCN (Meyer-Bernstein & Morin, 1996; Mistlberger, 2000; Rea & Pickard, 2000). A unique type of serotonergic drug termed mixed agonists/antagonists, due to their antagonistic actions at presynaptic axoaxonic heteroreceptors and agonistic actions at postsynaptic somatodendritic autoreceptors, have been shown to greatly potentiate photic phase shifts in the early or late subjective night (Rea *et al.*, 1995; Gannon, 2003; Sterniczuk *et al.*, 2008; Smith *et al.*, 2010).

Two of the most commonly researched examples of this class of serotonergic mixed agonists/antagonists are NAN-190 and BMY7378. Both NAN-190 and BMY7378 have been shown to potentiate photic phase shifts by as much as 250-300%, inducing phase shifts up to 8 hours in magnitude (Rea *et al.*, 1995; Byku & Gannon, 2000; Gannon, 2003). This potentiation has been shown to be the result of binding at the 5-HT_{1A} receptor, such that mice lacking this receptor do not show potentiated photic phase

shifts following NAN-190 pretreatment in the late subjective night (Smith *et al.*, 2010). However, 5-HT_{1A} receptors are found both in the raphe nuclei, in the form of somatodendritic autoreceptors (Hannon & Hoyer, 2008), and in the SCN, in the form of presynaptic axoaxonic heteroreceptors as well as postsynaptic receptors (Duncan *et al.*, 1999; Hannon & Hoyer, 2008). Systemic BMY7378 has been shown to lead to decreased release of serotonin at the SCN (Dudley *et al.*, 1998). This decreased 5-HT release coincides well with part of the suspected mechanism of action of this class of mixed serotonin agonist/antagonists, such that it likely represents the outcome of an agonistic action at autoreceptors in the median raphe to prevent serotonin release in the SCN. It is also presumed that there could be an antagonistic action at the RHT terminals or SCN cells to block serotonergic inhibition at these locations. The decreased inhibition to the SCN that would result from receptor binding at either or both of these locations would lead to increased excitation following photic input, thus allowing an overall potentiated effect of light on the circadian system. However, this is only speculation, and it is unknown exactly where or how this class of drug is acting in the brain to produce the observed behavioural effects.

The present study sought to determine the location of action of the serotonin mixed agonist/antagonist BMY7378. This study first examined the effect of BMY7378 pretreatment on the magnitude of phase shifts elicited by late subjective night light exposure, when administered systemically compared to administration directly into either the SCN or the median raphe nucleus (MRN). Next, this study attempted to determine the contribution of the serotonergic fibers connecting the MRN to the SCN in the

potentiation effect of BMY7378 administered systemically prior to a phase shifting light pulse in the late subjective night.

2.2 Experimental procedures

2.2.1 Animals and housing

A total of 62 male Syrian hamsters, obtained from Charles River Laboratories (Kingston, NY, USA), were initially housed in groups of two or three and maintained under a 14:10 light/dark cycle (lights on at 7:00 h and off at 21:00 h). Cage level luminance was approximately 300 lux. Animals were housed in a temperature- (21 ± 1°C) and humidity-controlled room, with *ad libitum* access to food and water. At the start of the experiment, or following surgery, each animal was transferred to an individual cage and maintained in its home environment, in a 14:10 light/dark cycle, for a minimum of 1 week. After this recovery period, or following an initial week of habituation to the new environment following arrival, hamsters were transferred to individual clear polycarbonate cages (45 × 20 × 22 cm) equipped with a running wheel (diameter of 14 cm), and maintained in constant darkness (DD) for the duration of the study. Cages were changed at least every 14 days (7-10 days prior to and following a manipulation). All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary, and adhered to the Canadian Council on Animal Care guidelines for the ethical use of animals.

2.2.2 Cannula implantation

Cannula implantation was performed at least 1 week following the arrival of the hamsters into the new environment, or once they weighed at least 110g. Each animal

received an intraperitoneal (i.p.) injection of the anesthetic sodium pentobarbital (CEVA; ~120 mg/kg), followed by a subcutaneous (s.c.) injection of the analgesic buprenorphine (~0.3 mg/kg). The hamsters were then stereotaxically implanted with a 9mm 22 gauge stainless steel cannula (Plastics One Inc., Roanoke, VA, USA) cemented to the skull with dental acrylic and jeweler's screws. The cannula was either aimed at the SCN region (coordinates: 0.3 mm anterior of bregma, 0.3 mm lateral of midline, 7.0 mm ventral to the skull) or the median raphe nucleus (at a 20° lateral angle to avoid obstruction of the cerebral aqueduct; coordinates: 4.4 mm posterior of bregma, 2.3 mm lateral of midline, 5.4 mm ventral to dura), with the incisor bar set to 2 mm below the interaural level. A dummy cannula was inserted to maintain patency, and both the dummy cannula and injection cannula extended 1 mm beyond the tip of the guide.

2.2.3 5,7-DHT lesion to the SCN

Animals received a peri-SCN injection of either a serotonin neurotoxin or vehicle control at least 1 week following their arrival, or once they weighed at least 110g. Each animal received an i.p. injection of desipramine hydrochloride (25 mg/kg dissolved in physiological saline; Sigma-Aldrigh, Oakville, ON, Canada) followed immediately by the anesthetic sodium pentobarbital (~70 mg/kg; CEVA Sante Animale), at least 30 min prior to the peri-SCN injection. Using a stereotaxic frame, a 26-gauge 1 µL Hamilton syringe was aimed at the SCN (10° lateral angle; coordinates: 0.3mm anterior to bregma, 1.25mm lateral of midline, 7.65mm ventral to dura; with the incisor bar set to 2 mm below the interaural level). Lesion animals (n = 10) received a 0.5 µL injection of 5,7-dihydroxytryptamine (5,7-DHT; ~24 µg free base/µL in 0.5% ascorbic acid in

physiological saline; Sigma Aldrigh, Oakville, ON, Canada) to lesion the serotonergic input into the SCN, and control animals (n = 4) received a 0.5 µL injection of vehicle (0.5% ascorbic acid in physiological saline). Injections took place over 5 min, and the syringe was left in place for a further 5 min to insure adequate dispersion of the injection. The burrhole used to deliver the injection was packed with bone wax, and the incision was sutured closed. Animals received a subcutaneous injection of Torbugesic (2mg/kg; Butorphanol; Wyeth Canada) as an analgesic near the end of the surgical procedure.

2.2.4 Drugs and reagents

The 5-HT_{1A} receptor mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378) was obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON). BMY7378 was dissolved in physiological saline. For experiment 1a and 2, BMY7378 (5 mg/kg) was injected systemically. The i.p. dose of BMY7378 was based upon the dose which produced the largest effect in previous work performed by Gannon (2003). For experiment 1b and 1c, BMY7378 (15.58 nmol/µL for some of Intra-SCN; 31.16 nmol/µL for some Intra-SCN and all Intra-MRN) was injected intra-cerebrally (0.5 µL), administered over 30 seconds via polyethylene 20 tubing using a 1 µL syringe, which was left in place for an additional 30 seconds before removing the injection cannula. Vehicle control injections (physiological saline) were based on a comparable volume by weight (experiment 1a and 2) or volume (experiment 1b and 1c). All injections were performed in constant darkness with the aid of night-vision goggles (BG15Alista, Richmond Hill, ON).

2.2.5 Activity rhythms

Wheel-running activity was continuously monitored using magnetic switches mounted on the running wheel, and connected to a computer running the Clocklab data collection software package (Coulbourn Instruments, Allentown, PA, USA). Actograms (graphical representation of wheel-running behavior) were generated and analyzed by Clocklab analysis software. Hamsters were allowed to free-run in constant darkness (DD) for a minimum of 7 days prior to the start of manipulations. Onset of running wheel activity for manipulation day was predicted using a regression line fit to the activity onsets for the 7-10 days prior to the testing day.

2.2.6 Experiment 1 – Enhanced photic phase shifts with BMY7378

This experiment examined the effects of BMY7378 administered systemically and intra-cerebrally on a brief exposure to dim light, as well as without light, on behavioral phase shifts of the circadian activity rhythm. Animals were housed in DD (0 lux) for the duration of this experiment. The dim light pulse was administered using a light-sealed box with a light bulb partially covered in aluminum foil, such that the light intensity at cage level was measured to be 40 lux using a light meter. In experiment 1a, hamsters (n = 9 at 40 lux, n = 5 at 0 lux) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse at two different intensities (40 lux followed by 0 lux) in the late subjective night, 6 h after their predicted activity onset (i.e., circadian time (CT) 18, where activity onset is designated to be CT12 by convention). In experiment 1b and 1c, hamsters (1b: n = 17 at 40 lux, n = 19 lux; 1c: n = 11 at 40 lux, n = 4 at 0 lux) were exposed to a

pretreatment of intra-cerebral micro-injection of either BMY7378 or vehicle control, followed 10 minutes later by a 15-minute light pulse at two different intensities (40 lux then 0 lux) at CT18. Animals received each injection treatment on separate occasions in a counterbalanced fashion for each of the light intensities.

Following the manipulations, actograms were analyzed using the automatic fitting function included in the Clocklab software, creating one line of best fit for the activity onsets of the 10 days prior to and including the manipulation day, and another line of best fit for the 10-12 days following the light pulse (excluding the first 3-5 onsets to avoid transient effects). The phase shift in the behavioral activity rhythm was calculated based on the horizontal difference between the predicted onsets from the two regression lines on the day following the manipulation.

2.2.7 Experiment 2 – Effect of BMY7378 without 5-HT fibers connecting MRN and SCN

This experiment examined the effects of BMY7378 administered systemically on a brief exposure to dim light, on behavioral phase shifts of the circadian activity rhythm. Animals (n = 14) were housed in DD (0 lux) for the duration of this experiment. The dim light pulse was administered as previously described. Hamsters were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute 40 lux light pulse at CT18.

Following the manipulations, actograms were analyzed using the automatic fitting function included in the Clocklab software, creating one line of best fit for the activity onsets of the 10 days prior to and including the manipulation day, and another line of best

fit for the 10 days following the light pulse (excluding the first 3 onsets to avoid transient effects). The phase shift in the behavioral activity rhythm was calculated based on the horizontal difference between the predicted onsets from the two regression lines on the day following the manipulation.

2.2.8 Perfusion and immunohistochemistry

All animals were euthanized with an overdose of sodium pentobarbital (Euthanyl; 240mg/mL, 0.7mL per hamster; dose: ~840mg/kg), and transcardially perfused using approximately 50 mL of phosphate buffered saline (PBS), followed by 50 mL of buffered 4% paraformaldehyde. Brains were extracted and postfixed in 4% paraformaldehyde overnight, and subsequently cryoprotected in a 20% sucrose solution for 24 hours. Brains were sliced through the anterior hypothalamus (Experiments 1b and 2) and the raphe nuclei (Experiments 1c and 2) using a cryostat, with alternate 35 μm sections through the SCN and MRN being collected for Nissl stain to verify cannula placements (Experiment 1b and 1c) or immunohistochemistry (IHC; Experiment 2) for lesion analysis.

Diaminobenzidine (DAB) immunohistochemistry was completed using a previously described method from our lab (see Antle *et al.*, 2008). Brain sections were exposed to a series of treatments at room temperature on a shaker tray unless otherwise indicated. Briefly, sections were rinsed for 30 minutes in 0.5% H_2O_2 in PBSx to inactivate endogenous peroxidase, followed by three 10-minute PBSx rinses, a 90-minute incubation in 10% normal goat serum in PBSx, and a 2-day incubation at 4°C in the primary antibody in 4% normal goat serum: for serotonin (rabbit anti-serotonin; 1: 2500; (#20080) Immunostar) in the raphe nuclei or the serotonin transporter (SERT; rabbit anti-

serotonin transporter; 1:2500; (#24330) Immunostar) in the SCN. Subsequently, sections were exposed to three 10-minute PBSx rinses, a 1-hour incubation in the secondary antibody biotinylated goat anti-rabbit (1:200; Vector Laboratories, Burlingame, CA), three 10min PBSx rinses, a 1-hour incubation in ABC (Vectastain Elite kit, Vector Laboratories, Burlingame, CA), and three final 10-minute PBSx rinses. Slices were then developed for approximately 5 minutes in 0.05% DAB and 0.02% NiCl in 25mL of 0.1M Tris buffer, activated with 80μL of 30% H_2O_2. Finally, slices were mounted on gelatin-coated slides, air-dried, dehydrated through an alcohol series, cleared with xylenes, and coverslipped with Permount.

2.2.9 Histological evaluation of intracranial implant sites

Cannula placements were examined after a minimum of 10 days following the final round of injections. Placements were assessed using alternate slices of Nissl stained tissue. Accurate placements were quantified using Image Pro Plus 7.0.0.591 (Media Cybernetics, Inc., Bethesda, MD), whereby the tip of the cannula guide was within 500μm of the SCN or MRN.

2.2.10 Quantification of 5,7-DHT lesion

Images of the DAB-labeled tissue were captured using the bright field setting on an Olympus BX51 microscope to determine the extent of the lesions. To determine baseline SERT immunoreactivity (IR), the mean relative optical density (ROD) was calculated for the ventral SCN, across both nuclei of animals that received a vehicle injection, using computerized image analysis software (ImageJ 1.42q; National Institutes of Health, Bethesda, MD). The same area containing the ventromedial SCN and a portion

of the retinorecipient region was used in all analyses to determine ROD. Two sections per animal at approximately the same rostrocaudal levels were chosen that displayed the densest SERT immunoreactivity (Morin & Blanchard, 1991; Yamakawa & Antle, 2010). The ROD of each SCN was calculated as a ratio over background control density from an unstained region adjacent to the SCN. The mean ROD of control animals was compared with lesioned animals to determine the extent of the 5-HT fiber loss in the SCN due to treatment with 5,7-DHT. Animals treated with 5,7-DHT that had no fibers in the SCN or just a few thick fibers along the margins of the SCN adjacent to the optic chiasm were deemed to be complete lesions and were retained for analysis, while animals treated with 5,7-DHT that had numerous fibers visible within the SCN, even if less than that observed in control animals, were deemed to be partial lesions and were excluded from the analysis.

2.2.11 Statistical analysis

For experiment 1, a 2x3 mixed Analysis of Variance (ANOVA) was used to determine if there was a significant difference in the magnitude of phase shift elicited by the drug and its vehicle followed by a 40 lux light pulse, between the systemic, Intra-SCN and Intra-MRN routes of administration. A second 2x3 mixed ANOVA was used to determine if there was a significant difference between the same treatment and condition groups followed by a 0 lux light pulse. A t-test was also used to determine if there was a significant difference in the magnitude of phase shift elicited between a BMY7378 dose of 15.58 nmol/μL and a dose of 31.16 nmol/μL when administered directly to the SCN with a subsequent 40 lux light pulse. For experiment 2, a 2x2 mixed ANOVA was used to

determine if there was a significant difference in the magnitude of phase shift elicited by the drug and its vehicle followed by a 40 lux light pulse, between control animals and those that received a 5,7-DHT lesion directly to their SCN. Statistical significance was set at $P<0.05$ for all tests, using SigmaPlot (Systat Software, Inc.; San Jose, CA, USA). All significant ANOVAs were followed up by Student-Newman-Keuls post hoc multiple comparisons. All means are reported as ± standard error of the mean (SEM).

2.3 Results

2.3.1 Experiment 1 – Enhanced photic phase shifts with BMY7378

BMY7378 significantly increased the magnitude of photic phase shifts induced in the late subjective night, when compared to vehicle control, but this effect was dependent upon route of administration (Treatment x Condition Interaction: $F_{(2,34)} = 16.969$, $p < 0.001$; Figure 2.1 and 2.2). Specifically, pretreatment with BMY7378 significantly potentiated the effect of late night light exposure when the drug was administered either systemically ($p <0.001$) or directly into the MRN ($p < 0.001$), however not when administered directly into the SCN ($p = 0.826$). There was no significant difference in the magnitude of phase shifts elicited by a dose of BMY7378 of 15.58 nmol/μL and a dose of 31.16 nmol/μL injected directly into the SCN ($t_{(15)} = 0.742$, $p = 0.470$), and thus the animals were combined into one group for the above analysis. There were also no significant differences in the magnitude of phase shifts elicited between the three routes of administration of saline prior to a 40 lux light pulse. BMY7378 administered without a subsequent light pulse in the late subjective night also significantly increased the magnitude of phase shift elicited, but this effect was also dependent upon the route of

administration (Treatment x Condition Interaction: $F_{(2,20)} = 28.158$, $p < 0.001$; Figure 2.3 and 2.4). Specifically, treatment with BMY7378 in the late subjective night induced significant phase advances when administered systemically ($p < 0.001$), but not when administered either directly into the SCN ($p = 0.332$) or directly into the MRN ($p = 0.354$). There were no significant differences between the magnitudes of phase shifts elicited by the administration of saline alone with any of the three routes of administration.

Figure 2.1 Representative actograms for systemic, intra-SCN and intra-MRN injections with light exposure.

Representative actograms of running wheel activity for hamsters receiving (A) a systemic injection of saline at CT17.25 (denoted by a diamond; ◆) followed by a 15-minute 40 lux light pulse at CT18 (denoted by a sun; ), and subsequently receiving a systemic injection of BMY7378 (5mg/kg) at CT17.25 (denoted by a diamond; ◆) and a light pulse at CT18 (denoted by a sun; ). Diagonal lines represent regression lines fit to activity onsets both before and after each treatment condition. Representative actograms of running wheel activity for a hamster receiving a cannula microinjection of saline 10 minutes prior to CT 18 followed by a light pulse at CT18, and subsequently an injection of BMY7378 (31.16nmol/μL) 10 min prior to CT18 followed by a light pulse at CT 18 administered (B) directly into the SCN or (C) directly into the MRN.

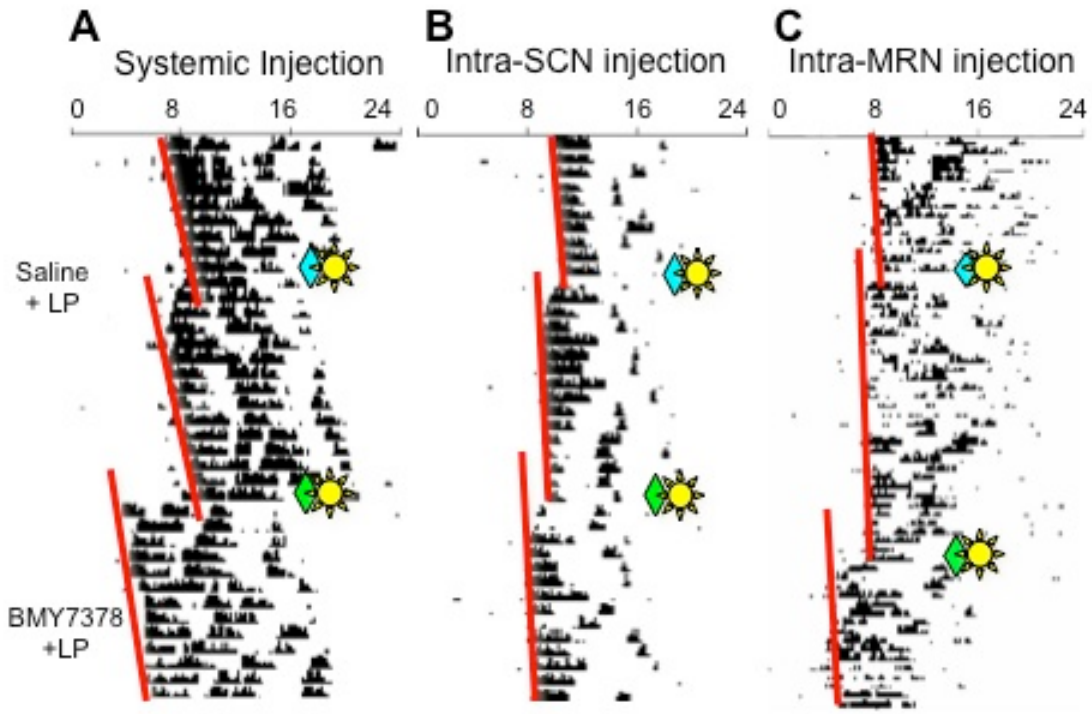

A
Systemic Injection
B
Intra-SCN injection
C
Intra-MRN injection
0
8
16
24
Saline
+ LP
BMY7378
+LP

Figure 2.2 Mean photic phase shifts for systemic, intra-SCN and intra-MRN injections.

Mean phase shifts (±SEM) for animals administered saline or BMY7378 at CT17.25 (systemic) or 10 minutes prior to CT18 (intra-SCN or intra-MRN), followed by a light pulse at CT18. (***) indicates significance at $p < 0.001$.

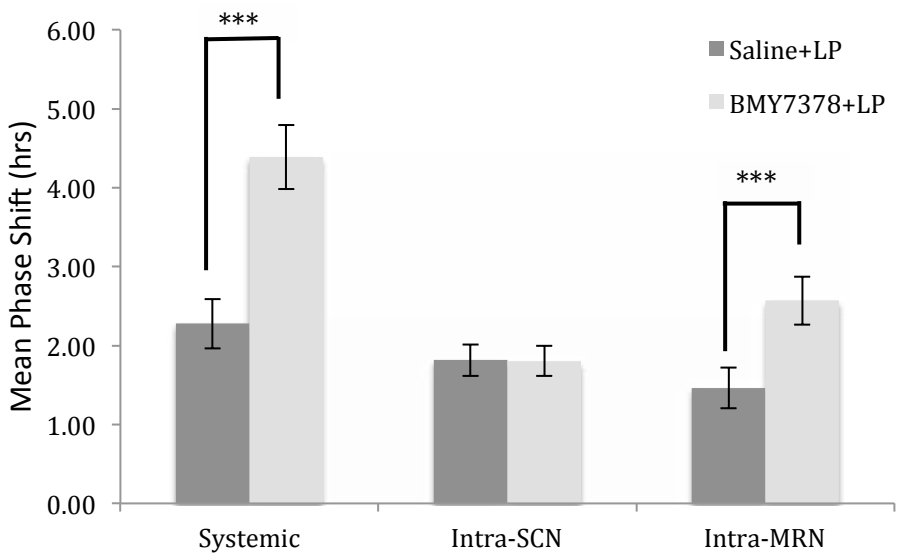

***
***
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Mean Phase Shift (hrs)
Saline+LP
BMY7378+LP
Systemic
Intra-SCN
Intra-MRN

Figure 2.3 Representative actograms for systemic, intra-SCN and intra-MRN injections without light exposure.

Representative actograms of running wheel activity for hamsters receiving (A) a systemic injection of saline at CT17.25 (denoted by a diamond; ◆), and on a subsequent date receiving a systemic injection of BMY7378 (5mg/kg) at CT17.25 (denoted by a diamond; ◆). Representative actograms of running wheel activity for a hamster receiving a cannula microinjection of saline 10 min prior to CT 18, and on a subsequent date receiving an injection of BMY7378 (31.16nmol/μL) 10 minutes prior to CT18 administered (B) directly into the SCN or (C) directly into the MRN. Diagonal lines represent regression lines fit to activity onsets both before and after each treatment condition.

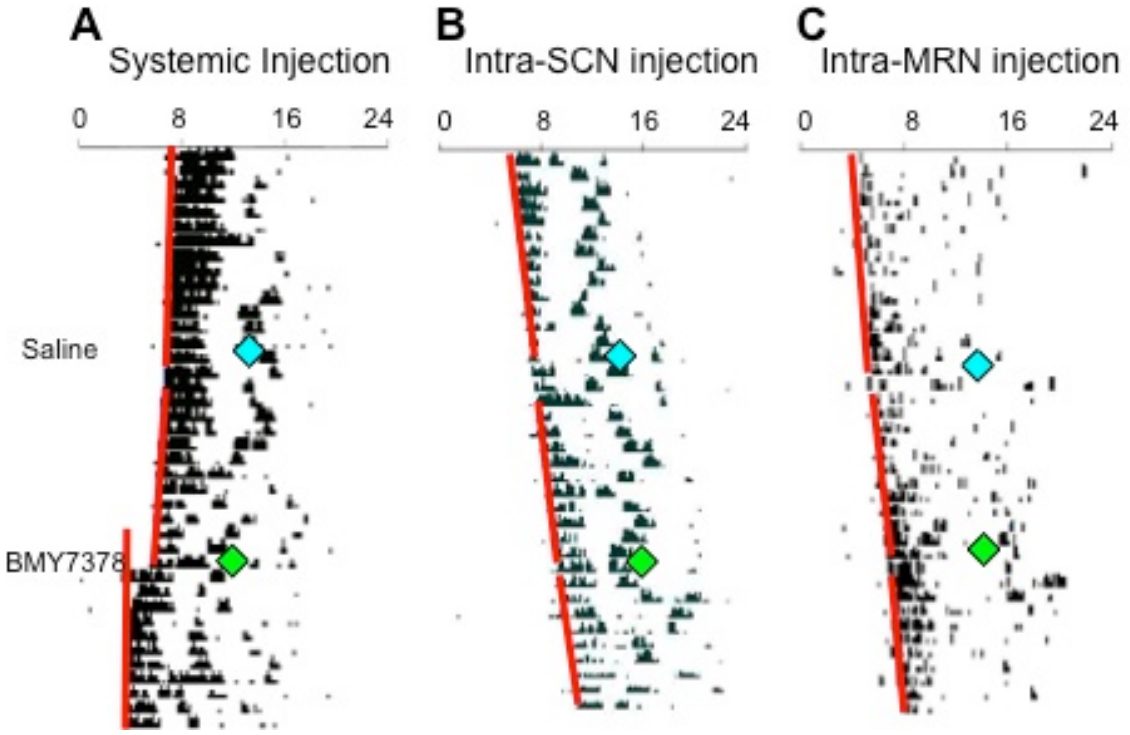
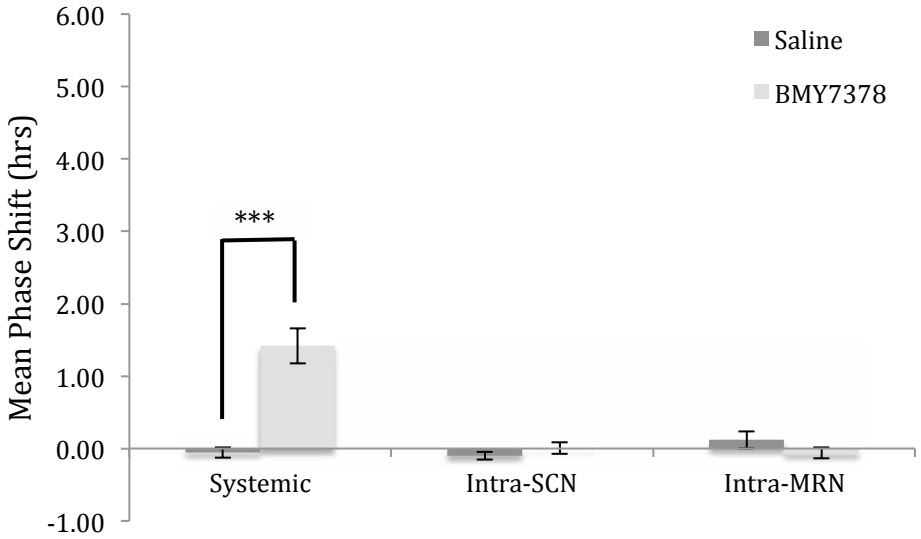

A
Systemic Injection
B
Intra-SCN injection
C
Intra-MRN injection
0
8
16
24
Saline
BMY7378

Figure 2.4 Mean phase shifts for systemic, intra-SCN and intra-MRN injections without light exposure.

Mean phase shifts (±SEM) for animals administered saline or BMY7378 at CT17.25 (systemic) or 10 minutes prior to CT18 (intra-SCN or intra-MRN) without subsequent light exposure. (***) indicates significance at $p < 0.001$.


6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Mean Phase Shift (hrs)
***
Saline
BMY7378
Systemic
Intra-SCN
Intra-MRN

2.3.2 Experiment 2 – Effect of BMY7378 without 5-HT fibers connecting MRN and SCN

The relative optical density of the SERT-immunoreactivity (-IR) in the ventromedial portion of the SCN was lower following 5,7-DHT treatment into the SCN (Lesion = 82.68 ± 1.27 percent of the mean Control ROD value; $t_{(12)} = 0.419$, $p < 0.001$; Figure 2.5). The mean ROD for the SCN of lesioned animals was 1.13 ± 0.02, while the mean ROD of the SCN for control animals was 1.37 ± 0.07. When fibers did remain in the 5,7-DHT-treated animals, they were visible in the ventromedial portion of the SCN. The dorsal and median raphe nuclei were examined in all animals included in the analysis to ensure there was not an appreciable loss of 5-HT cell bodies in either of these nuclei, indicating a lack of damage to the cell bodies and hopefully their processes to brain structures other than those 5-HT fibers specifically connecting the cells in the raphe nuclei to the SCN which were eliminated by 5,7-DHT (Figure 2.6).

Only those lesion animals judged to have complete or near complete loss of serotonin fibers in the SCN were used in the behavioural phase shift analysis. Systemic administration of BMY7378 significantly increased the magnitude of photic phase shifts induced in the late subjective night, when compared to vehicle control, in both control and lesioned animals (Main Effect of Treatment: $F_{(1,12)} = 46.969$, $p < 0.001$; Figure 2.7 and 2.8). However, there was no significant effect of 5,7-DHT injection to eliminate the 5-HT fibers connecting the raphe nuclei to the SCN on the magnitude of photic phase shifts elicited in the late subjective night (Main Effect of Condition: $F_{(1,12)} = 3.149$, $p < 0.101$).

Figure 2.5 Representative photomicrographs of SERT staining in the SCN with and without a 5,7-DHT lesion to the SCN.

Serotonin transporter (SERT)-labeled diaminobenzidine-stained SCN sections. (A) A representative SCN of an animal treated with a vehicle injection to the SCN (0.5% ascorbic acid in sterile saline). Dark arrows show a dense SERT/serotonergic fiber plexus input into the ventromedial SCN. (B) A representative SCN of an animal treated with a 5,7-DHT injection to the SCN. Dark arrows show a nearly complete loss of serotonin transporter fibers (and thus serotonergic input) into the SCN. 3V = 3rd Ventricle, OX = optic chiasm. Scale bar = 200μm.

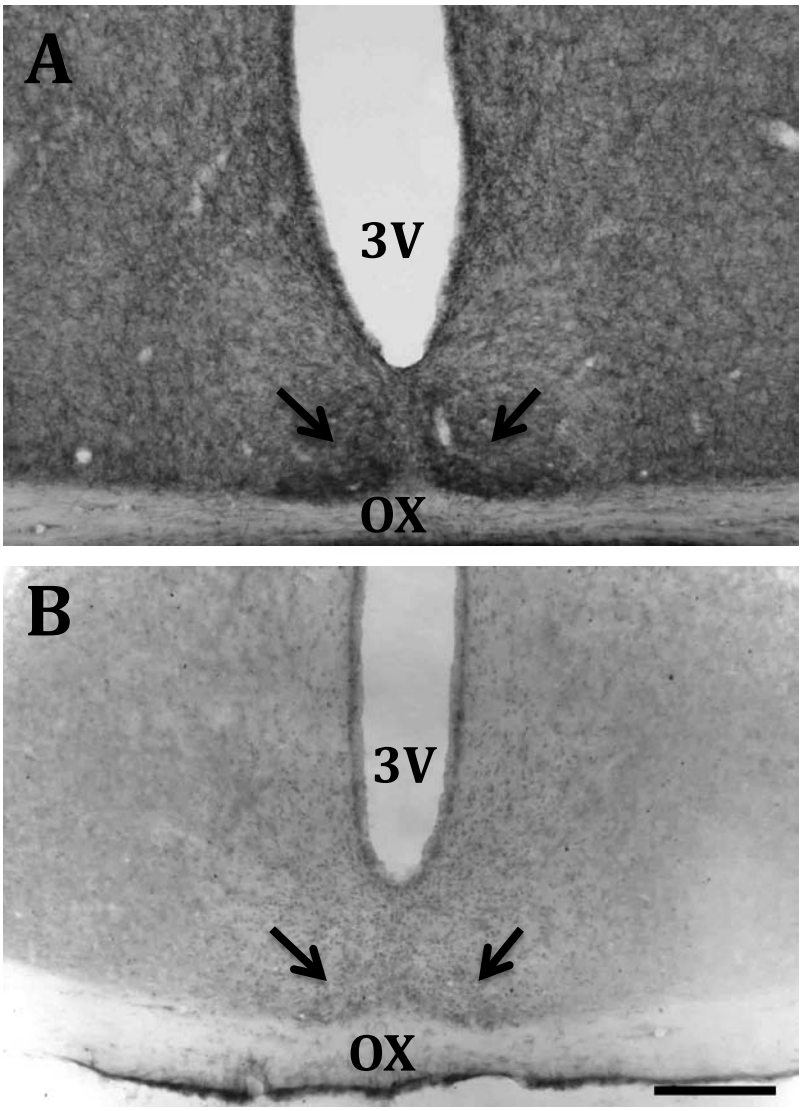

A
3V
OX
B
3V
OX

Figure 2.6 Representative photomicrographs of 5-HT staining in the raphe nuclei with and without a 5,7-DHT lesion to the SCN.

5-HT-labeled diaminobenzidine-stained raphe nuclei sections. Representative sections containing the (A) dorsal raphe and (C) median raphe nuclei of an animal treated with a vehicle injection to the SCN (0.5% ascorbic acid in sterile saline). Representative sections containing the (B) dorsal raphe and (D) median raphe nuclei of an animal treated with a 5,7-DHT injection to the SCN. Presence of 5-HT cell bodies in both conditions, in both structures, indicates that a 5,7-DHT lesion to the SCN eliminated fibers locally but did not damage the cell bodies from which those fibers originated. Scale bar = 200μm.

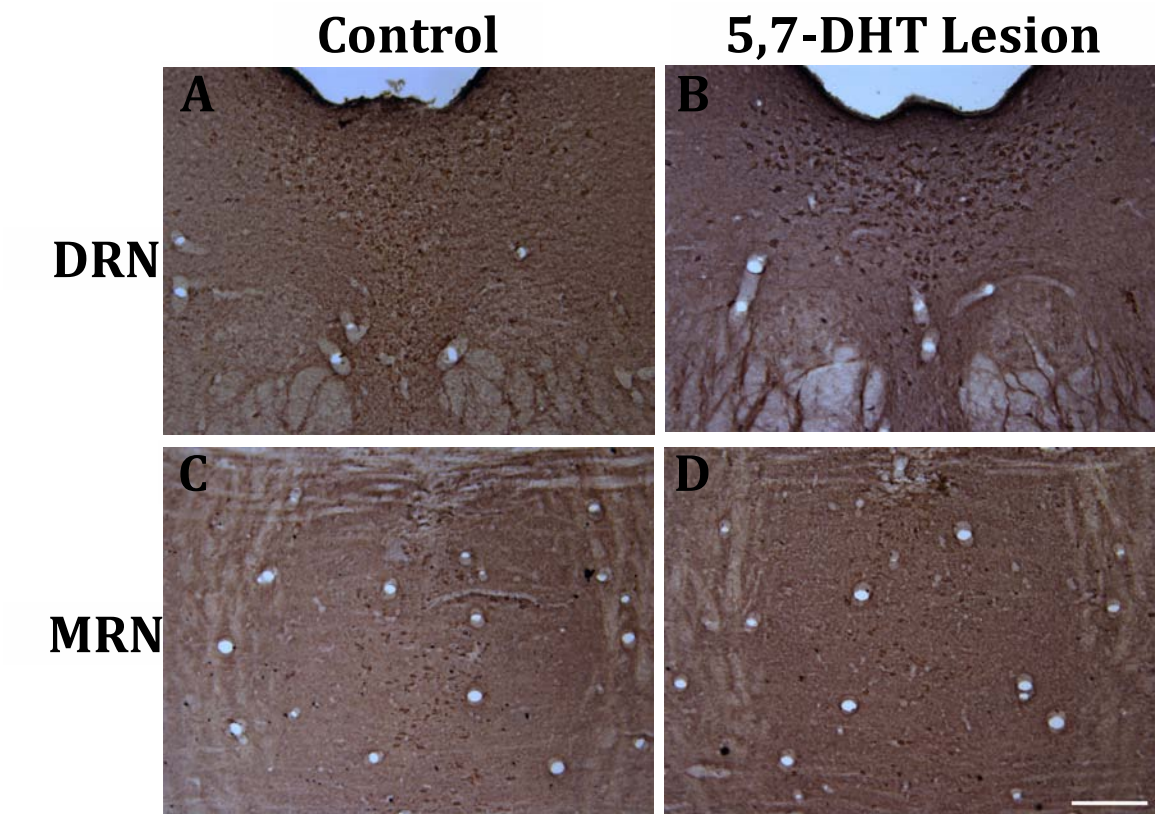

Control
5,7-DHT Lesion
A
B
DRN
C
D
MRN

Figure 2.7 Representative actograms with and without a 5,7-DHT lesion to the SCN. Representative actograms of running wheel activity for (A) a Control and (B) a 5,7-DHT lesioned hamster receiving a systemic injection of saline at CT17.25 (denoted by a diamond; ◆) followed by a 15-minute 40 lux light pulse at CT18 (denoted by a sun; ), and on a subsequent date receiving a systemic injection of BMY7378 (5mg/kg) at CT17.25 (denoted by a diamond; ◆) and a light pulse at CT18 (denoted by a sun; ). Diagonal lines represent regression lines fit to activity onsets both before and after each treatment condition.

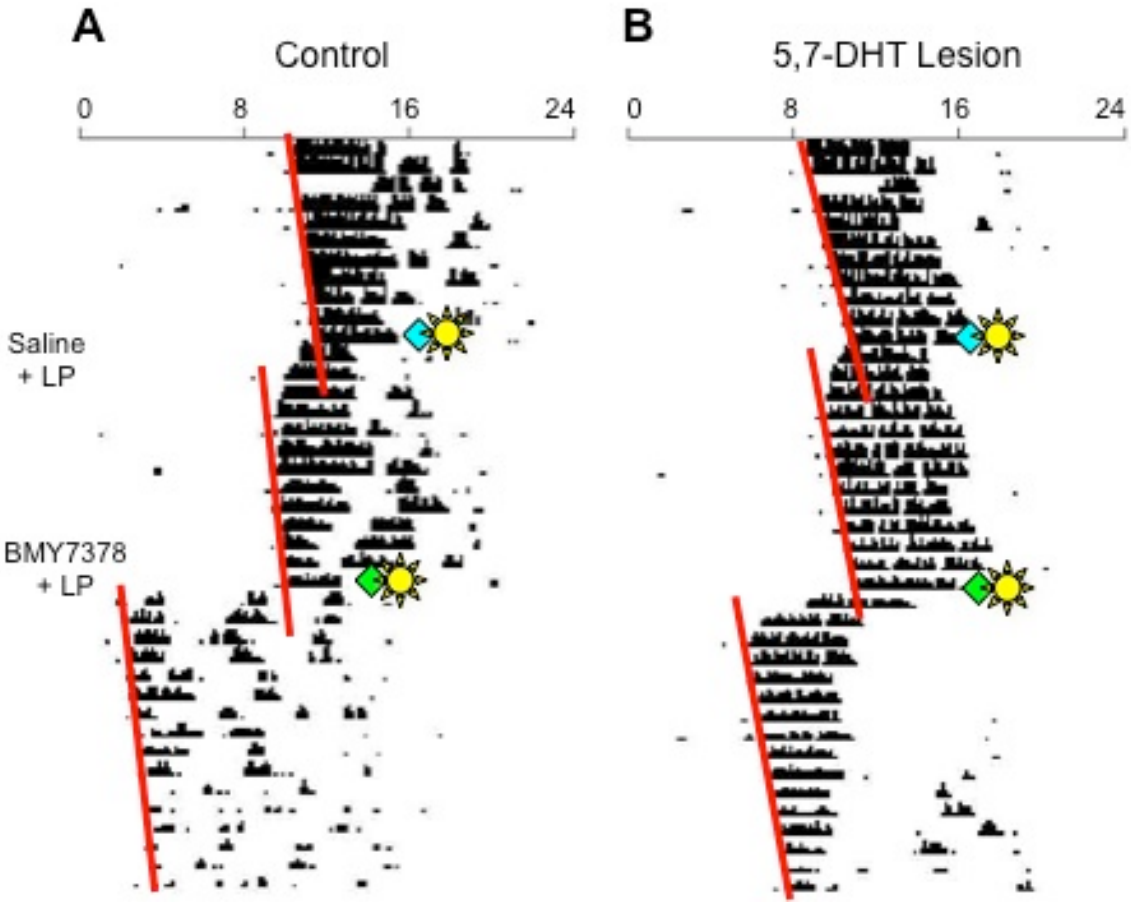
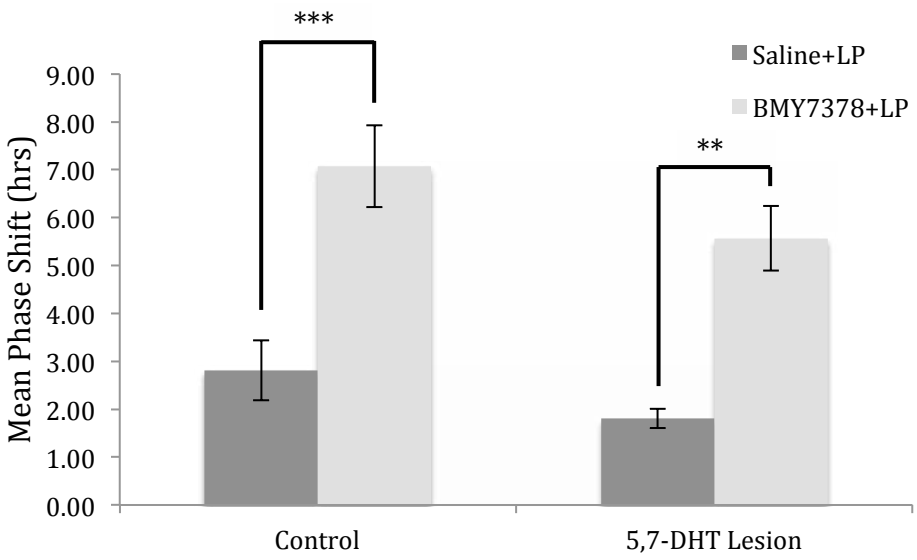

A
Control
0
8
16
24
Saline
+ LP
BMY7378
+ LP
B
5,7-DHT Lesion
0
8
16
24

Figure 2.8 Mean photic phase shifts with and without a 5,7-DHT lesion to the SCN. Mean phase shifts (±SEM) for Control and 5,7-DHT lesioned animals administered a systemic injection of saline or BMY7378 (5mg/kg) at CT17.25, followed by a light pulse at CT18. *** and ** indicate significance at $p < 0.001$ and $p < 0.01$, respectively.


***
**
Saline+LP
BMY7378+LP
Mean Phase Shift (hrs)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Control
5,7-DHT Lesion

2.4 Discussion

In this study, we show that BMY7378 significantly potentiates photic phase advances in the hamster circadian activity rhythm. It appears that this effect is mediated by the activity of BMY7378 directly on cells in the MRN, but not directly on cells in the SCN itself. BMY7378 was also shown to produce a phase advance when injected without a subsequent light exposure, and this effect appeared not to be mediated by activity directly on cells in either the SCN or the MRN. It was also shown that the serotonergic fibers connecting the raphe nuclei to the SCN are not necessary to observe the potentiation of photic phase advances following systemic administration of BMY7378 prior to light exposure in the late subjective night.

BMY7378 significantly potentiates photic phase advances when administered systemically. It has been proposed that mixed agonists/antagonists, such as BMY7378, could enhance photic phase shifts by decreasing the serotonergic output of the raphe through somatodendritic autoreceptor activation, while simultaneously blocking any further serotonergic activity at presynaptic heteroreceptors on the RHT or postsynaptic receptors on cells of the SCN (Gannon, 2003). Serotonin has been shown to inhibit photic responses in the SCN by inhibiting neurotransmitter release from retinal terminals (Pickard *et al.*, 1999) as well as by inhibiting activity on SCN cells themselves (Rea *et al.*, 1994; Ying & Rusak, 1994; Antle *et al.*, 2003). The hypothesized mechanism of action of mixed agonists/antagonists is consistent with the observation that systemic administration of BMY7378 increases the magnitude of the phase shift. Reducing the levels of serotonin released from the raphe nuclei and preventing the effects of the

serotonin that is still released into the synapses with SCN cells should have an overall effect of disinhibition at the level of the SCN. This disinhibition would then lead to an increased effect of glutamate release following stimulation of the retinal ganglion cells by light exposure, and thus an increase in the magnitude of the phase shift elicited.

Our finding that BMY7378 administered directly into the MRN is able to induce photic phase advances that are significantly greater than those induced by light alone, is consistent with the first portion of the above proposed mechanism of action. BMY7378 injected into the MRN would lead to activation of the 5-HT_{1A} somatodendritic autoreceptors, and thus a decrease in serotonin release at all axon terminals of those cells, including those which form synapses with cells in the SCN. However, our finding that BMY7378 administered directly to the SCN does not result in potentiated photic phase advances is inconsistent with the second portion of the above proposed mechanism of action. The fact that intra-SCN injections of BMY7378 do not potentiate photic phase shifts disproves the proposed mechanism – that these drugs work in part by blocking the effects of serotonin at presynaptic heteroreceptors on RHT terminals or postsynaptic receptors on the cells of the SCN.

Another serotonergic drug, the 5-HT_{1A} agonist MKC-242, which also potentiates photic phase shifts when administered systemically (Moriya *et al.*, 1998), has also been found to retain its ability to increase the magnitude of light-induced phase shifts when administered directly to the raphe nuclei (Takahiro Moriya; personal communication). However, the 5-$HT_{1A/7}$ agonist 8-OH-DPAT, which attenuates photic phase shifts when administered systemically, was found to retain its ability to attenuate photic shifts when

administered directly to the SCN (Antle *et al.*, 2003). Thus, it would appear that serotonergic drugs which act as agonists at the postsynaptic receptors in the SCN, such as 8-OH-DPAT, lead to an attenuation of photic phase shifts likely by increasing inhibition at the level of the SCN, whereas those which produce their effect by acting as agonists at the autoreceptors in the raphe nuclei, such as MKC-242 and BMY7378, instead lead to a potentiation of photic phase shifts; this likely occurs by reducing serotonergic inhibition at the level of the SCN.

Although we demonstrated that BMY7378 does not potentiate the effects of light by binding to receptors directly in the SCN, this drug ultimately has to induce some sort of change to the workings of the cells in the SCN, by some other route, in order to adjust the magnitude of the phase shift elicited. In fact, along with the behavioural effects observed in terms of the potentiation of photic phase shifts, the 5-HT_{1A} mixed agonist/antagonist NAN-190 (which appears to function very similarly to BMY7378) has been shown to potentiate the effects of artificial activation of the light pathway without retinal input. The effects of injecting NMDA or gastrin-releasing peptide directly into the SCN, both of which have been shown to induce light-like phase shifts (Piggins *et al.*, 1995), can be potentiated by systemic NAN-190 pretreatment (Sterniczuk *et al.*, 2008).

The most parsimonious explanation for this indirect effect on the SCN would be that following binding to the autoreceptors in the raphe nuclei, BMY7378 would reduce the amount of serotonin released at its terminals in the SCN, thus reducing inhibition on the cells of the retinorecipient region of the SCN and allowing for an increased excitation along the photic pathway. In fact, systemic administration of BMY7378 in the late

subjective night has been shown to lead to a decrease in the amount of serotonin released onto the SCN region (Dudley *et al.*, 1998). However, the present study indicates that the direct connection from the MRN to the SCN is not strictly necessary for the potentiating effect of BMY7378, although the magnitude of the potentiation may not be quite to the full degree of that observed with systemic administration. Therefore, the potentiation effect on the behavioural activity rhythms observed with BMY7378 pretreatment must be in large part the result of some other indirect pathway from the raphe nuclei to the SCN, and the decrease in the amount of serotonin released onto the SCN may be irrelevant to this phenomenon.

The most likely candidate for the mechanism of action of BMY7378 is the result of the indirect pathway connecting the raphe nuclei to the SCN by way of the intergeniculate leaflet (IGL) in the lateral geniculate nucleus (Morin, 1994; Harrington, 1997; Morin, 1999). The IGL receives direct retinal input as well as serotonergic input, and this pathway has been shown to modulate the effects of light on the circadian system (Morin, 1994). Ablation of the IGL has been shown to alter some circadian-related behaviours (Harrington & Rusak, 1986). Serotonergic projections from the raphe nuclei synapse on cells containing several different neuropeptides and neurotransmitters within the IGL, including neuropeptide Y (NPY), enkephalin (ENK) and GABA (Harrington, 1997).

Activation of autoreceptors in the raphe nuclei by BMY7378 likely causes a reduction in the amount of serotonin released onto the IGL, leading to an overall disinhibition of the cells that then project from the IGL to the SCN. In fact, BMY7378

appears to interact with NPY released from the cells of the IGL in order to produce the photic potentiation effect observed, such that NPY antagonism in combination with BMY7378 leads to an even larger increase in the magnitude of phase shift elicited by light exposure than with only BMY7378 and light exposure (Lall & Harrington, 2006). NPY normally reduces the effects of light on phase advances of the circadian activity rhythm (Harrington, 1997), and thus it is not unexpected that blocking its effect led to an even greater disinhibition at the level of the SCN. However, the effect of NPY alone cannot fully explain the increase in phase shifts that are induced by systemic administration of BMY7378, because a disinhibition of the NPY cells in the IGL would be expected to attenuate, not potentiate, photic phase shifts. Thus it is likely that BMY7378 leads to an effect upon the cells of the IGL that is more complex than simply disinhibiting NPY cells. However, exactly which cell population within the IGL is having its activity altered by BMY7378 has yet to be elucidated.

A complete understanding of the pharmacology and location of action for 5-HT_{1A} mixed agonists/antagonists, such as BMY7378, to modulate circadian sensitivity to light, is pertinent in today's fast paced world. This knowledge could allow for the eventual development of chronobiotics specifically designed to rapidly resynchronize circadian rhythms. Effective management, or even prevention, of the chronic desynchronization inherent to rotating shift work, jet lag, or circadian rhythm disorders, could play a substantial part in the elimination of the numerous adverse health effects which are associated with this type of chronic desynchrony.

3. Potentiation of photic phase shifts by the serotonergic mixed agonist/antagonist BMY7378 in mice: Behaviour and *Period* gene expression

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Running Head: BMY7378 and photic phase shifts in mice

Author Contributions:

Victoria Smith:
- Experimental design
- Laboratory Research: Predictions for manipulation times, injections, light pulse manipulations, phase shift calculations, perfusions, tissue collection, *in situ* hybridization, microscopy, ROD analysis
- Manuscript Preparation: Entered and organized data; ran statistical analyses, produced figures, composed manuscript

Dr. Michael Antle:
- Assisted with experimental design, and trained me on *in situ* hybridization techniques
- Provided laboratory, equipment and financial support necessary to carry out experiments
- Manuscript Preparation: Provided feedback and editing of the manuscript

Abstract

The 5-HT_{1A} mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378) has been shown to greatly potentiate photic phase shifts in hamsters. It has yet to be determined if this effect is also observed in mice with systemic BMY7378 pretreatment. The intracellular changes in the cells of the suprachiasmatic nuclei (SCN), which are associated with such enhanced phase shifting, are also unknown. Here we examine the behavioural and underlying *Period* gene expression involved with photic phase shifts induced in the late subjective night following BMY7378 pretreatment. Administration of BMY7378 to mice had no effect on the magnitude of phase shift elicited by exposure to dim light in the late subjective night. BMY7378 also had no effect on the phase of the circadian activity rhythm of mice without subsequent light exposure. The light-induced expression of the circadian clock components *Period1* and *Period2* were also not affected by BMY7378 pretreatment, during the late subjective night. These findings suggest that although BMY7378 potentiates photic phase shifts in hamsters, this drug does not function in the same way in mice.

Key Words: Circadian, Suprachiasmatic Nucleus, BMY7378, Mice, *Period* gene expression

3.1 Introduction

Endogenously generated daily oscillations in physiology and behaviour are evident throughout phylogeny, from prokaryotes to mammals (Sharma, 2003). Such circadian oscillations in mammals are controlled by the circadian clock, located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972), and are entrained to the daily environmental light/dark cycle by photic input to the eye (Daan & Pittendrigh, 1976; Rusak & Zucker, 1979). Light information is relayed from the intrinsically photosensitive retinal ganglion cells in the retina, along the retinohypothalamic tract (RHT) to the SCN, where it can alter the expression of circadian rhythms (Berson, 2003; Hirota & Fukada, 2004). The SCN also receives dense serotonergic innervation from the median raphe nucleus (Meyer-Bernstein & Morin, 1996). Serotonin (5-HT) has been shown to modulate the effects of light by dampening the excitatory signals sent to the cells of the SCN as a result of light exposure (Meyer-Bernstein & Morin, 1996; Mistlberger, 2000). A unique class of serotonergic compounds, termed mixed agonists/antagonists, are capable of modulating the effects of light on the circadian system by enhancing the phase shifting effect that light has during the subjective night (Gannon, 2003).

These unique drugs proposed to act as antagonists at presynaptic axoaxonic heteroreceptors or postsynaptic receptors on the cells of the SCN, as well as agonists at postsynaptic somatodendritic autoreceptors. When administered during the early or late subjective night, these drugs are known to greatly potentiate photic phase shifts in hamsters, inducing shifts in the activity rhythm of up to 8 hours in magnitude (Rea *et al.*,

1995; Gannon, 2003). The potentiation effects produced by this class of drug is presumed to be the result of the combination of an agonistic action at the median raphe to prevent serotonin release onto the SCN, along with an antagonistic action at the RHT terminals or SCN cells to block serotonergic inhibition at these locations. The resulting overall decreased inhibition to the SCN is suspected to lead to increased excitation following photic input, thus allowing an overall potentiated phase shifting effect.

Two of the most commonly researched examples of this class of serotonergic mixed agonists/antagonists are NAN-190 and BMY7378. Both NAN-190 and BMY7378 have been shown to potentiate photic phase shifts in hamsters by as much as 250-300% (~8 hours in magnitude; Rea *et al.*, 1995; Byku & Gannon, 2000; Gannon, 2003). NAN-190 has been demonstrated to have a similar effect in mice (Smith *et al.*, 2010). The 5-HT_{1A} agonist MKC-242, which has also been shown to greatly enhance photic phase shifts in both mice and hamsters (Moriya *et al.*, 1998; Takahashi *et al.*, 2002), was also found to lead to increased expression of both *mPer1* and *mPer2* mRNA in the SCN 3 hours following exposure to light (Takahashi *et al.*, 2002). These findings indicate a mechanism of action involving an extended activation of the *Period* genes normally induced in the SCN following phase shifting light exposure.

The present study sought to first determine if the potentiation effect previously observed following BMY7378 pretreatment in hamsters was reproducible in mice. Subsequently, this study attempted to explore the molecular underpinnings of the photic phase shift potentiation previously observed with BMY7378 in hamsters, by examining

the regionally-specific expression of the *Period* genes involved in the transduction of the incoming light signal into phase shifts of the behavioural output of the circadian clock.

3.2 Experimental procedures

3.2.1 Animals and housing

A total of 17 male C57BL/6J mice were used, obtained from the University of Calgary Life and Environmental Science Animal Resource Centre. Mice were individually housed in Nalgene Type L clear polycarbonate cages (30.3 cm long × 20.6 cm wide × 26 cm high; Nalg Nunc International, Rochester, NY), equipped with a stainless steel running wheel (diameter of 24.2 cm). Animals were housed in a temperature- (21 ± 1°C) and humidity-controlled room, with *ad libitum* access to food and water. Cages were changed at least every 14 days (7-10 days prior to, and following a manipulation). All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary, and adhered to the Canadian Council on Animal Care guidelines for the ethical use of animals.

3.2.2 Drugs and reagents

The 5-HT_{1A} mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378) was obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON). BMY7378 was dissolved in physiological saline. Injections were administered intraperitoneally (i.p.), with the dose of BMY7378 being 5 mg/kg and vehicle control injections (physiological saline) being a comparable volume based on weight. The dose of BMY7378 was based upon the lowest dose which produced the largest effect in hamsters in previous work performed by

Gannon (2003). All injections were performed in constant darkness with the aid of night-vision goggles (BG15Alista, Richmond Hill, ON).

3.2.3 Activity rhythms

Wheel-running activity was continuously monitored using magnetic switches mounted on the running wheel, and connected to a computer running the Clocklab data collection software package (Coulbourn Instruments, Allentown, PA, USA). Actograms (graphical representation of wheel-running behavior) were generated and analyzed by Clocklab analysis software. Mice were allowed to freerun in constant darkness (DD) for a minimum of 7 days prior to the start of manipulations. Onset of running wheel activity for manipulation day was predicted using a regression line fit to the activity onsets for the 7-10 days prior to the testing day.

3.2.4 Experiment 1 – Phase shifts following BMY7378 in mice

This experiment examined the effects of BMY7378 on a brief exposure to dim light in the late subjective night on behavioral phase shifts of the activity rhythm. Animals were housed in DD (0 lux) for the duration of this experiment. The dim light pulse was administered using a light-sealed box with a light bulb partially covered in aluminum foil such that the light intensity at cage level was measured as 40 lux (as measured by a light meter). Mice (n = 17) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse in the late subjective night at one of two intensities (40 lux or 0 lux), 10 h after their predicted activity onset (i.e., circadian time (CT) 22, where activity onset is designated to be CT12 by convention). Animals received each injection treatment on

separate occasions in a counterbalanced fashion. Following the manipulations, actograms were analyzed using the automatic fitting function included in the Clocklab analysis software, creating one line of best fit for the activity onsets of the 10 days prior to and including the manipulation day, and another line of best fit for the 10 days following the light pulse (excluding the first 3 onsets to avoid transient effects). The phase shift in the behavioral activity rhythm was calculated based on the horizontal difference between the predicted onsets from the two regression lines on the day following the manipulation.

3.2.5 Experiment 2 – *Period* gene expression following BMY7378 pretreatment

This experiment examined the effects of BMY7378 on a brief exposure to dim light in the late subjective night on light-induced *Period1* and *Period2* mRNA expression in the SCN. Animals were housed in DD (0 lux) for the duration of this experiment. The 40 lux light pulse was administered as described previously. Mice (n = 11) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute 40 lux light pulse at CT22. Animals were euthanized with an i.p. injection of sodium pentobarbital (Euthanyl; 240 mg/mL, 0.3 mL per mouse), 90 minutes after the start of the light pulse (i.e. at CT23.5). The animal's head was wrapped in aluminum foil to prevent the retinas from being exposed to light prior to and during the perfusion. Animals were then transcardially perfused using approximately 50 mL of phosphate buffered saline (PBS), followed by 50 mL of buffered 4% paraformaldehyde. Brains were extracted and postfixed in 4% paraformaldehyde overnight, and subsequently cryoprotected in a 20% sucrose solution for 24 hours. Brains were sliced

through the anterior hypothalamus using a cryostat, with alternate 35 μm sections through the SCN being collected for *in situ* hybridization.

3.2.6 *In situ* hybridization

For *mPer1*and *mPer2 in situ* hybridization, all instruments and trays were cleaned with RNase Zap (Ambion, Austin, TX) and all solutions were prepared with RNase-free water. Free floating tissue sections were processed using the probe and digoxigenin (DIG) protocol of Yan and Silver (2002). First, tissue was treated with proteinase K (1 mg/mL, 0.1M Tris buffer pH 8.0; 50 mM EDTA; 10 minutes) at 37°C. This reaction was stopped by adding 2 mL of 4% paraformaldehyde. Following rinses in saline sodium citrate (300mM NaCl, 30mM sodium Citrate) the tissue was treated with 0.25% acetic anhydride in 0.1M triethanolamine for 10 minutes. The sections were then incubated in 1.5 mL hybridization buffer [50% formamide, 60mM sodium citrate, 600mM NaCl, 10% dextran sulphate, 1% N-laurylsarcosine, 25 mg/mL tRNA, 1X Denhardt's, 0.25mg/mL salmon sperm DNA] containing the DIG-labeled *mPer1* or *mPer2* (mPer1 and mPer2 plasmids courtesy of Dr. L. Yan, Michigan State University, East Lansing, MI) antisense cRNA probes (0.1 μg/mL; for details see Hamada et al., 2001) for 16 hours at 60°C.

After two 30-minute high-stringency posthybridization washes (50% formamide/saline sodium citrate at 60°C), sections were treated with RNase A. After more high-stringency washes, the tissue was then processed for immunodetection with a nucleic acid detection kit (Roche, Indianapolis, IN). The sections were incubated in 1.0% of blocking reagent in buffer 1 (100 mM Tris-HCl buffer, 150 mM NaCl, pH 7.5) for 1 hour at room temperature. Sections were then incubated at 4°C in alkaline phosphatase-

conjugated Dig antibodies diluted 1:5000 in buffer 1 for 3 days. On the following day, sections were washed in buffer 1 twice (15 minutes each), and incubated in buffer 3 (100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl and 50 mM $MgCl_2$) for 5 minutes. They were then incubated in a solution containing nitroblue tetrazolium salt (0.34 mg/mL) and 5-bromo-4-chrolo-3-indolyl phosphate toluidinium salt (0.18 mg/mL; Roche) for 16 hours. The colourimetric reaction was stopped by immersing the sections in buffer 4 (10 mM Tris-HCl containing 1 mM EDTA, pH 8.0). The tissue was then mounted on gelatin-coated slides, air-dried, briefly dehydrated in an alcohol series, cleared with xylenes, and coverslipped with Permount.

3.2.7 Quantification of gene expression

Images of the digoxigenin-labeled tissue were captured via an Olympus BX51 microscope equipped with a monochrome QICam Fast 1394 digital video camera (QImaging, Burnaby, BC, Canada). Bilateral calculations of the relative optical density (ROD) of *mPer1* and *mPer2* mRNA expression were performed at the mid-caudal level of the SCN using computerized image analysis software (ImageJ 1.42q; National Institutes of Health, Bethesda, MD). RODs were calculated based on the grey level of the SCN expressed as a ratio over the grey level of adjacent non-SCN tissue in the same frame. Separate counts were obtained for two regions of the SCN: core and shell, delineated based on the general shape of the GRP-IR and VP-IR regions, respectively, as previously identified by our lab in Smith *et al.* (2010). A core/shell analysis has important heuristic value, as it provides as starting point for more detailed analyses of SCN specialization (Antle & Silver, 2005), although it is important to keep in mind that such

an approach, while more detailed than a simple whole SCN analysis, still represents an oversimplification of SCN heterogeneity (Morin, 2007).

3.2.8 Statistical analysis

For experiment 1, a two-tailed independent samples *t*-test was used to determine if there was a significant difference in the magnitude of photic phase shifts elicited by the two different pretreatment conditions (BMY7378 or vehicle control). A second two-tailed paired *t*-test was used to determine if there was a significant difference in the magnitude of the phase shifts induced by each of the two pretreatment conditions without a subsequent light pulse. For experiment 2, a mixed two-way analysis of variance (ANOVA) was used, one for each of *mPer1* and *mPer2*, to determine if there was a significant difference in the expression induced in the core and shell regions of the SCN by a light pulse in animals pretreated with each of the two pretreatments conditions. Statistical significance was set at $P<0.05$ for all tests, using SigmaPlot Version 11.2.0.5 (Systat Software, Inc.; San Jose, CA). All significant ANOVAs were followed up by Student-Newman-Keuls post hoc multiple comparisons. All means are reported as ± standard error of the mean (SEM) in figures.

3.3 Results

3.3.1 Experiment 1 – Phase shifts following BMY7378 in mice

BMY7378 had no significant effect on the magnitude of photic phase shifts induced in the late subjective night, when compared to vehicle control ($t_{(21)} = 0.186$, $p = 0.854$; Figure 3.1 and 3.2). BMY7378 administered without a subsequent light pulse in the late subjective night also had no significant effect on the magnitude of phase shift

elicited ($t_{(10)} = 0.239$, $p = 0.816$; Figure 3.2). In fact, essentially no phase shifts were elicited by either BMY7378 or saline.

Figure 3.1 Representative actograms of mice pretreated with saline or BMY7378 prior to a late subjective night light pulse.

Representative actograms of running wheel activity for mice receiving an i.p. injection of (A) Saline, denoted by a diamond (◆), or (B) BMY7378 denoted by a diamond (◆), at CT21.25, followed by a 15 min 40 lux light pulse at CT22, denoted by a sun (☀). Diagonal lines represent regression lines fit to activity onsets both before and after exposure to a light pulse.

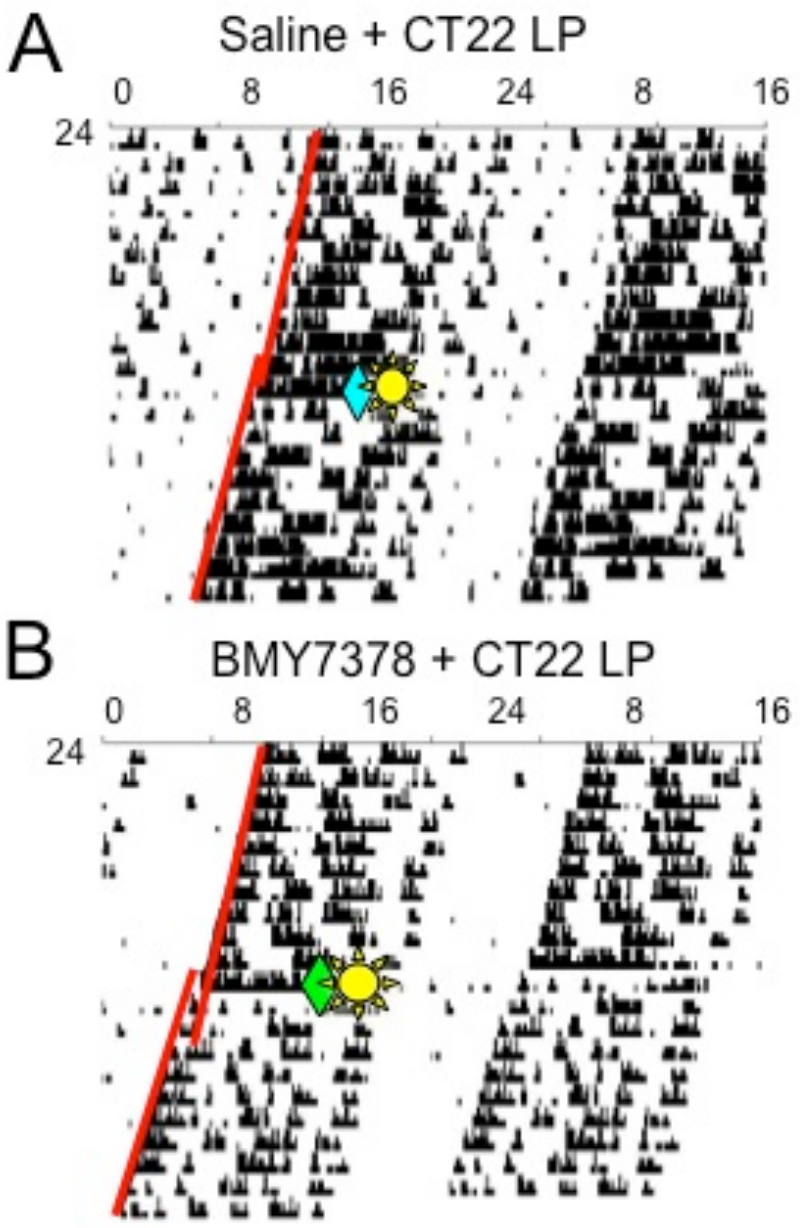
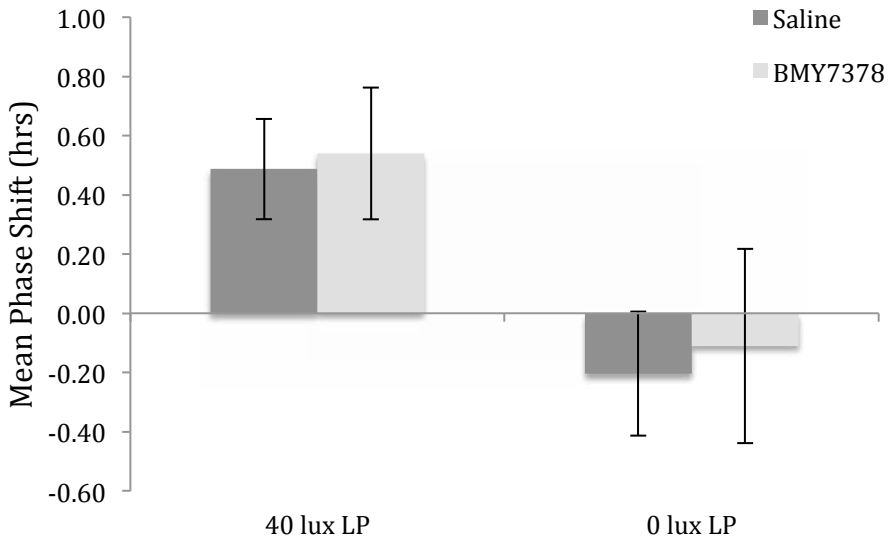

A
Saline + CT22 LP
0
8
16
24
8
16
24
B
BMY7378 + CT22 LP
0
8
16
24
8
16
24

Figure 3.2 Mean phase shifts for mice pretreated with saline or BMY7378.

Mean phase shifts (± SEM) of mice treated with either saline or BMY7378 at CT21.25, followed by a 40 lux or 0 lux light pulse at CT22.


Saline
BMY7378
Mean Phase Shift (hrs)
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
40 lux LP
0 lux LP

3.3.2 Experiment 2 – *Period* gene expression following BMY7378 pretreatment

Representative examples of the *mPer1* and *mPer2* expression for each drug treatment condition are presented in Figure 3.3. No significant difference was observed in the amount of either *mPer1* or *mPer2* mRNA expression in BMY7378 pretreated animals relative to saline pretreated mice (Treatment x SCN Region Interaction: $F_{(1,9)} = 0.911$, $p = 0.365$ and Treatment x SCN Region Interaction: $F_{(1,9)} = 2.030$, $p = 0.188$, respectively; Figure 3.4). As expected, the majority of gene expression was observed in the core region of the SCN in all animals (SCN Region: $F_{(1,9)} = 238.790$, $p < 0.001$ for *mPer1*; SCN Region: $F_{(1,9)} = 56.485$, $p < 0.01$ for *mPer2*).

Figure 3.3 Representative photomicrographs of *mPer1* and *mPer2* in the SCN of mice pretreated with saline or BMY7378.

Representative examples of SCN tissue slices indicating (A,B) *mPer1* and (C,D) *mPer2* cells at CT23.5 and stained using *in situ* hybridization following a 15-minute 40 lux light pulse in the late subjective night (CT22), pretreated at CT21 with an i.p. injection of either (A,C) saline or (B, D) BMY7378. Scale bar = 200μm.

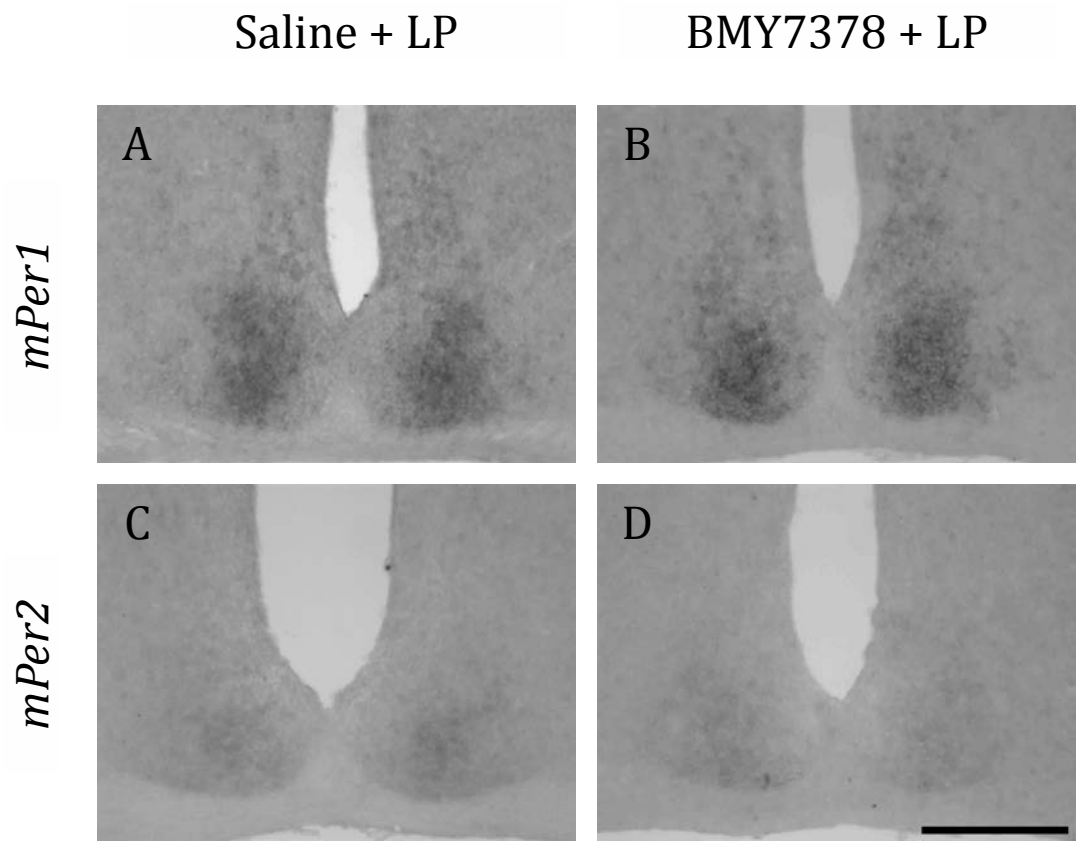
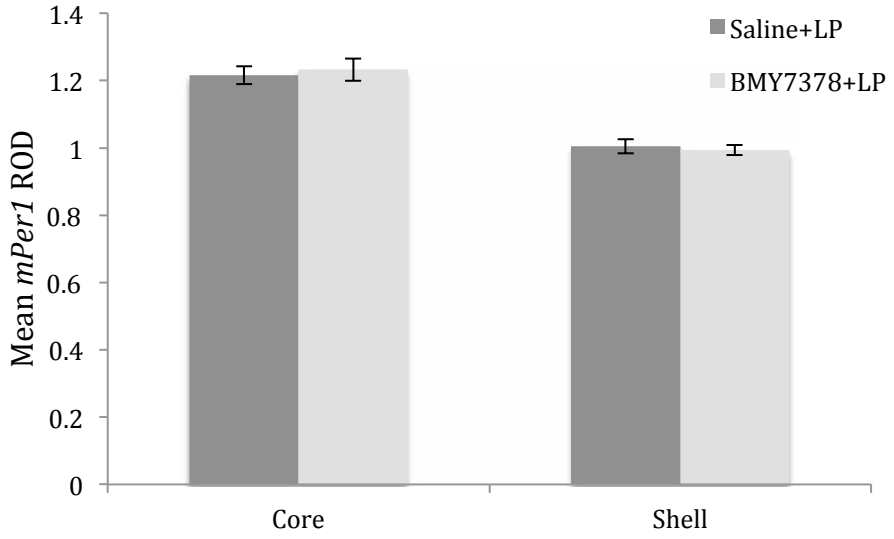

Saline + LP
BMY7378 + LP
A
B
C
D
mPer1
mPer2

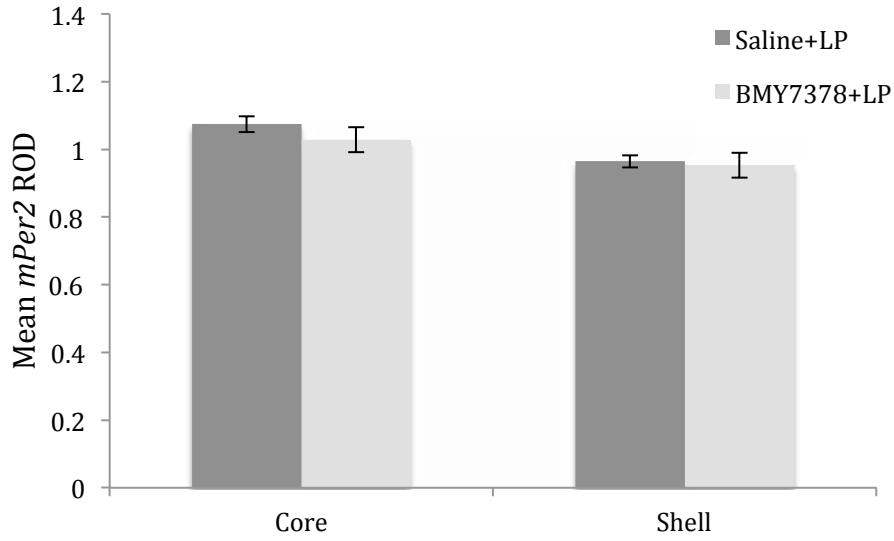
Figure 3.4 Mean ROD of *mPer1* and *mPer2* in the SCN of mice.

Mean relative optical density (ROD) of (A) *mPer1* and (B) *mPer2* in the core and shell regions of the SCN of animals following a 15-minute 40 lux light pulse in the late subjective night pretreated with either vehicle control (saline) or BMY7378.

A


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mean mPer1 ROD
Saline+LP
BMY7378+LP
Core
Shell

B


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mean mPer2 ROD
Saline+LP
BMY7378+LP
Core
Shell

3.4 Discussion

This was the first known study to examine circadian responses to the 5-HT_{1A} mixed agonist/antagonist BMY7378 in mice. The first objective of this experiment was to reproduce, in mice, the phase shift potentiation effects of BMY7378 that have been documented in hamsters (Byku & Gannon, 2000; Gannon, 2003), as well as the effects observed previously with NAN-190 in both hamsters and mice (Rea *et al.*, 1995; Smith *et al.*, 2010). Contrary to what was observed previously in hamsters following BMY7378 pretreatment, no significant potentiation of photic phase advances was observed in mice. Also unlike what has previously been observed in hamsters, BMY7378 had no significant effect on circadian phase without subsequent light exposure in the late subjective night. Not surprising, considering the lack of effect on behavioural phase shifts, there was also no significant effect of BMY7378 pretreatment on the amount of *mPer1* or *mPer2* mRNA expression induced by light in the late subjective night.

As expected, based on work by Yan and Silver (2002), *mPer1* gene expression was highly induced in the core region of the SCN following light exposure in the late subjective night. Also, the negligible amount of *mPer2* expression we observed throughout the SCN following late night light exposure coincides with previous work demonstrating essentially no *mPer2* expression 90 min following exposure to light (Yan & Silver, 2002). However, the extent of the expression of *mPer1* and *mPer2* did not appear to be altered in either the core or shell regions of the SCN by BMY7378 pretreatment. Our present findings parallel those we found previously with NAN-190 in mice, such that NAN-190 also had no effect on the expression of either *mPer1* or *mPer2*

(Smith *et al.*, 2010). However, NAN-190 was able to significantly potentiate photic phase shifts in the late subjective night, whereas BMY7378 did not have this same effect in mice despite previously being shown to potentiate photic phase shifts in hamsters (Gannon, 2003; Smith *et al.*, 2010).

The 5-HT_{1A} receptor agonist MKC-242, which is also able to potentiate photic phase shifts in both mice and hamsters (Moriya *et al.*, 1998; Takahashi *et al.*, 2002), was shown to have no significant effect on either *mPer1* or *mPer2* when examined 1 hour following light exposure in the early subjective night (Takahashi *et al.*, 2002). However, MKC-242 was found to increase the expression of both *mPer1* and *mPer2* 3 hours following early subjective night light exposure, thus prolonging the normal light-induced *Period* gene expression in the mouse (Takahashi *et al.*, 2002). Therefore, it appears that BMY7378 administration in mice does not work via the same mechanism as MKC-242, in terms of either behavioural phase shifts or *Period* gene expression. However, BMY7378 does potentiate photic phase shifts in hamsters, and thus it is possible that in this species the mechanism by which BMY7378 produces its effect could be similar to that of MKC-242. Future studies would need to examine the time-course of light-induced *Period* gene expression following BMY7378 pretreatment in hamsters.

By understanding the precise pharmacology responsible for the modulation of the circadian system's sensitivity to light stimuli which is observed in hamsters with the class of drugs termed mixed agonists/antagonists, including BMY7378, it may be possible to eventually develop chronobiotics specifically targeted for the rapid resynchronization of circadian rhythms. Effectively managing or even preventing the chronic

desynchronization between the circadian system and the external environment, such as that observed with shift work, jet lag, or circadian rhythm disorders, could play a large role in the elimination of the adverse health effects associated with this sort of desynchrony. Thus, it is of great importance in today's 24-h society to more fully understand the pharmacology behind drugs such as BMY7378.

4. Molecular underpinnings of photic phase shift potentiation with BMY7378

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Author Contributions:

Victoria Smith:
- Experimental design
- Laboratory Research: Predictions for manipulation times, light pulse manipulations, injections, perfusions, tissue collection, immunohistochemistry, microscopy, cell counting and ROD analysis
- Manuscript Preparation: Entered and organized data; ran statistical analyses, produced figures, composed manuscript

Ryan Jeffers:
- Laboratory Research: assisted with portions of the cFos and JunB immunohistochemistry and tissue mounting

Dr. Michael Antle:
- Assisted with experimental design
- Provided laboratory, equipment and financial support necessary to carry out experiments
- Manuscript Preparation: provided feedback and editing of the manuscript

Abstract

The 5-HT_{1A} mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378) has been shown to greatly potentiate photic phase shifts in hamsters. The underlying mechanism and intracellular changes in the suprachiasmatic nucleus (SCN) by which this potentiation is accomplished has yet to be fully determined. Here we examine the effect of BMY7378 on numerous proteins expressed in the SCN following light exposure in the late subjective night (CT18). BMY7378 administration increased the amount of several photoinducible genes in the SCN at specific time points following light exposure in the late subjective night. The amount of phosphorylated ERK was shown to be increased 60 minutes following light exposure, while the amount of cFos was increased at 180 minutes, indicating an extended action of these two light-induced proteins in the SCN following BMY7378 pretreatment. The amount of CREM at 240 minutes, JunB at 6 hours, and PER1 at 6 hours were also found to increase with BMY7378 administration prior to light exposure in the late subjective night. However, P-ERK expression up to 45 minutes after light exposure, as well as P-ERK and P-CREB at 120 minutes were all unaffected by BMY7378. Also unaffected by BMY7378 treatment were cFos expression up to 120 minutes and JunB up to 180 minutes following light exposure. These findings suggest that BMY7378 may potentiate photic phase shifts at least partly by upregulating the activity of some, but not all, genes and biochemical pathways involved in coupling the light signal to the output of the circadian clock, particularly those which are active many hours after the light signal reaches the SCN.

Key Words: Circadian, Suprachiasmatic Nucleus, 5-HT, BMY7378, cFos, JunB, PER1, P-ERK, P-CREB, CREM

4.1 Introduction

Daily oscillations in physiology and behaviour are evident throughout phylogeny, from prokaryotes to mammals (Sharma, 2003). In mammals, such endogenously generated circadian oscillations are controlled by the circadian clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972). The circadian system is entrained to the daily environmental light/dark cycle by photic input relayed from the intrinsically photosensitive retinal ganglion cells in the retina, along the retinohypothalamic tract (RHT) to the retinorecipient cells of the SCN (Daan & Pittendrigh, 1976; Rusak & Zucker, 1979; Berson, 2003; Hirota & Fukada, 2004). This signal then alters the expression of circadian rhythms in the endogenously rhythmic region of the SCN (Berson, 2003; Hirota & Fukada, 2004).

Along with inducing shifts in the behavioural activity rhythms, aptly timed light exposure in the subjective night results in the expression of numerous genes in the cells located in the retinorecipient cells in the core region of the SCN, including immediate-early genes (IEGs) such as *c-fos* and *JunB*, along with the clock genes *Per1* and *Per2* (Kornhauser *et al.*, 1996a; Guido *et al.*, 1999a; Guido *et al.*, 1999b; Edelstein *et al.*, 2003; Hamada *et al.*, 2004). Although it is thought that the light-induced expression of IEGs could be an early event in the response cascade which ultimately leads to photic phase alterations of the circadian pacemaker (Rea, 1992), the induction of the IEG *c-fos* is not sufficient, or even required to induce phase shifts in the circadian activity rhythm (Honrado *et al.*, 1996).

Second-messenger signaling pathways, including the pathway leading to the phosphorylation of the extracellular signal-regulated kinase (ERK), serve to couple incoming photic input to the transcription of IEGs and ultimately the *Period* genes (Sanada *et al.*, 2002; Butcher *et al.*, 2003). Similarly, the phosphorylated form of cAMP-response-element-binding protein (CREB) is also activated immediately following light exposure. CREB subsequently binds to the cAMP response element (CRE) of the promoter region of several genes, including *c-fos*, *Per1*, and *Per2*, inducing their transcription (Dunlap *et al.*, 2004; Antle & Silver, 2005). These cascades are activated extremely rapidly by light, before an increase in IEG mRNA is even observed (Dziema *et al.*, 2003).

The phase shifting effects of light are consistently accompanied by the colocalized expression of the phosphorylated forms of ERK (P-ERK) and CREB (P-CREB; von Gall *et al.*, 1998; Dziema *et al.*, 2003). Another type of CRE-binding proteins, encoded by the cAMP response element modulator (CREM) gene, have been shown to act as either activators or repressors of genes with CRE promotor regions when they dimerize with each other or with the Fos and Jun families of IEGs (Lalli *et al.*, 1996; Sassone-Corsi, 1998; De Cesare *et al.*, 1999). The inducible cAMP early repressor (ICER), a truncated product transcribed along with the C-terminal segment of CREM, functions as a powerful repressor of cAMP-induced transcription and has been shown to increase in the rat SCN following a phase-shifting light pulse (Molina *et al.*, 1993; Lalli *et al.*, 1996; Lamas *et al.*, 1996; Schwartz *et al.*, 2005).

Serotonin (5-HT) has been shown to modulate the effects of light by dampening the excitatory signals sent to the cells of the SCN as a result of light exposure (Meyer-Bernstein & Morin, 1996; Mistlberger, 2000). A class of serotonergic compounds, termed mixed agonists/antagonists due to their presumed actions as antagonists at presynaptic axoaxonic heteroreceptors as well as agonists at postsynaptic somatodendritic autoreceptors, are known to modulate the effects of light on the circadian system (Gannon, 2003). When drugs of this class, including BMY7378, are administered during the early or late subjective night either before or after phase shifting light exposure, they are known to greatly potentiate photic phase shifts in the hamster, inducing shifts in the activity rhythm of up to 8h in magnitude (Rea *et al.*, 1995; Gannon, 2003; Kessler *et al.*, 2008; Lungwitz & Gannon, 2009; Smith *et al.*, 2010). The potentiation effects produced by this class of drug was originally presumed to be the result of the combination of an agonistic action at the median raphe to prevent serotonin release onto the SCN, along with an antagonistic action at the RHT terminals or SCN cells to block serotonergic inhibition at these locations. This decreased inhibition to the SCN is suspected to lead to increased excitation following photic input, thus allowing an overall potentiated phase shifting effect.

As with shifts in the behavioural output of circadian rhythms, the serotonergic system has also been shown to modulate the effects of photic input on the light-induced gene expression in the cells of the SCN. Both agonists and antagonists of the serotonergic system are known to alter circadian activity rhythms and also modulate the light-induced gene expression in the SCN (Glass *et al.*, 1994; Amir *et al.*, 1998; Takahashi *et al.*,

2002). Serotonergic agonists which attenuate light-induced phase shifts can also attenuate the subsequent cFos protein expression observed in the SCN (Amir *et al.*, 1998). Conversely, the selective 5-HT_{1A} receptor agonist MKC-242, which has the ability to potentiate photic phase advances and delays, has been found to concomitantly prolong the light-induced expression of *mPer1* and *mPer2* in the mouse SCN during the late subjective night (Moriya *et al.*, 1998; Takahashi *et al.*, 2002). When exogenously applied, the serotonergic mixed agonist/antagonist NAN-190, which has also been shown to potentiate photic phase shifts, leads to an attenuation of the cFos and P-CREB expression normally induced by a light pulse (Recio *et al.*, 1996; Smith *et al.*, 2010).

The precise molecular underpinnings of the photic phase shift potentiation observed with serotonergic mixed agonists/antagonists such as NAN-190 and BMY7378, both in terms of gene expression and biochemical pathways leading to that gene expression, are still largely unknown. Thus the present study sought to determine the molecular underpinnings of the potentiating effects of BMY7378 on light-induced phase shifts in hamsters. Both the regionally-specific expression and time course of several different proteins and genes involved in the transduction of the light signal into the circadian clock, were examined. The proteins and genes of specific interest included: the phosphorylated forms of the proteins ERK and CREB, which are involved in the biochemical cascades that regulate changes in clock gene expression; CREM, which could act as an activator or repressor of genes with CRE promotor regions; cFos and JunB, the protein product of the immediate-early genes *c-fos* and *JunB*; and PER1, the protein product of the clock gene *Period1* which is a core component of the

transcription/translation feedback loop at the heart of the circadian clock. In general, it is hypothesized that all genes and proteins will be expressed to a greater degree in the ventrolateral core region of the SCN compared to the dorsomedial shell. However, more specifically, it was hypothesized that the activation of several biochemical cascade components will be prolonged or delayed, allowing for an increased response to photic stimuli. It was also expected that the number of cells expressing the AP1 complex components cFos and JunB following BMY7378 pretreatment would be decreased, as previously described (Recio *et al.*, 1996; Smith *et al.*, 2010). Conversely, it was hypothesized that pretreatment with BMY7378 will augment the expression of IEGs and PER1 6 hours following the exposure to light, corresponding to BMY7378's ability to potentiate photic phase shifts when administered up to 6 hours after light exposure (Kessler *et al.*, 2008; Lungwitz & Gannon, 2009).

4.2 Experimental procedures

4.2.1 Animals and housing

A total of 94 male Syrian hamsters, obtained from Charles River Laboratories (Kingston, NY, USA), were initially housed in groups of two or three and maintained under a 14:10 light/dark cycle (lights on at 7:00 h and off at 21:00 h). Cage level luminance was approximately 300 lux. Animals were housed in a temperature- (21 ± 1°C) and humidity-controlled room, with *ad libitum* access to food and water. Following an initial week of habituation to the new environment following arrival, hamsters were transferred to individual clear polycarbonate cages (45 × 20 × 22 cm) equipped with a running wheel (diameter of 14 cm), and maintained in constant darkness (DD) for the

duration of the study. All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary, and adhered to the Canadian Council on Animal Care guidelines for the ethical use of animals.

4.2.2 Drugs and reagents

The 5-HT_{1A} receptor mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378) was obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON). BMY7378 was dissolved in physiological saline. BMY7378 (5 mg/kg) was injected systemically, with the dose being based upon the dose which produced the largest effect in previous work performed by Gannon (2003). Vehicle control injections (physiological saline) were based on a comparable volume by weight. All injections were performed in constant darkness with the aid of night-vision goggles (BG15Alista, Richmond Hill, ON).

4.2.3 Activity rhythms

Wheel-running activity was continuously monitored using magnetic switches mounted on the running wheel, and connected to a computer running the Clocklab data collection software package (Coulbourn Instruments, Allentown, PA, USA). Actograms (graphical representation of wheel-running behavior) were generated and analyzed by Clocklab analysis software. Hamsters were allowed to free-run in constant darkness (DD) for a minimum of 7 days prior to the start of manipulations. Onset of running wheel activity for manipulation day was predicted using a regression line fit to the activity onsets for the 7-10 days prior to the testing day.

4.2.4 Experiment 1 – Effects of BMY7378 on biochemical cascade components

This experiment examined the effects of BMY7378, administered systemically with a brief exposure to dim light, on the phosphorylation of the biochemical cascade components ERK and CREB, as well as the expression of the CREM protein within the SCN. Animals were housed in DD (0 lux) for the duration of this experiment. The dim light pulse was administered using a light-sealed box with a light bulb partially covered in aluminum foil, such that the light intensity at cage level was measured to be 40 lux using a light meter. In experiment 1a, hamsters (n = 24) were exposed to a pretreatment of an intraperitoneal (i.p.) injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse in the late subjective night, 6 hours after their predicted activity onset (i.e., CT 18, where activity onset is designated to be CT12 by convention). Hamsters were then euthanized at 4 different durations following the beginning of the light pulse (15 minutes, 30 minutes, 45 minutes, 60 minutes), and SCN issue was stained for P-ERK. In experiment 1b, hamsters (n = 6) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse at CT18. Hamsters were then euthanized 120 minutes following the start of the light pulse, and SCN tissue was stained for P-ERK and P-CREB. In experiment 1c, hamsters (n = 6) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse at CT18, and one hamster was not exposed to either an injection or a light pulse. Hamsters were then euthanized 240 minutes following CT18, and SCN tissue was stained for CREM.

4.2.5 Experiment 2 – Effect of BMY7378 on immediate-early gene and period protein expression

This experiment examined the effects of BMY7378, administered systemically with a brief exposure to dim light, on the expression of the protein forms of the immediate-early genes *cFos* and *JunB*, as well as the protein form of the *Period1* gene within the SCN. Animals were housed in DD (0 lux) for the duration of this experiment. The dim light pulse was administered as previously described. In experiment 2a, hamsters (n = 37) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse in the late subjective night, 6 hours after their predicted activity onset (i.e., CT 18, where activity onset is designated to be CT12 by convention). Hamsters were then euthanized at 4 different durations following the beginning of the light pulse (60 minutes, 90 minutes, 120 minutes, 180 minutes), with SCN tissue being stained for cFos and JunB. In experiment 2b, hamsters (n = 21) were exposed to a pretreatment of an i.p. injection of either BMY7378 or vehicle control, followed 45 minutes later by a 15-minute light pulse at CT18. Hamsters were then euthanized 6 hours following the start of the light pulse, with SCN tissue being stained for JunB or PER1.

4.2.6 Perfusion and immunohistochemistry

All animals were euthanized with an overdose of sodium pentobarbital (Euthanyl; 240mg/mL, 0.7mL per hamster; dose: ~840mg/kg), the head was wrapped in aluminum foil to prevent the retinas from being exposed to light prior to and during the perfusion, and animals were then transcardially perfused using approximately 50 mL of phosphate

buffered saline (PBS), followed by 50 mL of buffered 4% paraformaldehyde. Brains were extracted and postfixed in 4% paraformaldehyde overnight, and subsequently cryoprotected in a 20% sucrose solution for 24 hours. Brains were sliced through the anterior hypothalamus using a cryostat, with alternate 35 μm sections through the SCN being collected for immunohistochemistry (IHC).

4.2.6.1 Diaminobenzidine (DAB)

Diaminobenzidine IHC was completed using a previously described method from our lab (see Antle *et al.*, 2008). Brain sections were exposed to a series of treatments at room temperature on a shaker tray unless otherwise indicated. Briefly, sections were rinsed for 30min in 0.5% H_2O_2 in PBS with 0.3% Triton X-1900 (PBSx) to inactivate endogenous peroxidase, followed by 3 10-minute PBSx rinses, a 90-minute incubation in 1% normal goat serum in PBSx (P-ERK, P-CREB) or 10% normal goat serum in PBSx (CREM-1, mPER1), and a 2-day incubation at 4°C in the primary antibody for P-ERK (rabbit anti-Phospho-p44/42 MAP Kinase (Thr202/Tyr204); 1:600; Cell Signaling Technology, Inc., Danvers, MA), P-CREB (rabbit anti-Phospho CREB; 1:250; Cell Signaling Technology, Inc., Danvers, MA), CREM-1 (rabbit anti-CREM-1; 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), or PER1 (rabbit anti-mPER1: 1:10,000; EMD Millipore, Billerica, MA). Subsequently, sections were exposed to 3 10-minute PBSx rinses, a 1h incubation in the secondary antibody biotinylated goat anti-rabbit (1:200; Vector Laboratories, Burlingame, CA), 3-10min PBSx rinses, a 1-hour incubation in ABC (Vectastain Elite kit, Vector Laboratories, Burlingame, CA), 3 10-minute PBSx rinses. Slices were then developed for approximately 5min in 0.05% DAB and 0.02%

NiCl in 25mL of 0.1M Tris buffer activated with 80μL of 30% H_2O_2. Finally, slices were mounted on gelatin-coated slides, air-dried, dehydrated through an alcohol series, cleared with xylenes, and coverslipped with Permount.

4.2.6.2 Fluorescent triple-label

Sections were triple-labeled for cFos (goat anti-Fos; 1:20,000; Santa Cruz Biotechnology, Santa Cruz, CA), vasopressin (VP; guinea-pig anti-VP; 1:5,000; Peninsula Laboratories, San Carlos, CA), and Calbindin (CalB; mouse anti-CalB; 1:20,000; Sigma) or cFos, VP, and JunB (mouse anti-JunB; 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA). IHC staining was completed using a previously described method from our lab (see Sterniczuk *et al.*, 2008). Briefly, brain sections were exposed to three 10-minute washes in 0.1M PBS, followed by a 1-hour incubation in a blocking buffer (2% normal donkey serum, in PBSx), after which the tissue was incubated for 48 hours at 4°C in the primary antibodies diluted in blocking buffer. Tissue was then washed using six 10-minute rinses in 0.1% PBSx, and left overnight in 0.1% PBSx. Finally, the brain sections were washed with another six 10-minute 0.1% PBSx rinses, incubated for 1 hour in the following secondary antibodies: CY-2 donkey anti-mouse, CY-3 donkey anti-rabbit, and CY-5 donkey anti-guinea pig (each at 1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and exposed to three final 10-minute washes in 0.1M PBS. Sections were then mounted on gelatin-coated slides, air dried, dehydrated in a series of alcohol rinses, cleared with xylenes, and coverslipped with Krystalon.

4.2.7 Quantification of protein expression

Images of the immunofluorescently-labeled tissue were captured via an Olympus BX51 microscope equipped with filters for each of the three cyanine dyes and a monochrome QICam Fast 1394 digital video camera (QImaging, Burnaby, BC). Bilateral counts of cFos-immunoreactive (cFos-IR) or JunB-IR cells were performed using computerized image analysis software (ImagePro Plus 5.1.2.59; Media Cybernetics, Inc., Bethesda, MD or ImageJ 1.42q; National Institutes of Health, Bethesda, MD), and were manually counted by the researcher who was blind to the treatment conditions. Cell counting was completed at three levels of the SCN centered around the mid-caudal level, identified by the presence of the GRP subnucleus. The sections 140μm anterior and posterior to the mid-caudal level were included in the analysis. At the mid-caudal level, separate cell counts were obtained for two regions of the SCN: shell (delineated by VP-IR) and core (delineated by GRP-IR). Images of the diaminobenzidine-labeled tissue were captured using the bright field setting of the same microscope. Bilateral counts of CREM- and PER1-labeled cells were performed using the same techniques as described above, at three rostral-caudal levels of the SCN, whereas bilateral counts of P-CREB and bilateral calculations of the relative optical density (ROD) of P-ERK expression were performed at four levels of the SCN (with sections 140μm and 280μm anterior, and 140μm posterior to the mid-caudal level being included in the analysis), using ImageJ on images converted to greyscale. The grey level of the SCN was expressed as a ratio over the grey level of adjacent non-SCN tissue in the same frame. At the mid-caudal level, cell counts and ROD were calculated for two SCN regions (core, shell) being delineated

based on the general shape of each region identified previously while performing the fluorescent triple-label cFos cell counts.

4.2.8 Statistical analysis

For experiment 1a, one 2x4 mixed Analysis of Variance (ANOVA) was used to determine if there was a significant difference in the amount of light-induced P-ERK elicited by BMY7378 or saline vehicle at each of four rostral-caudal levels, followed by a 40 lux light pulse, at each of the four time points following the start of the light pulse (15 minutes, 30 minutes, 45 minutes, 60 minutes). For experiment 1b, one 2x4 mixed ANOVA was used, for each of P-ERK and P-CREB, to determine if there was a significant difference in the amount of light-induced phosphorylation elicited by BMY7378 or saline at each of the four rostral-caudal levels, 120 minutes following a light pulse. For experiment 1c, a 2x3 mixed ANOVA was used to determine if there was a significant difference between the amount of CREM elicited by BMY7378 or saline at the three rostral-caudal levels, 240 minutes following a light pulse. A 2x2 mixed ANOVA was also used to determine if there was a significant difference in the amount of CREM elicited by BMY7378 or saline in the core and shell regions of the SCN at the mid-caudal level. For experiment 2a, one 2x3 mixed ANOVA was used to determine if there was a significant difference in the amount of light-induced cFos expression elicited by BMY7378 or saline at each of the four time points following the start of the light exposure (60 minutes, 90 minutes, 120 minutes, 180 minutes). One 2x3 mixed ANOVA was also used for each of the four time points (60 minutes, 90 minutes, 120 minutes, 180 minutes), to determine if there was a significant difference in the amount of light-induced

JunB expression elicited by BMY7378 or saline. A 2x2 mixed ANOVA was also used to determine if there was a significant difference in the amount of cFos elicited by BMY7378 or saline in the core and shell regions of the SCN at the mid-caudal level, and another 2x2 mixed ANOVA was used similarly for JunB expression. For experiment 2b, one 2x3 mixed ANOVA was used, for each of JunB and PER1, to determine if there was a significant difference in the amount of light-induced protein expression was elicited by BMY7378 or saline 6 hours following light exposure. A 2x2 mixed ANOVA was also used to determine if there was a significant difference in the amount of JunB elicited by BMY7378 or saline in the core and shell regions of the SCN at the mid-caudal level, and another 2x2 mixed ANOVA was used similarly for PER1 expression. Statistical significance was set at $P<0.05$ for all tests, using SigmaPlot Version 11.2.0.5 (Systat Software, Inc.; San Jose, CA, USA). All significant ANOVAs were followed up by Studen-Newman-Keuls post hoc multiple comparisons. All means are reported as ± standard error of the mean (SEM) in figures.

4.3 Results

4.3.1 Experiment 1 – Effects of BMY7378 on biochemical cascade components

BMY7378 had no significant effect on the magnitude of P-ERK induced in the SCN 15, 30 or 45 minutes following a late subjective night light pulse, when compared to vehicle control (Main Effect of Treatment @ 15 minutes: $F_{(1,12)} = 0.110$, $p = 0.757$; Figures 4.1 and 4.3A; Main Effect of Treatment @ 30 minutes: $F_{(1,12)} = 1.014$, $p = 0.371$; Figure 4.2 and 4.3B; Main Effect of Treatment @ 45 minutes: $F_{(1,12)} = 0.00161$, $p = 0.970$; Figure 4.4 and 4.6A). BMY7378 did however induce a trend towards significantly

increased amounts of P-ERK at some levels of the SCN 60 minutes following a late subjective night light pulse (Treatment x Level Interaction: $F_{(3,12)} = 3.448$, $p = 0.052$; Figure 4.5 and 4.6B). Specifically, BMY7378 led to increased P-ERK expression at the Mid-Caudal ($p < 0.05$) and Caudal ($p < 0.05$) levels of the SCN, with a trend towards an increase at the Rostral-Mid ($p = 0.067$) level of the SCN as well.

There was no effect of BMY7378 pretreatment on either P-ERK (Main Effect of Treatment: $F_{(1,12)} = 0.0335$, $p = 0.864$; Figure 4.7 and 4.9A) or P-CREB (Main Effect of Treatment: $F_{(1,12)} = 1.059$, $p = 0.362$; Figure 4.8 and 4.9B) when they were examined 120 minutes following light exposure in the late subjective night. There was also no effect of treatment on P-ERK expression when examined at the mid-caudal level across the five time points following light exposure (Treatment x Time after LP Interaction: $F_{(3,16)} = 0.652$, $p = 0.593$; Figure 4.10). As expected, significantly different amounts of P-ERK were observed across the four levels of the SCN at 15, 45, 60, and 120 minutes following the light pulse (Main Effect of Level @ 15 minutes: $F_{(3,12)} = 9.087$, $p < 0.01$; Main Effect of Level @ 45 minutes: $F_{(3,12)} = 4.039$, $p < 0.05$; Main Effect of Level @ 60 minutes: $F_{(3,12)} = 5.114$, $p < 0.05$; Main Effect of Level @ 120 minutes: $F_{(3,12)} = 3.509$, $p < 0.05$), with higher levels being generally observed at the more mid-levels of the SCN when compared to the Rostral or Caudal levels. Significantly different amounts of P-CREB-IR cells were also observed across the four levels of the SCN at 120 minutes following light exposure in the late subjective night (Main Effect of Level @ 120 minutes: $F_{(3,12)} = 26.868$, $p < 0.001$), also with higher amounts of expression being observed at the more Mid-levels of the SCN as compared to Rostral or Caudal levels.

Although there did appear to be CREM protein expression at CT22 without any exposure to light, the amount of expression did appear to be increased following light exposure at CT18. Pretreatment with BMY7378 was shown to increase the amount of CREM-IR cells observed throughout the SCN following late subjective night light exposure when compared to saline pretreatment (Main Effect of Treatment: $F_{(1,8)} = 8.132$, $p < 0.05$; Figure 4.11 and 4.12). Again, as expected, there was also significantly different amounts of CREM observed across the three levels of the SCN (Main Effect of Level: $F_{(2,8)} = 31.973$, $p < 0.001$), with the Mid-Caudal levels demonstrating higher levels of expression than the Rostral or Caudal levels of the SCN. At the Mid-Caudal level of the SCN, BMY7378 was shown to increase the amount of CREM protein expression observed (Main Effect of Treatment: $F_{(1,8)} = 63.469$, $p < 0.01$). There was also significantly more CREM protein expression observed in the core region of the SCN compare to the shell region (Main Effect of Region: $F_{(1,4)} = 207.482$, $p < 0.001$), as expected.

Figure 4.1 Photomicrographs of P-ERK expression 15-minutes following the start of the light exposure.

Representative SCN tissue slices indicating P-ERK expression at CT18.25, stained using DAB immunohistochemistry, following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

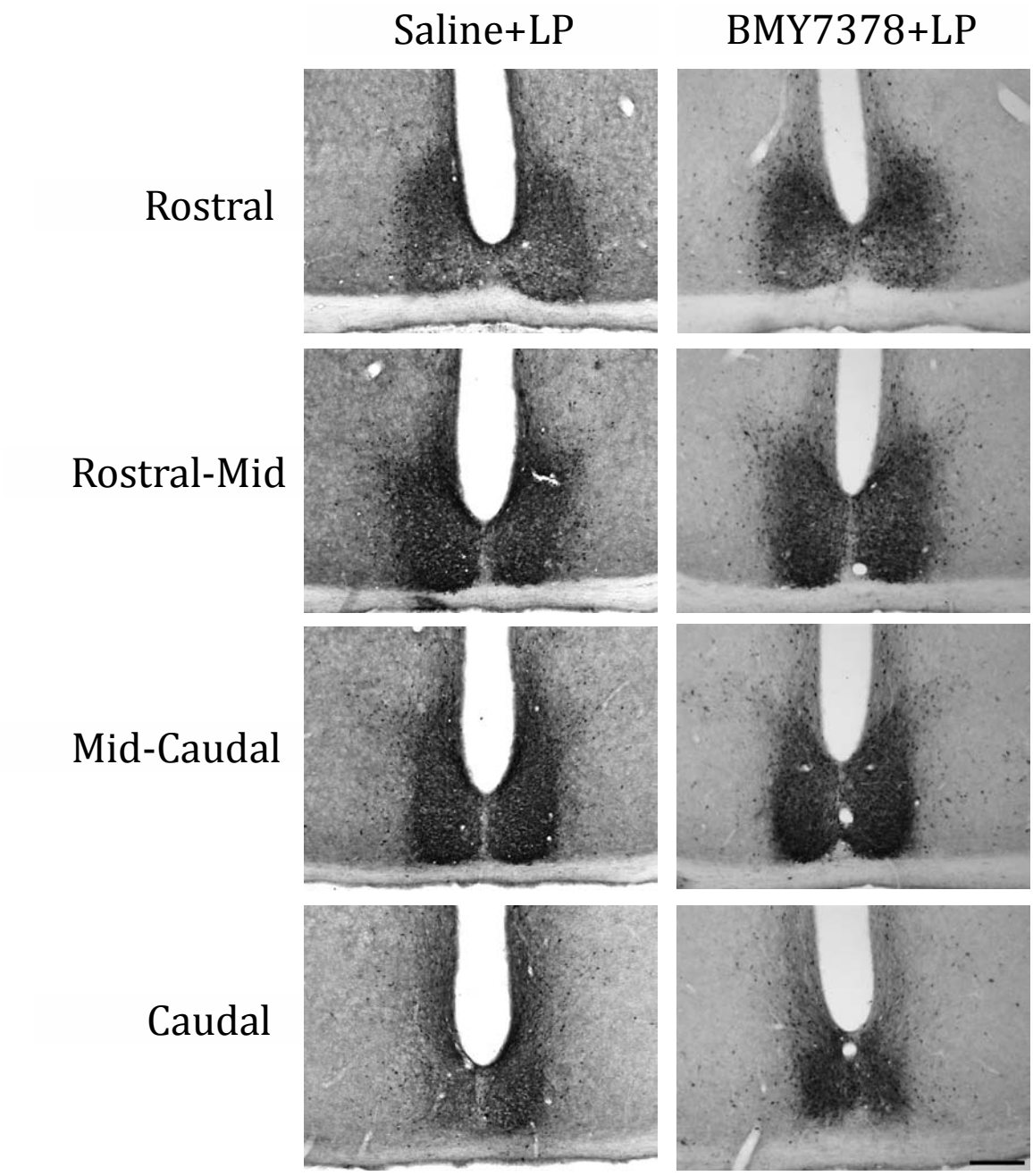

Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.2 Photomicrographs of P-ERK expression 30-minutes following the start of the light exposure.

Representative SCN tissue slices indicating P-ERK expression at CT18. 5 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

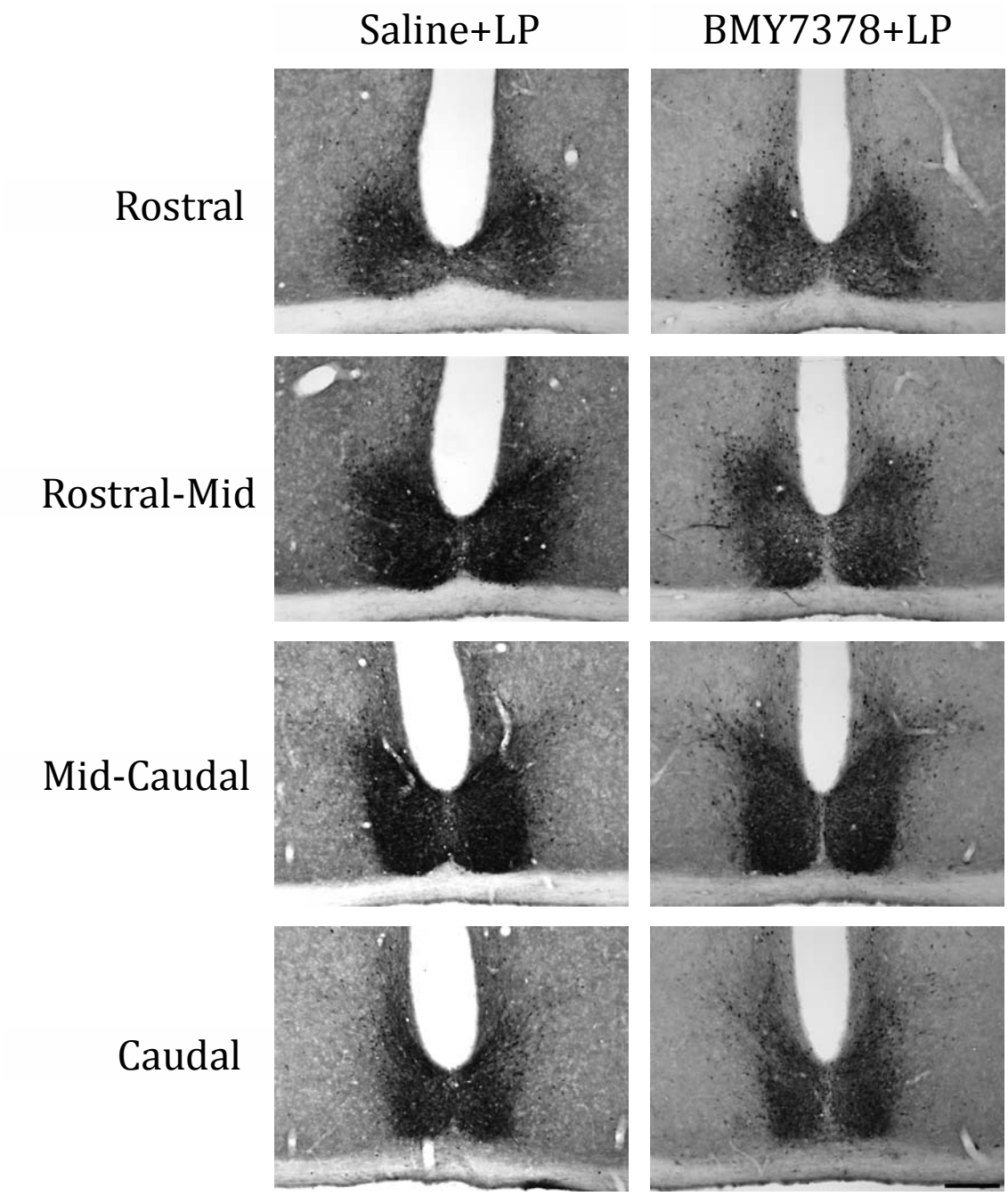

Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.3 Mean P-ERK ROD at 15 and 30 minutes following the start of light exposure.

Mean ROD of P-ERK expression (± SEM) at four rostral-caudal levels of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at (A) CT18.25 or (B) CT18.5.

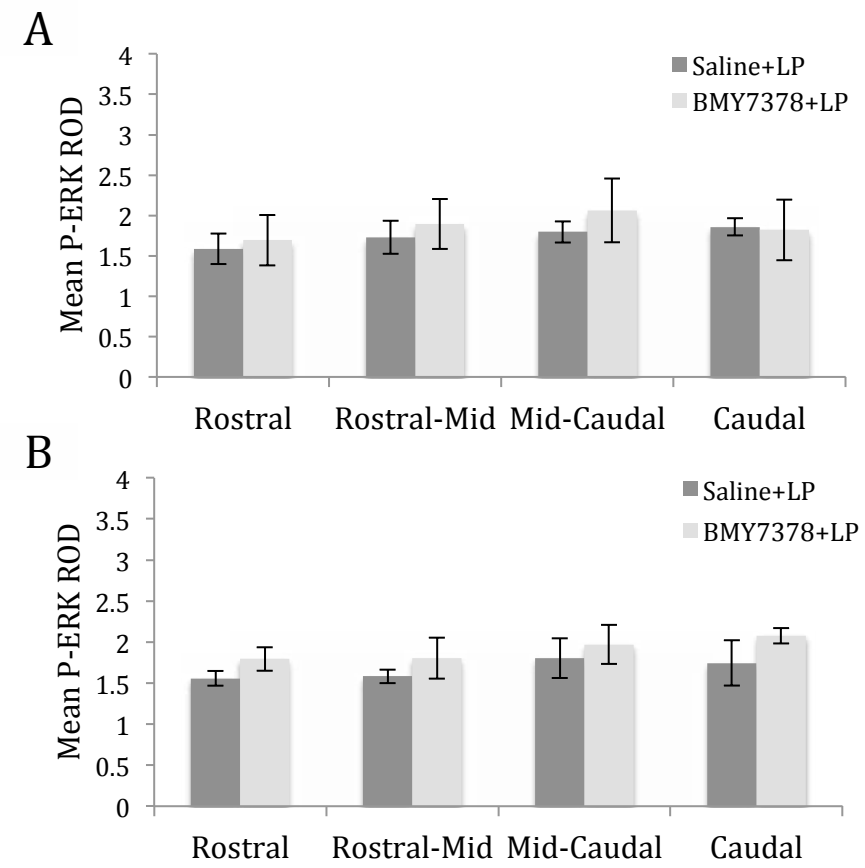

A
Mean P-ERK ROD
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal
B
Mean P-ERK ROD
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.4 Photomicrographs of P-ERK expression 45-minutes following the start of the light exposure.

Representative SCN tissue slices indicating P-ERK expression at CT18.75 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

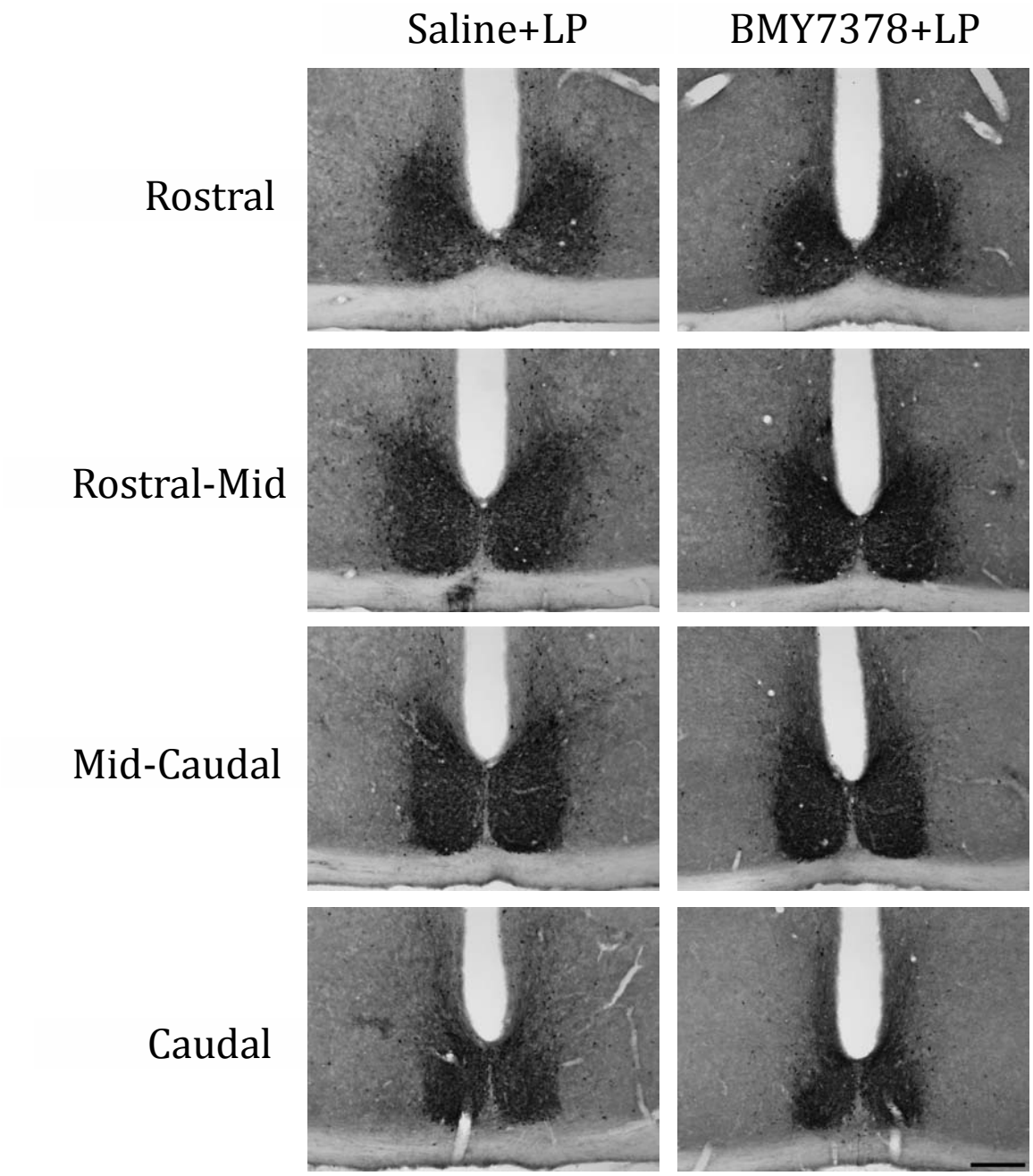

Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.5 Photomicrographs of P-ERK expression 60-minutes following the start of the light exposure.

Representative SCN tissue slices indicating P-ERK expression at CT19 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

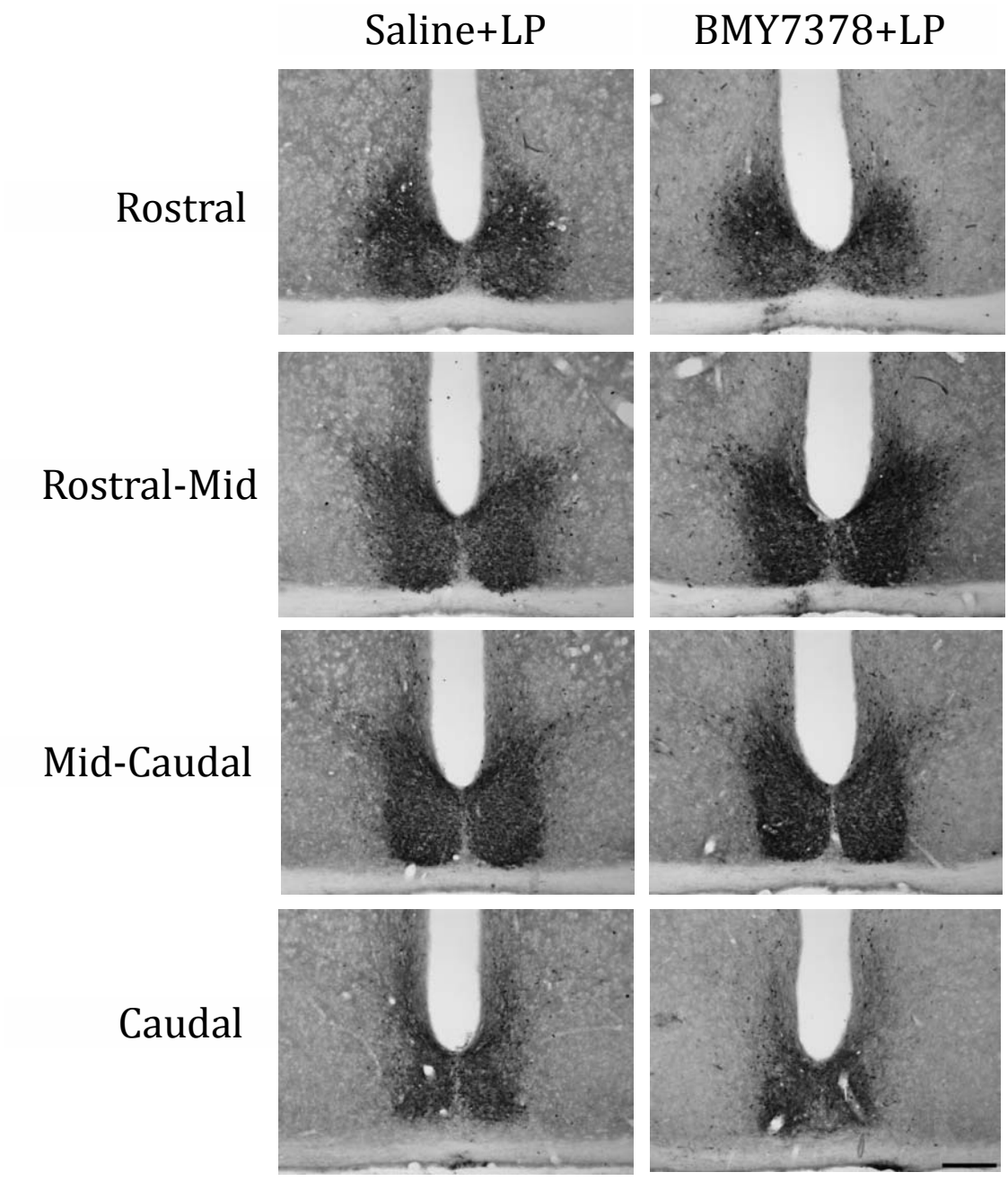

Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.6 Mean P-ERK ROD at 45 and 60 minutes following the start of light exposure.

Mean ROD of P-ERK expression (± SEM) at four rostral-caudal levels of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at (A) CT18.75 or (B) CT19. (*) indicates a statistical significance of $p<0.05$.

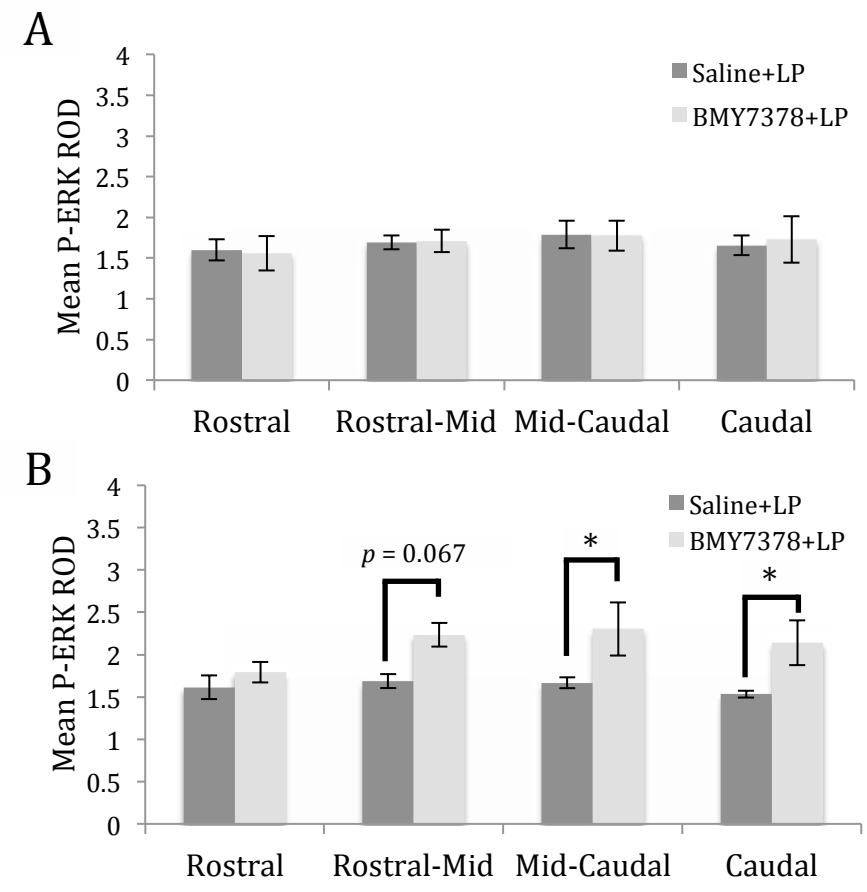

A
Mean P-ERK ROD
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal
B
Mean P-ERK ROD
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline+LP
BMY7378+LP
p = 0.067
*
*
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.7 Photomicrographs of P-ERK expression 120-minutes following the start of the light exposure.

Representative SCN tissue slices indicating P-ERK expression at CT20 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

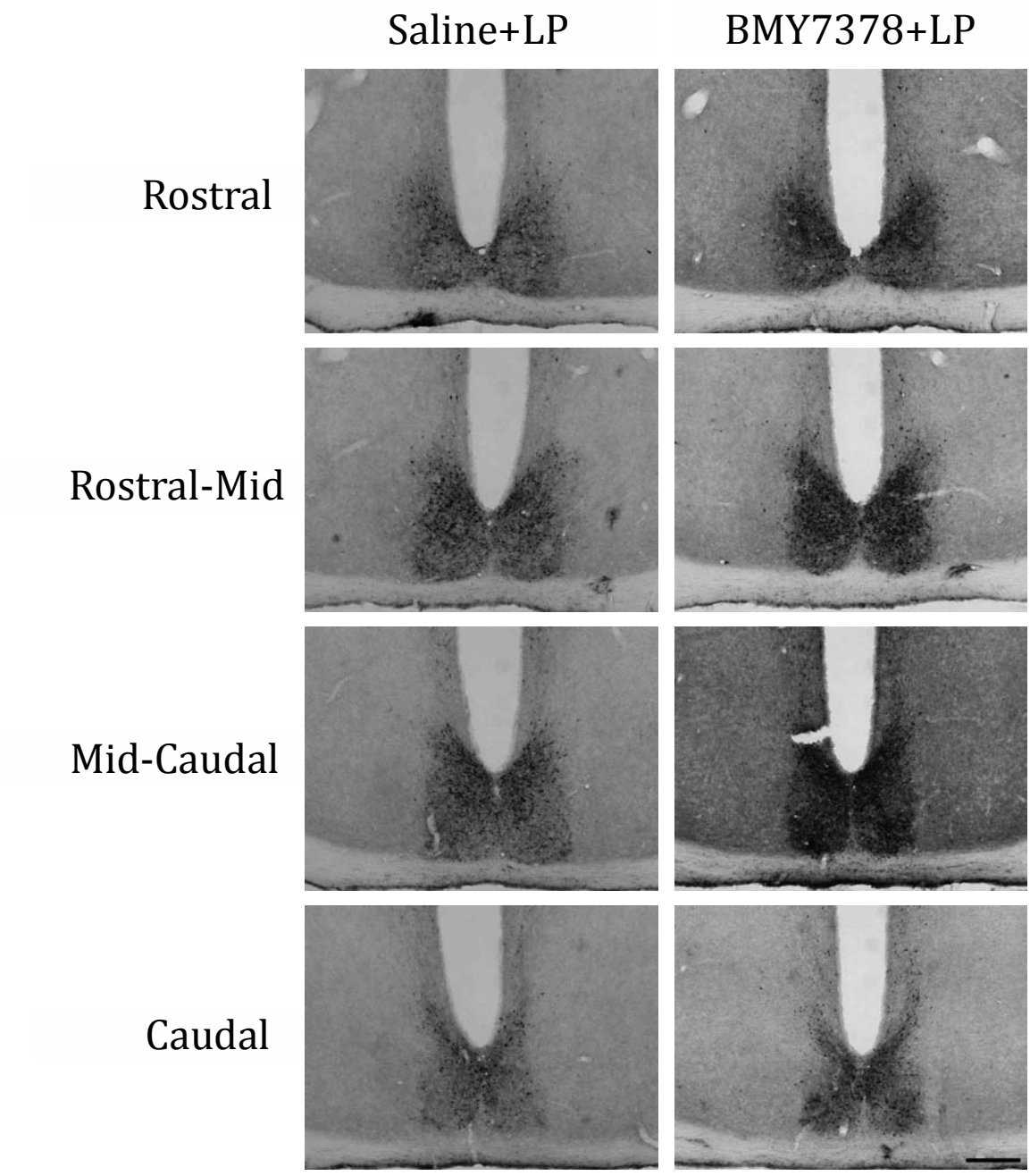

Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.8 Photomicrographs of P-CREB expression 120-minutes following the start of the light exposure.

Representative SCN tissue slices indicating P-CREB-expressing cells at CT20 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

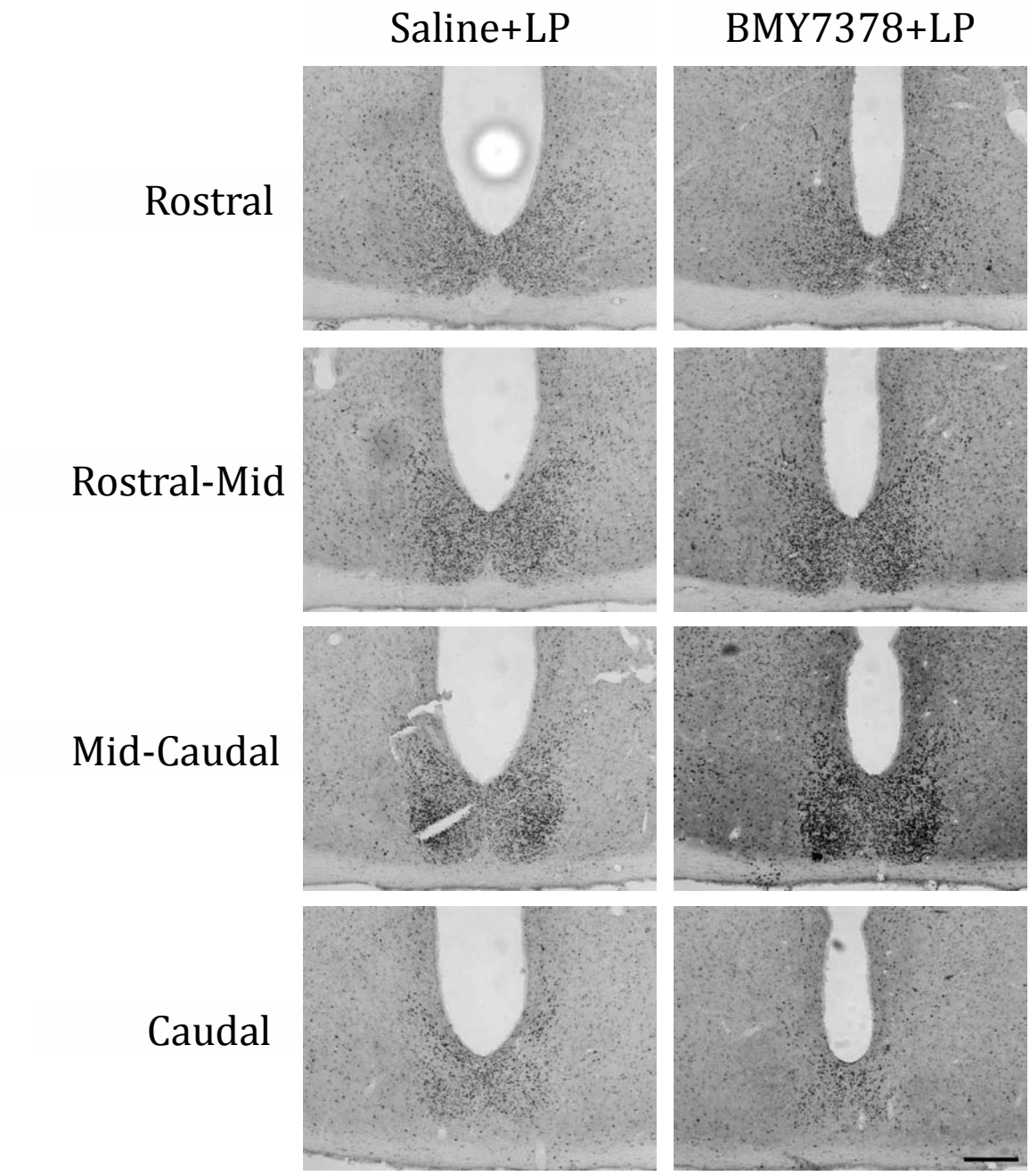

Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.9 Mean P-ERK ROD and mean number of P-CREB–IR cells 120 minutes following the start of the light exposure.

Mean (A) ROD of P-ERK expression (± SEM) or (B) mean number of P-CREB-expressing cells at four rostral-caudal levels of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT20.

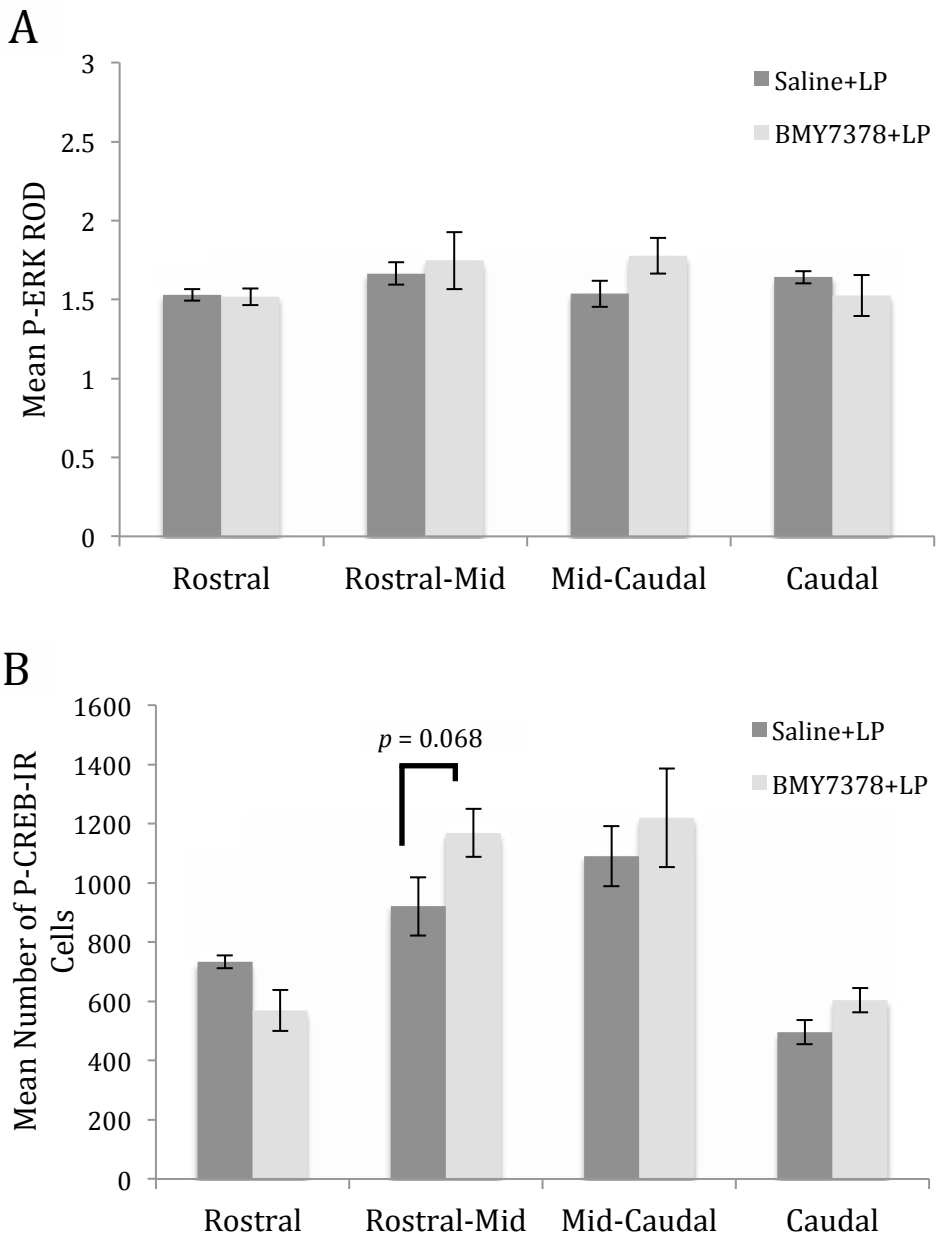

A
Mean P-ERK ROD
3
2.5
2
1.5
1
0.5
0
Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal
B
Mean Number of P-CREB-IR Cells
1600
1400
1200
1000
800
600
400
200
0
p = 0.068
Saline+LP
BMY7378+LP
Rostral
Rostral-Mid
Mid-Caudal
Caudal

Figure 4.10 Mean P-ERK ROD 15, 30, 45, 60 and 120 minutes following the light exposure.

Mean ROD of P-ERK expression (± SEM) in the SCN at five amounts of time following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25.

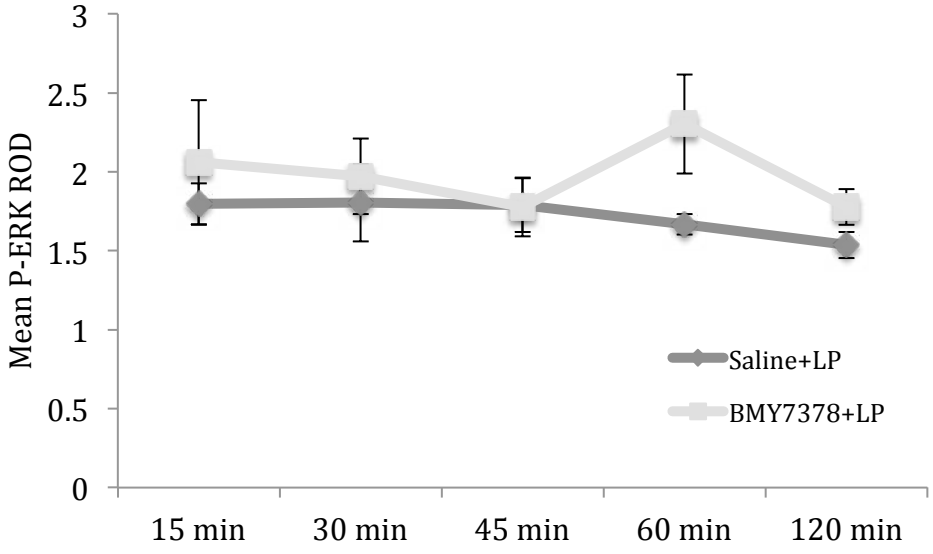

3
2.5
2
1.5
1
0.5
0
Mean P-ERK ROD
15 min
30 min
45 min
60 min
120 min
Saline+LP
BMY7378+LP

Figure 4.11 Photomicrographs of P-CREM expression 240 minutes following the start of the light exposure.

Representative SCN tissue slices indicating CREM-expressing cells at CT22 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. The right-hand column indicates CREM expression at CT22 of a hamster that was not pre-treated with an injection or a light pulse. Scale bar = 200μm.

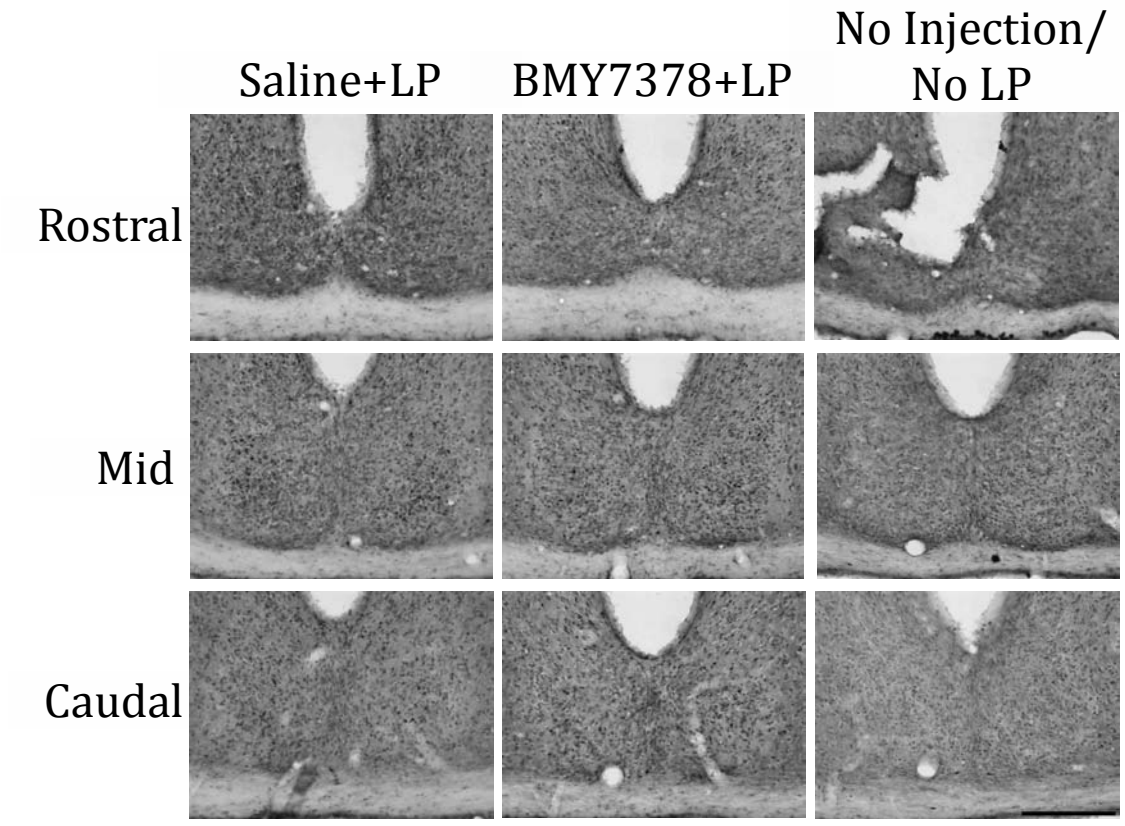
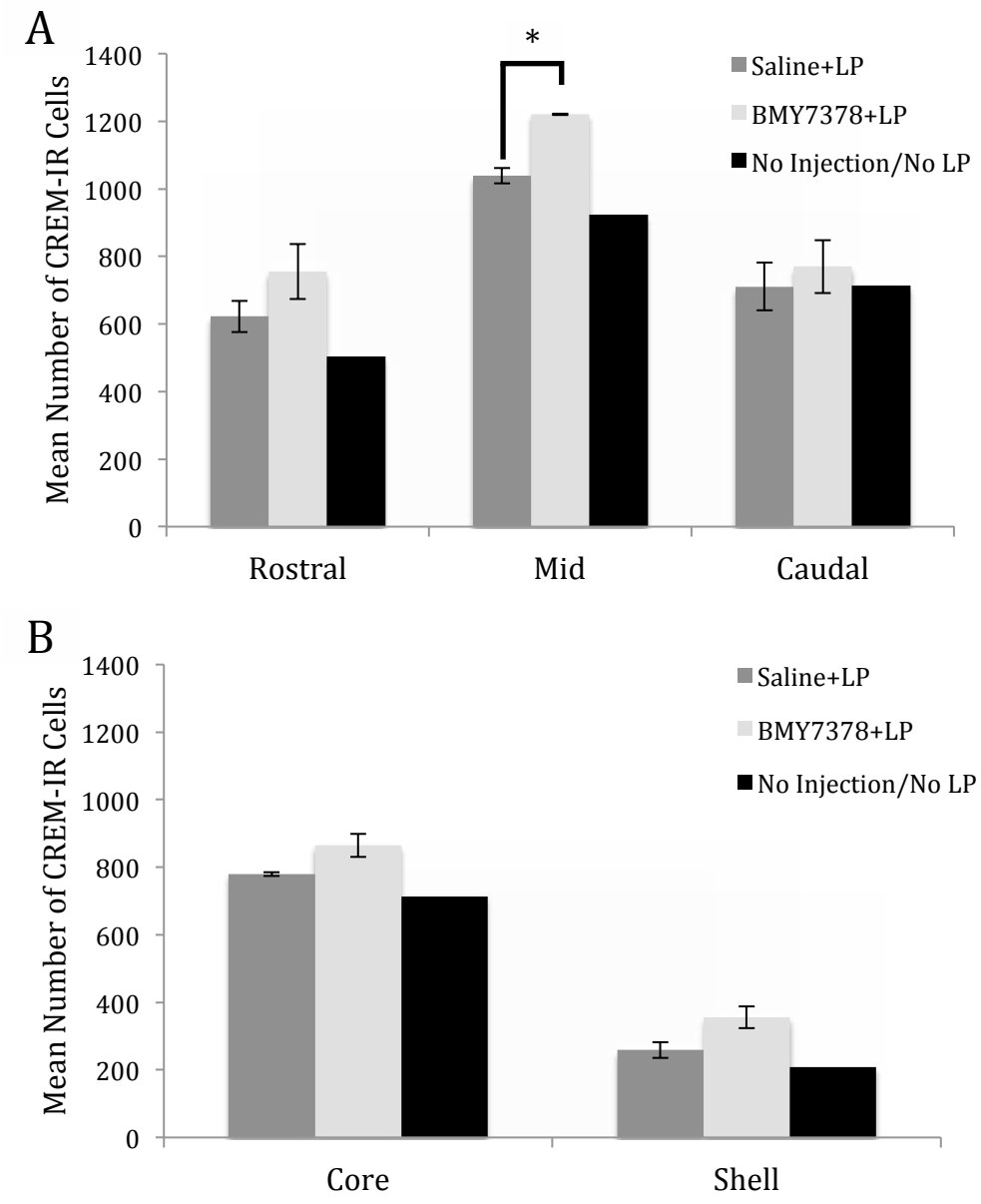

Saline+LP
BMY7378+LP
No Injection/
No LP
Rostral
Mid
Caudal

Figure 4.12 Mean number of P-CREM–IR cells 240 minutes following the start of the light exposure.

Mean number of CREM-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25, or with no injection or light exposure (n = 1). Hamsters were euthanized at CT22. (*) indicates a statistical significance of $p<0.05$.


A
Mean Number of CREM-IR Cells
1400
1200
1000
800
600
400
200
0
*
Saline+LP
BMY7378+LP
No Injection/No LP
Rostral
Mid
Caudal
B
Mean Number of CREM-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
No Injection/No LP
Core
Shell

4.3.2 Experiment 2 – Effect of BMY7378 on immediate-early gene and period protein expression

BMY7378 was shown to have no significant effect on the amount of light-induced cFos expression observed in the SCN 60 or 120 minutes following late subjective night light exposure (Main Effect of Treatment @ 60 minutes: $F_{(1,10)} = 0.960$, $p = 0.372$; Figure 4.13 and 4.14A; Main Effect of Treatment @ 120 minutes: $F_{(1,10)} = 0.833$, $p = 0.413$; Figure 4.15 and 4.16A). However, BMY7378 did induce a trend towards lower cFos expression 90 minutes following the late night light exposure (Main Effect of Treatment @ 90 minutes: $F_{(1,10)} = 3.390$, $p = 0.095$; Figure 4.17 and 4.18A), and significantly increased the amount of cFos expression 180 minutes following light exposure (Main Effect of Treatment @ 180 minutes: $F_{(1,10)} = 17.405$, $p < 0.001$; Figure 4.19 and 4.20A), with higher levels being observed particularly at the Mid ($p < 0.01$) and Caudal ($p < 0.01$) levels.

Specifically, BMY7378 also significantly increased the amount of light-induced cFos expression at the Mid-Caudal level (Main Effect of Treatment @ 180 minutes: $F_{(1,10)} = 6.760$, $p < 0.05$; Figure 4.20B), but without any effect on the relative expression pattern within the core and shell regions of the SCN at that level (Treatment x Region Interaction: $F_{(1,8)} = 0.0000343$, $p = 0.995$). As expected, higher amounts of cFos expression were observed at the Mid-Caudal level than the Rostral of Caudal levels of the SCN at all durations following light exposure (Main Effect of Level @ 60 minutes: $F_{(2,8)} = 43.561$, $p < 0.001$; Main Effect of Level @ 90 minutes: $F_{(2,8)} = 71.175$, $p < 0.001$; Main Effect of Level @ 120 minutes: $F_{(2,8)} = 106.105$, $p < 0.001$; Main Effect of Level @ 180

minutes: $F_{(2,8)} = 91.240$, $p < 0.001$). Increased amounts of cFos were also found in the core region of the SCN compared to the shell region at all durations following light exposure (Main Effect of Region @ 60 minutes: $F_{(2,8)} = 346.314$, $p < 0.001$; Figure 4.14B; Main Effect of Region @ 90 minutes: $F_{(2,8)} = 73.638$, $p < 0.001$; Figure 4.18B; Main Effect of Region @ 120 minutes: $F_{(2,8)} = 1130.446$, $p < 0.001$; Figure 4.16B; Main Effect of Region @ 180 minutes: $F_{(2,8)} = 58.084$, $p < 0.001$; Figure 4.20B). There was also no effect of treatment on cFos expression when examined at the mid-caudal level across the four time points following light exposure (Treatment x Time after LP Interaction: $F_{(3,16)} = 1.335$, $p = 0.282$; Figure 4.21).

BMY7378 was found to have no observable effects on the amount of JunB expression at 60, 90, 120, or 180 minutes following light exposure, either across three rostral-caudal levels or between the core and shell regions of the SCN at the mid-caudal level. However, as expected, all time points indicated higher overall levels of JunB protein expression at the Mid-Caudal level of the SCN compared to the Rostral and Caudal levels (Main Effect of Level @ 60 minutes: $F_{(2,8)} = 87.284$, $p < 0.001$; Figure 4.22 and 4.23A; Main Effect of Level @ 90 minutes: $F_{(2,8)} = 71.788$, $p < 0.001$; Figure 4.24 and 4.25A; Main Effect of Level @ 120 minutes: $F_{(2,8)} = 160.821$, $p < 0.001$; Figure 4.26 and 4.27A; Main Effect of Level @ 180min: $F_{(2,8)} = 120.041$, $p < 0.001$; Figure 4.28 and 4.29A), as well as higher levels of JunB in the core compared to the shell region of the SCN at all four time points (Main Effect of Region @ 60 minutes: $F_{(2,8)} = 335.827$, $p < 0.001$: Figure 4.23B; Main Effect of Region @ 90 minutes: $F_{(2,8)} = 264.916$, $p < 0.001$;

Figure 4.25B; Main Effect of Region @ 120 minutes: $F_{(2,8)} = 139.171$, $p < 0.001$; Figure 4.27B; Main Effect of Region @ 180 minutes: $F_{(2,8)} = 173.068$, $p < 0.001$; Figure 4.29B).

There was a significant interaction between the type of pretreatment injection and the three rostral-caudal levels of the SCN when examining JunB protein expression 6 hours following light exposure in the late subjective night (Treatment x Level Interaction: $F_{(2,8)} = 5.742$, $p < 0.05$; Figure 4.30 and 4.31A). Specifically, BMY7378 elicited an increased magnitude of JunB protein expression at both the Rostral ($p < 0.05$) and Mid-Caudal ($p < 0.001$) levels of the SCN. As expected, higher levels of JunB expression were observed at the Mid-Caudal level when compared to the Rostral ($p < 0.001$) or Caudal ($p < 0.001$) levels of the SCN. When the JunB expression at the Mid-Caudal level of the SCN was examined in terms of the amount of expression in the core and shell regions, BMY7378 induced significantly more JunB expression (Main Effect of Treatment: $F_{(1,4)} = 20.169$, $p < 0.05$; Figure 4.31B), however increased expression was observed in both the core ($p <0.01$) and shell ($p < 0.05$) regions. Again as expected, more JunB expression was observed in the core region of the SCN as compared the that observed in the shell, irrespective of the type of pretreatment ($F_{(1,4)} = 625.576$, $p < 0.001$). There was also no effect of treatment on JunB expression when examined at the mid-caudal level across the four time points following light exposure (Treatment x Time after LP Interaction: $F_{(3,16)} = 0.144$, $p = 0.933$; Figure 4.32).

Finally, BMY7378 was also found to increase the magnitude of PER1 expression in the SCN when observed 6 hours following light exposure in the late subjective night (Main Effect of Treatment: $F_{(1,26)} = 5.941$, $p < 0.05$; Figure 4.33 and 4.34A). Specifically,

BMY7378 led to increased PER1 expression in the Rostral ($p < 0.05$) and Mid-Caudal ($p < 0.01$) levels, but not at the Caudal level ($p = 0.497$) of the SCN. When the Mid-Caudal level was examined more closely for core and shell regional differences, BMY7378 increased the amount of PER1 expression (Main effect of Treatment: $F_{(1,13)} = 6.438$, $p < 0.05$; Figure 4.34B), with the increase being observed in the Core ($p < 0.01$) but not in the shell ($p = 0.153$) region.

Figure 4.13 Photomicrographs of cFos expression 60 minutes following the start of the light exposure.

Representative SCN tissue slices indicating cFos-expressing cells at CT19 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

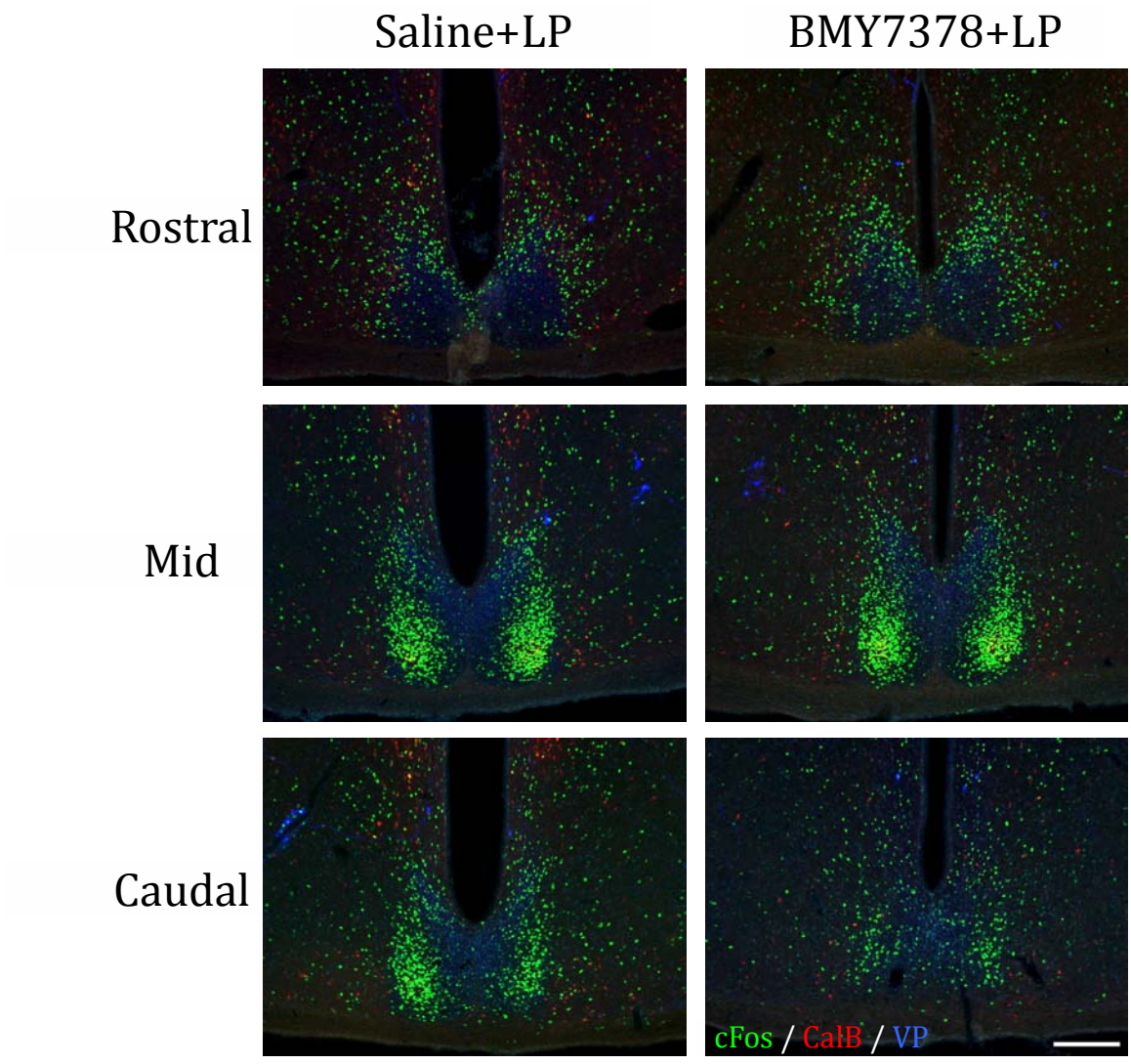

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
cFos / CalB / VP

Figure 4.14 Mean number of cFos–IR cells 60 minutes following the start of the light exposure.

Mean number of cFos-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT19.

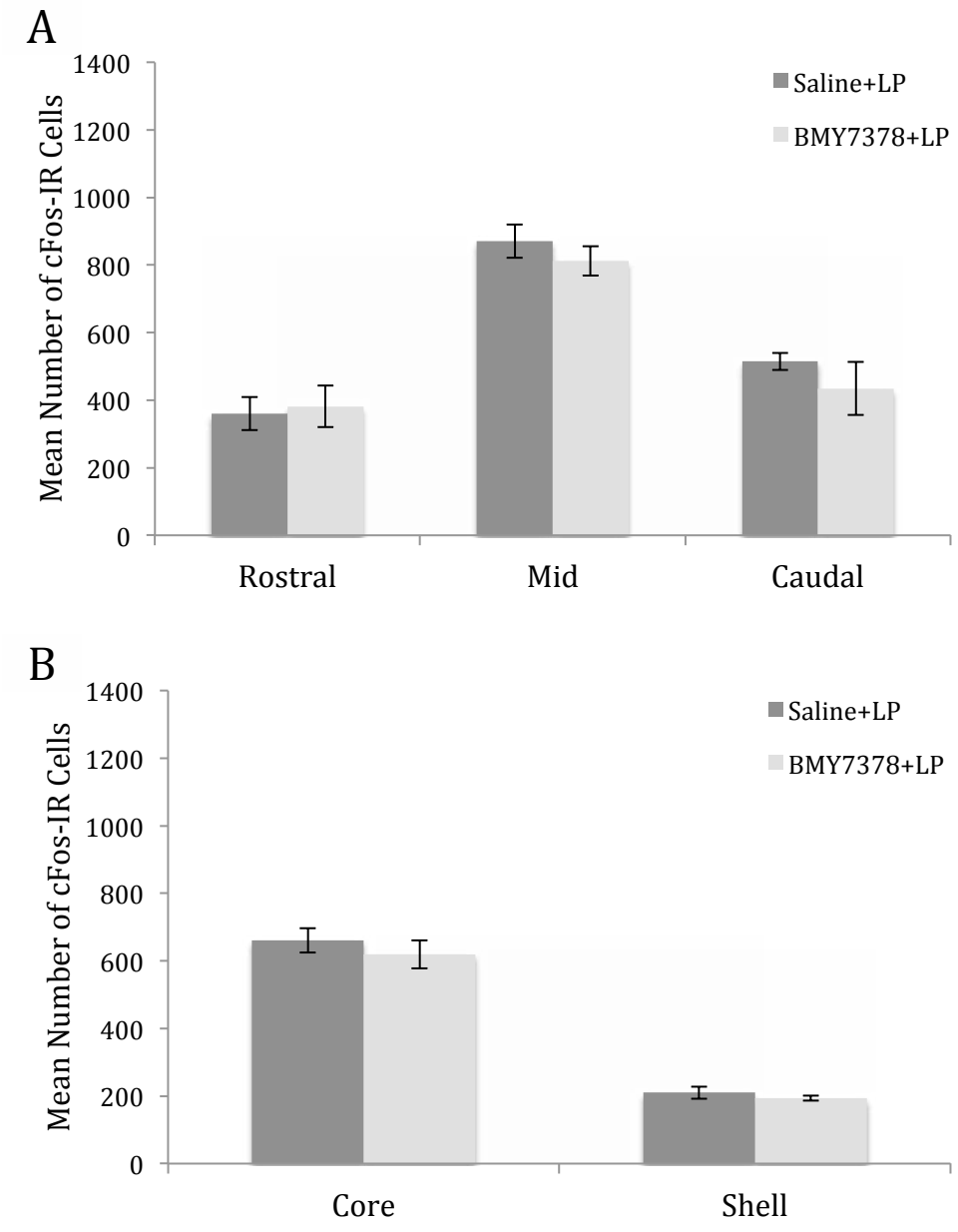

A
Mean Number of cFos-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
Mean Number of cFos-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.15 Photomicrographs of cFos expression 90 minutes following the start of the light exposure.

Representative SCN tissue slices indicating cFos-expressing cells at CT19.5 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

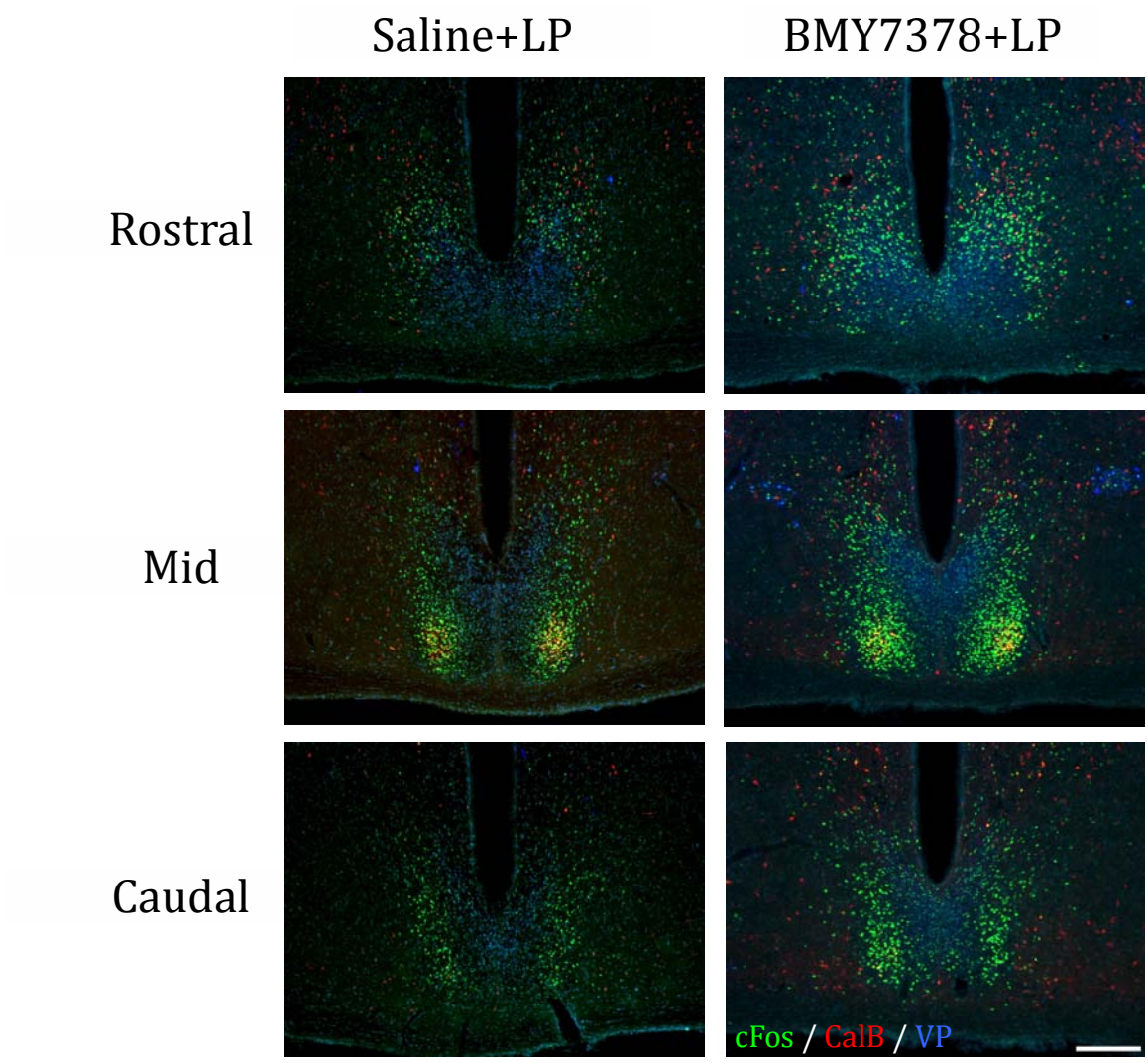

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
cFos / CalB / VP

Figure 4.16 Mean number of cFos–IR cells 90 minutes following the start of the light exposure.

Mean number of cFos-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT19.5. There is a trend towards a main effect of treatment across the rostral-caudal levels ($p = 0.095$).

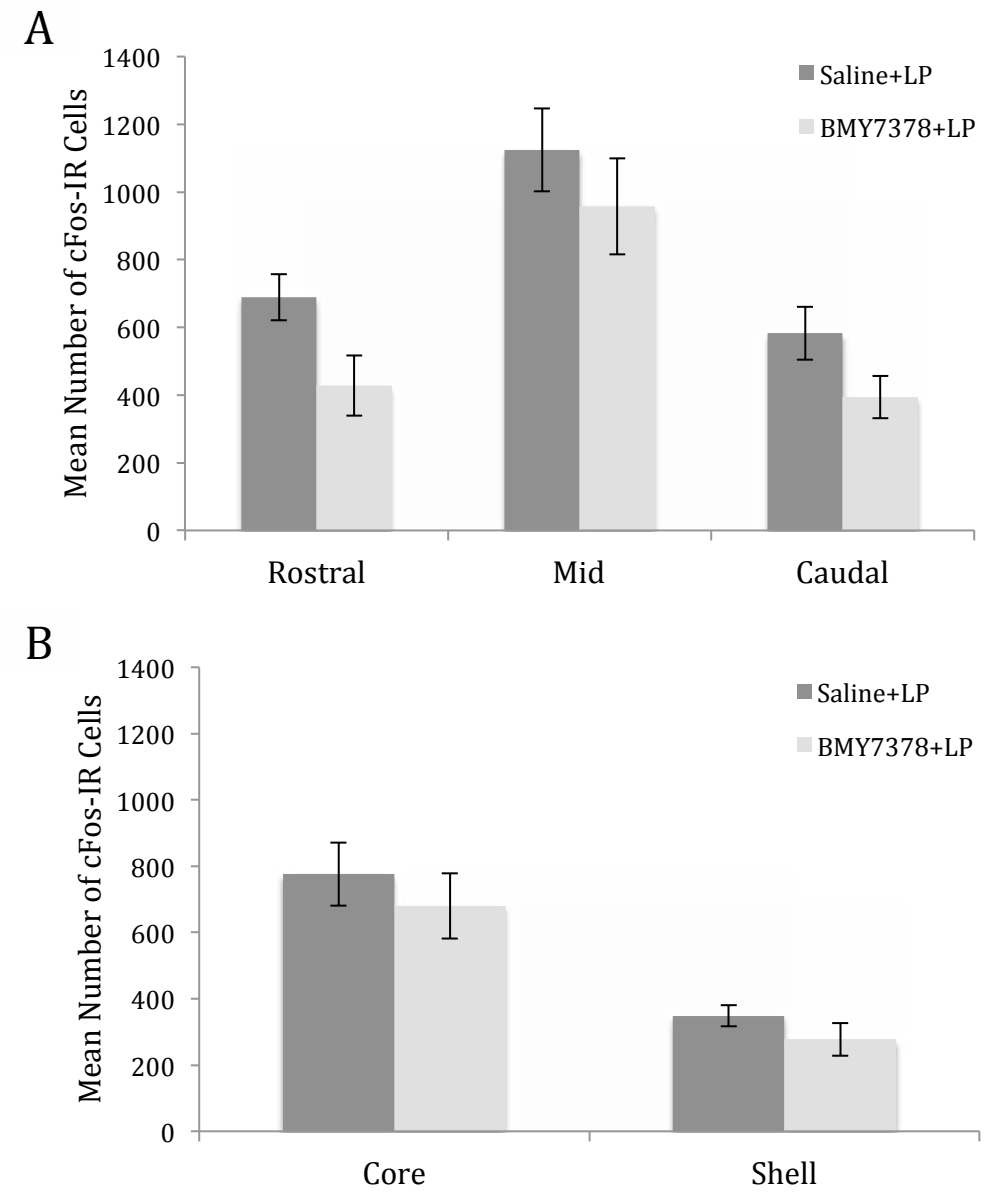

A
1400
1200
1000
800
600
400
200
0
Mean Number of cFos-IR Cells
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
1400
1200
1000
800
600
400
200
0
Mean Number of cFos-IR Cells
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.17 Photomicrographs of cFos expression 120 minutes following the start of the light exposure.

Representative SCN tissue slices indicating cFos-expressing cells at CT20 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

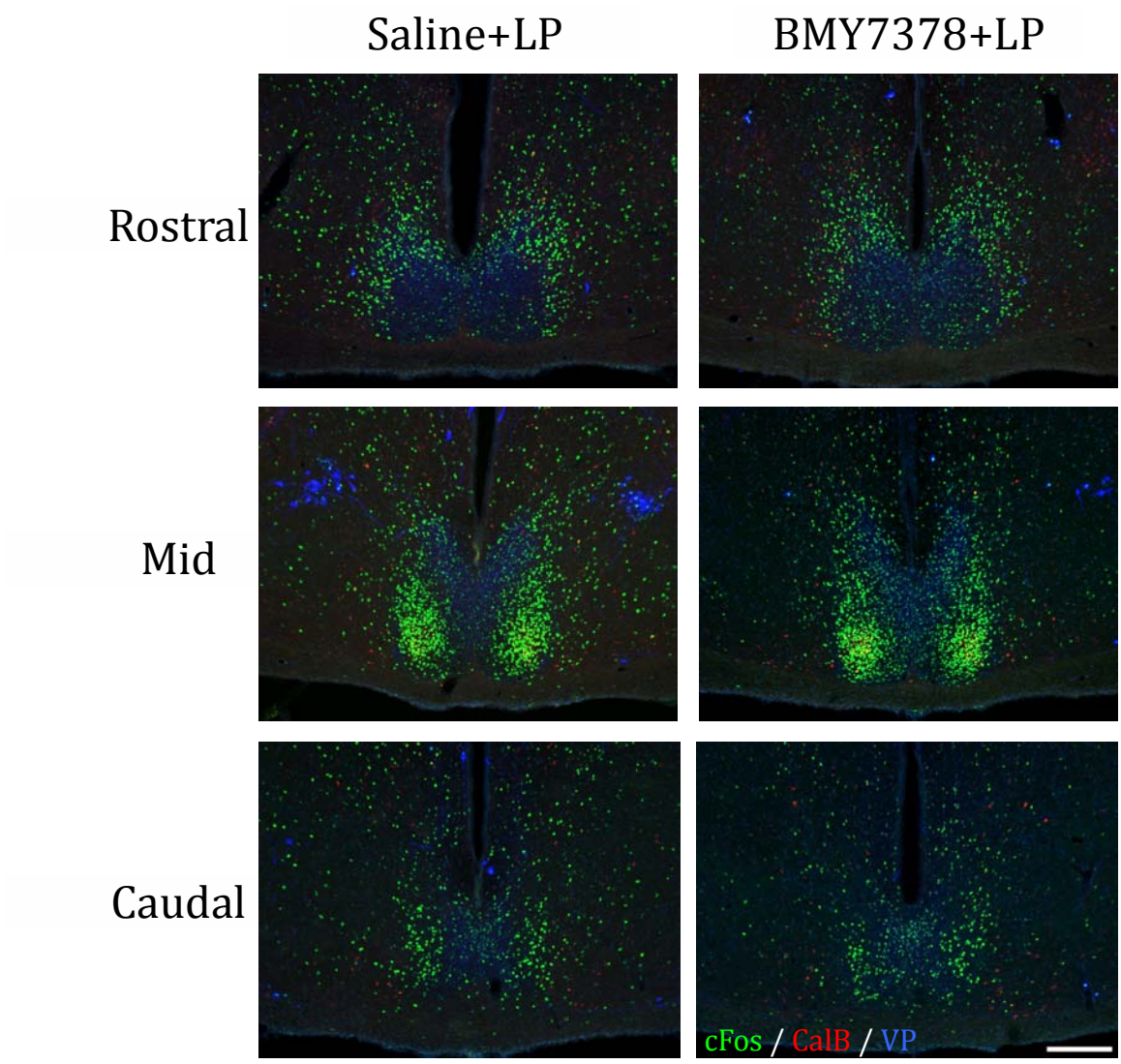

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
cFos / CalB / VP

Figure 4.18 Mean number of cFos–IR cells 120 minutes following the start of the light exposure.

Mean number of cFos-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT20.

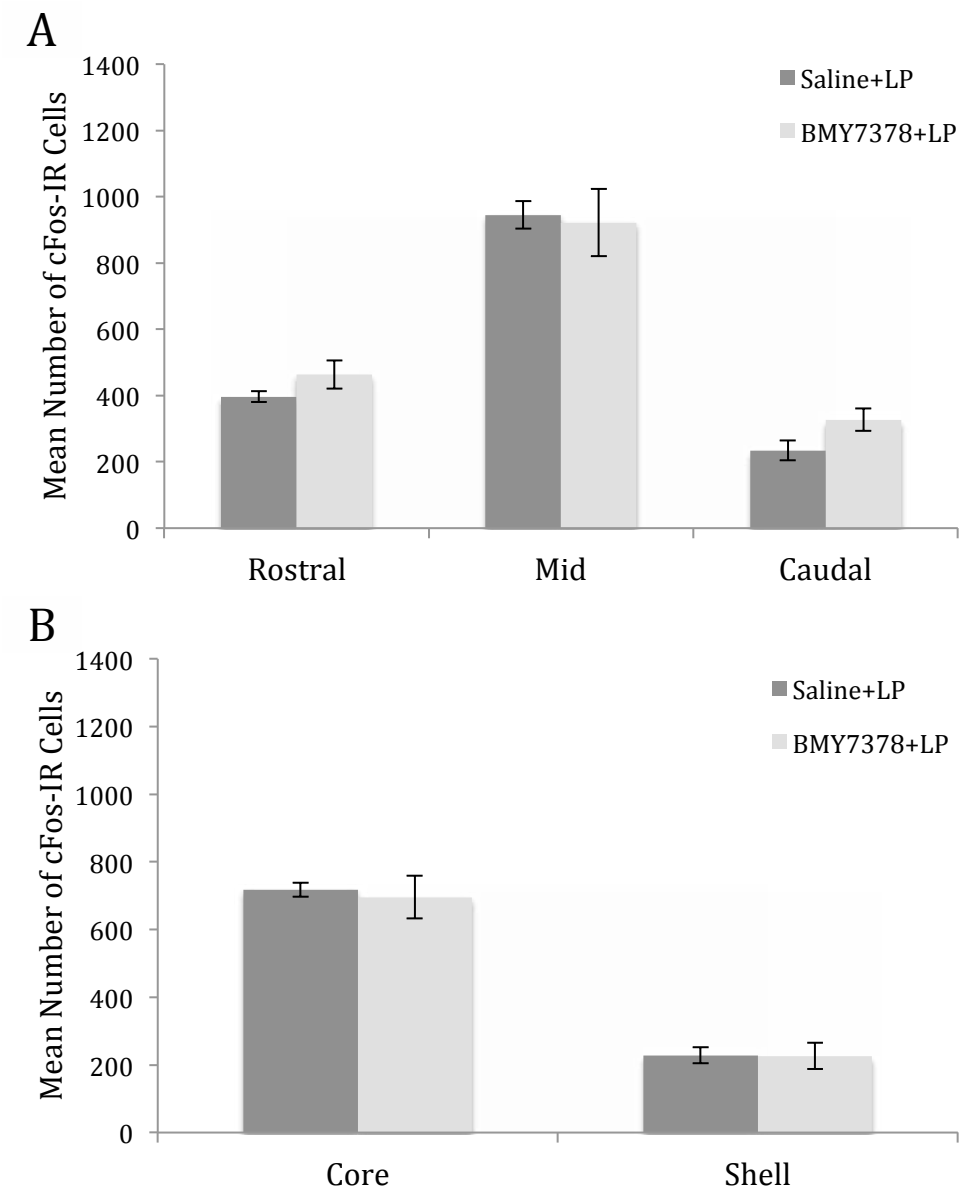

A
Mean Number of cFos-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
Mean Number of cFos-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.19 Photomicrographs of cFos expression 180 minutes following the start of the light exposure.

Representative SCN tissue slices indicating cFos-expressing cells at CT21 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

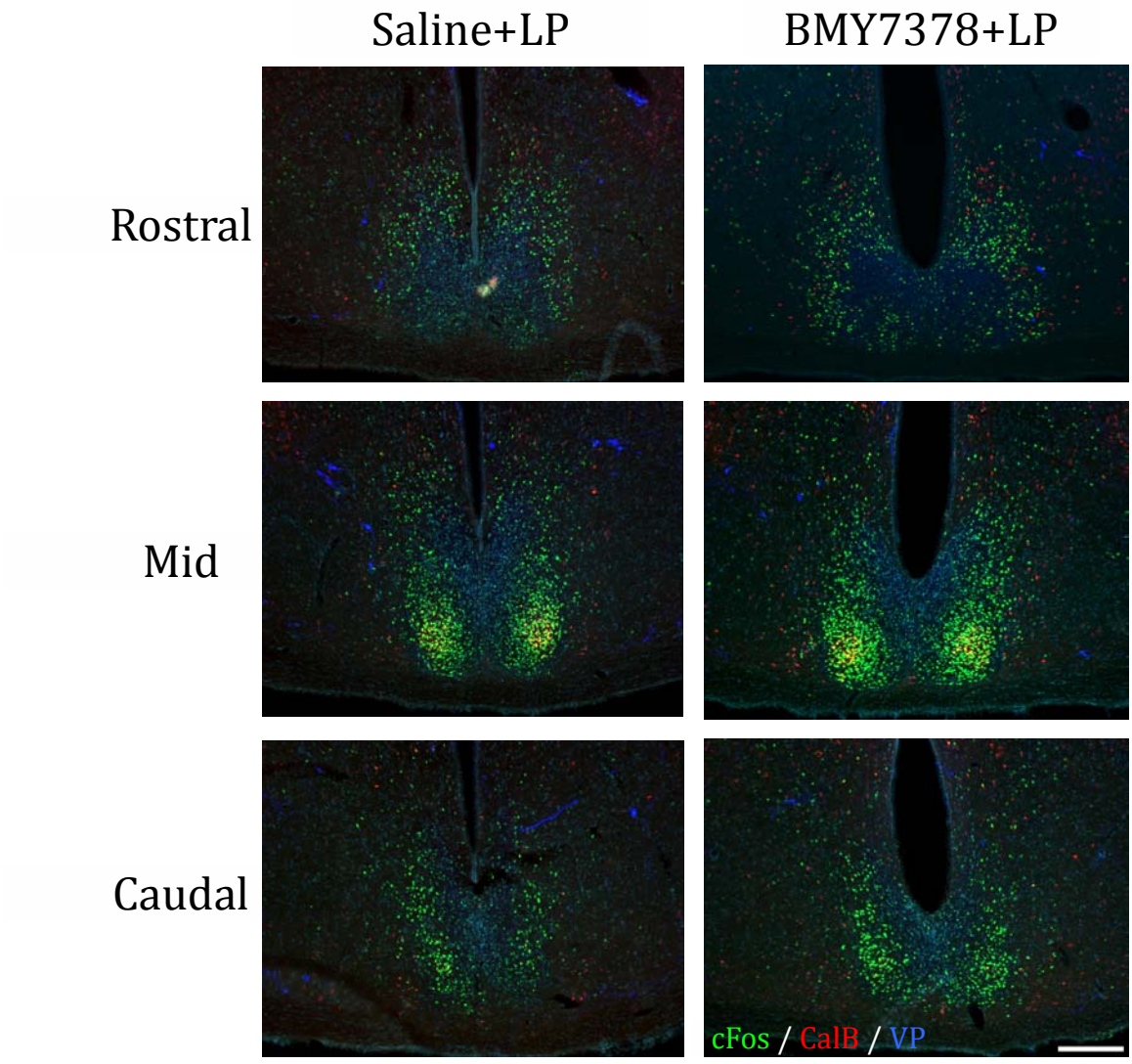

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
cFos / CalB / VP

Figure 4.20 Mean number of cFos–IR cells 180 minutes following the start of the light exposure.

Mean number of cFos-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT21. There was a main effect of treatment across the three rostral-caudal levels as well as across the core and shell. (**) indicates a statistical significance of $p<0.01$.

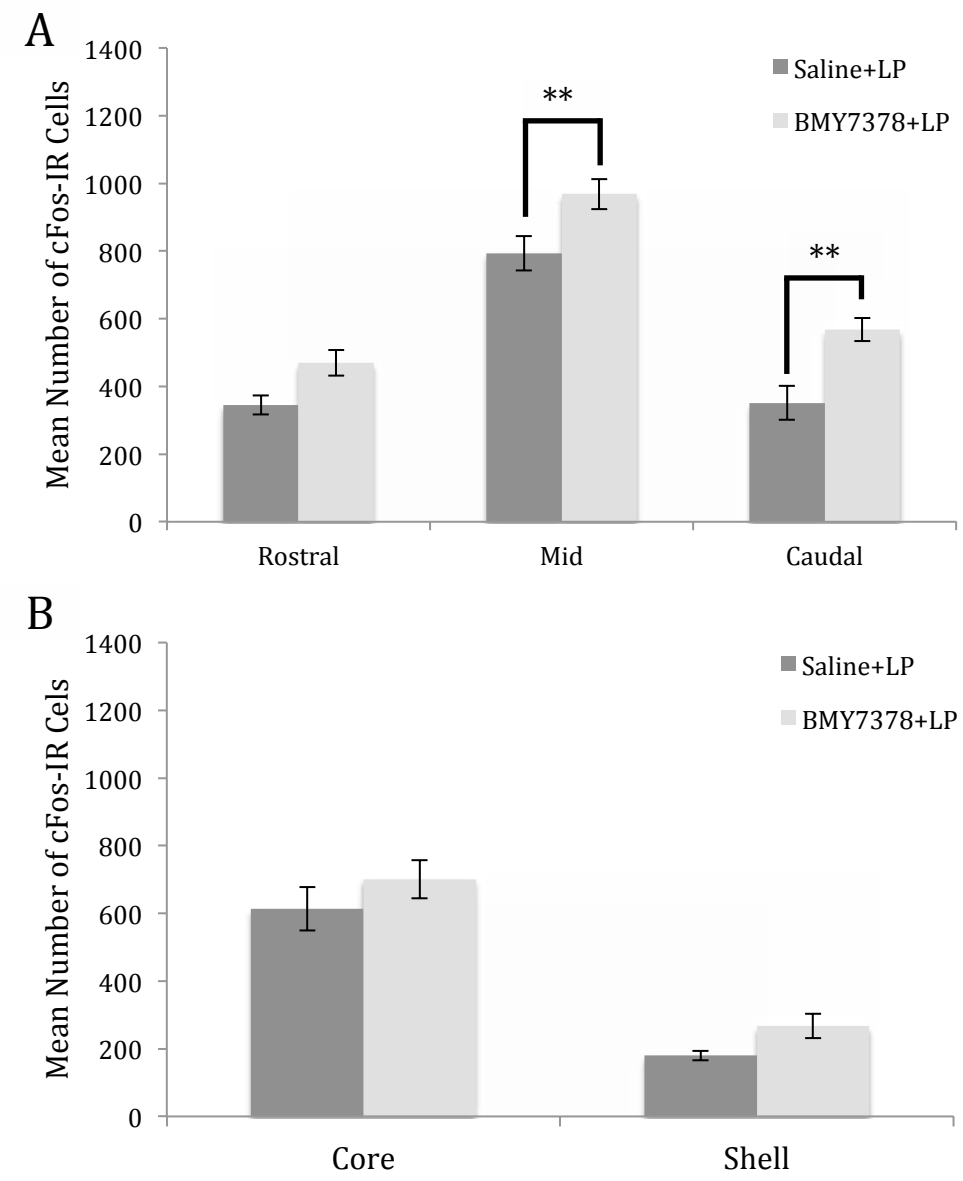

A
Mean Number of cFos-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
**
**
Rostral
Mid
Caudal
B
Mean Number of cFos-IR Cels
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.21 Mean number of cFos–IR cells 60, 90, 120, and 180 minutes following the start of the light exposure.

Mean number of cFos-expressing cells (± SEM) at four times following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25.

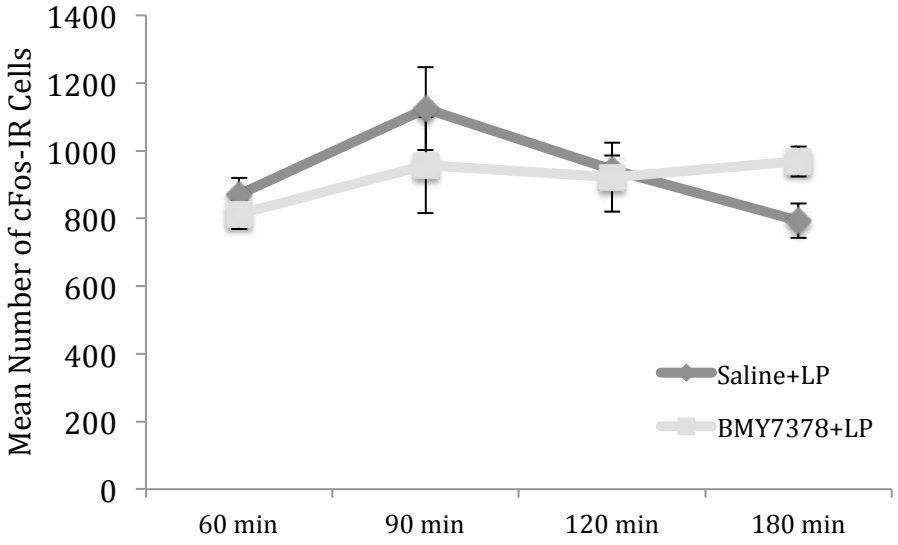

Mean Number of cFos-IR Cells
1400
1200
1000
800
600
400
200
0
60 min
90 min
120 min
180 min
Saline+LP
BMY7378+LP

Figure 4.22 Photomicrographs of JunB expression 60 minutes following the start of the light exposure.

Representative SCN tissue slices indicating JunB-expressing cells at CT19 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

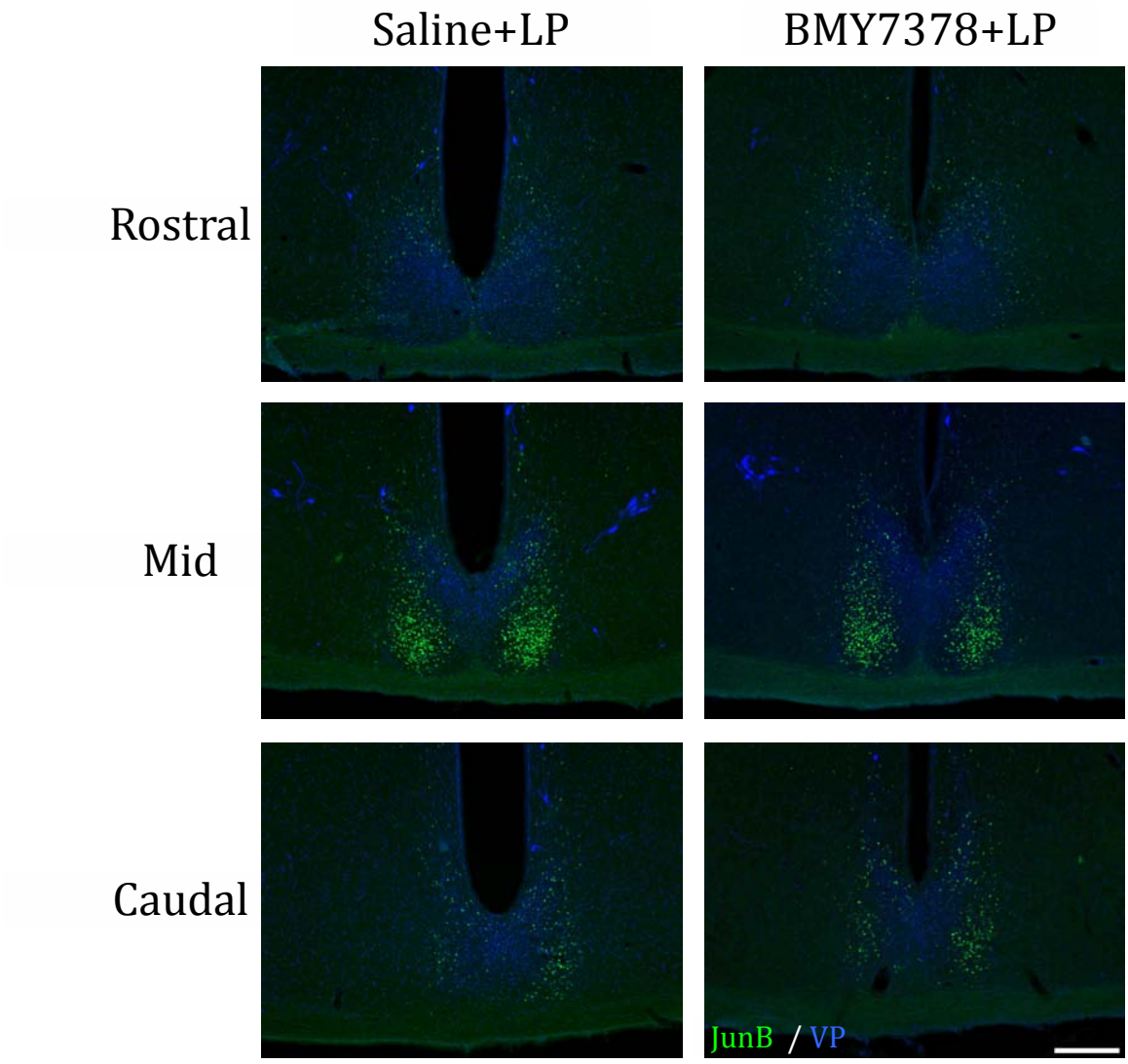

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
JunB / VP

Figure 4.23 Mean number of JunB–IR cells 60 minutes following the start of the light exposure.

Mean number of JunB-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT19.

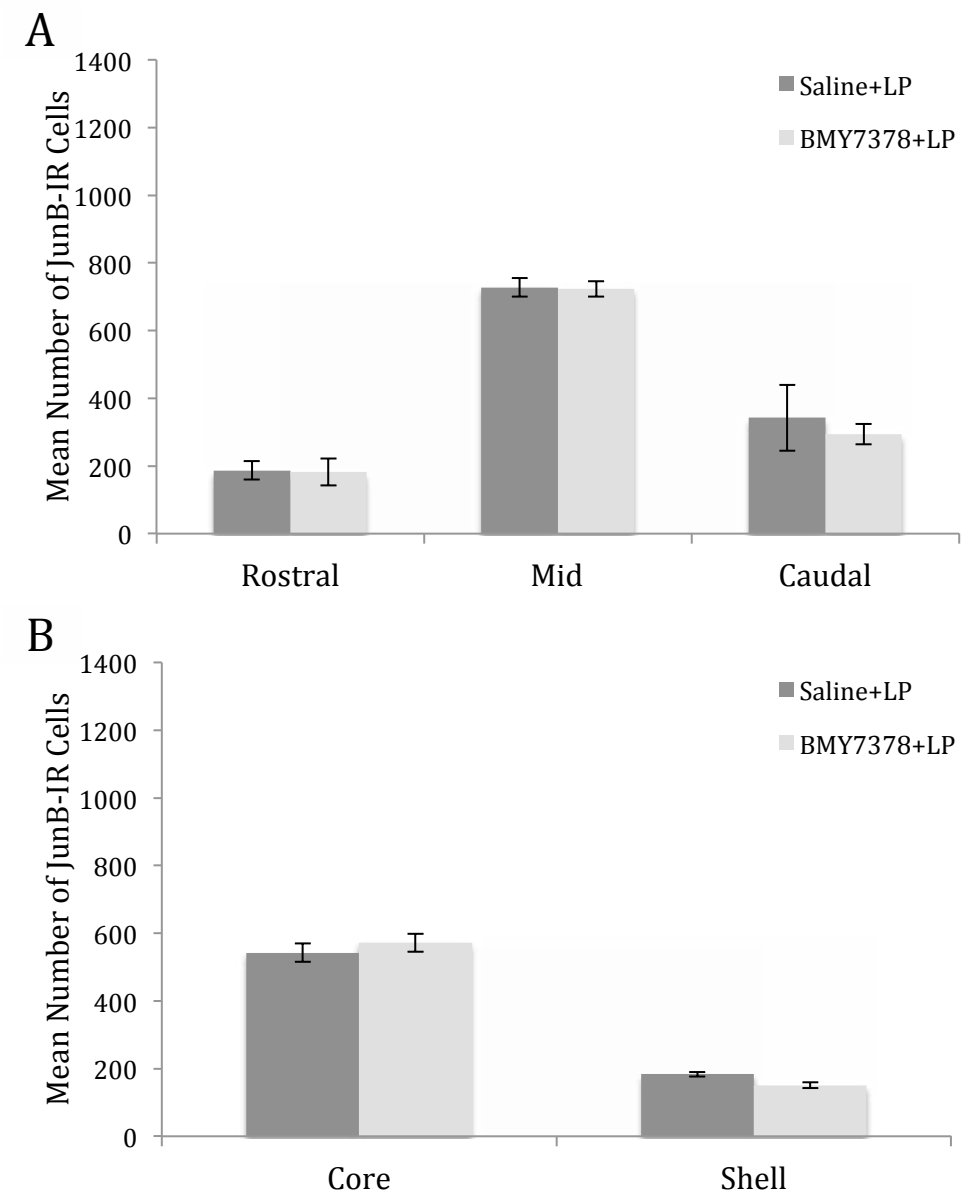

A
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.24 Photomicrographs of JunB expression 90 minutes following the start of the light exposure.

Representative SCN tissue slices indicating JunB-expressing cells at CT19.5 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

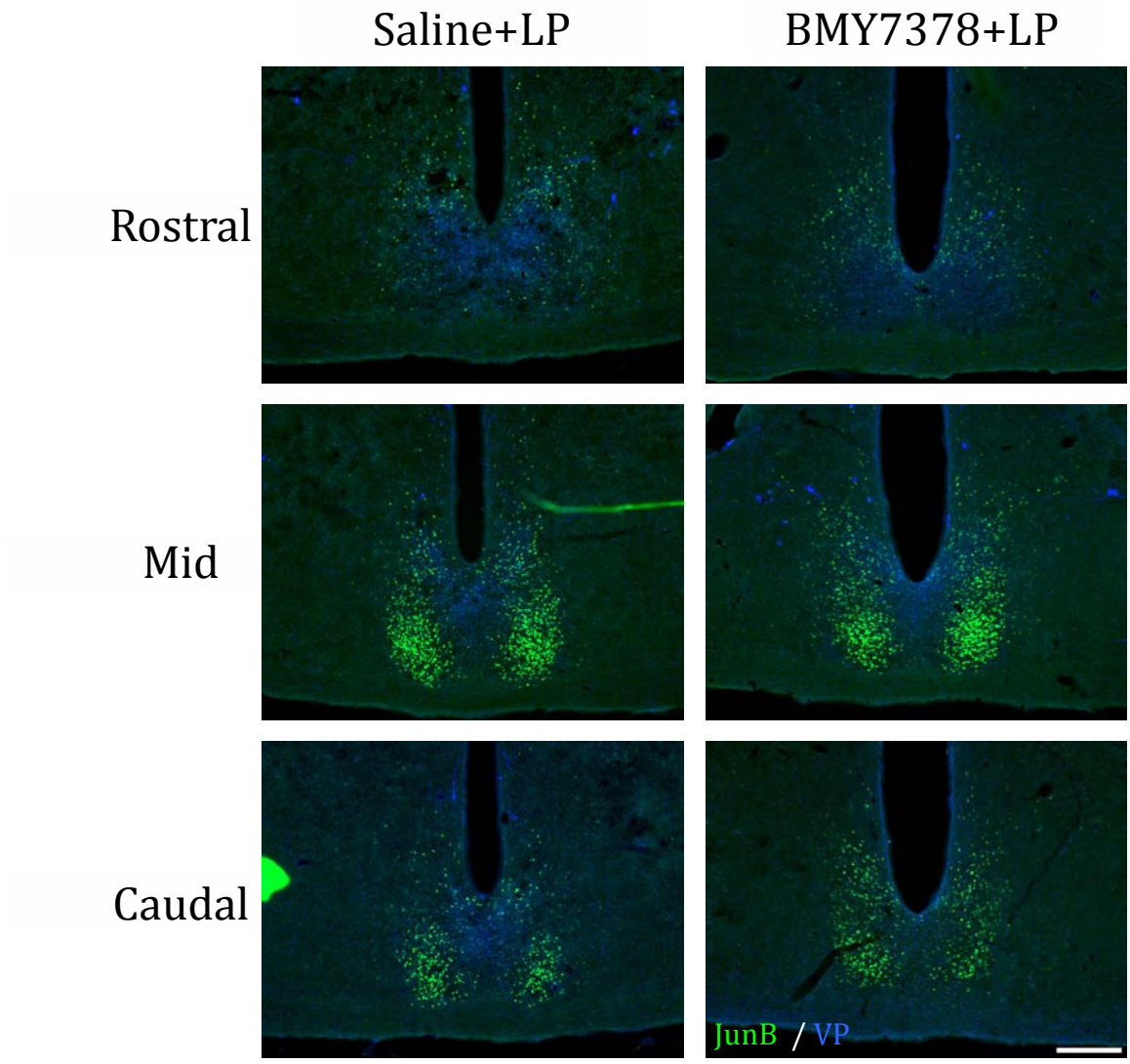

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
JunB / VP

Figure 4.25 Mean number of JunB–IR cells 90 minutes following the start of the light exposure.

Mean number of JunB-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT19.5.

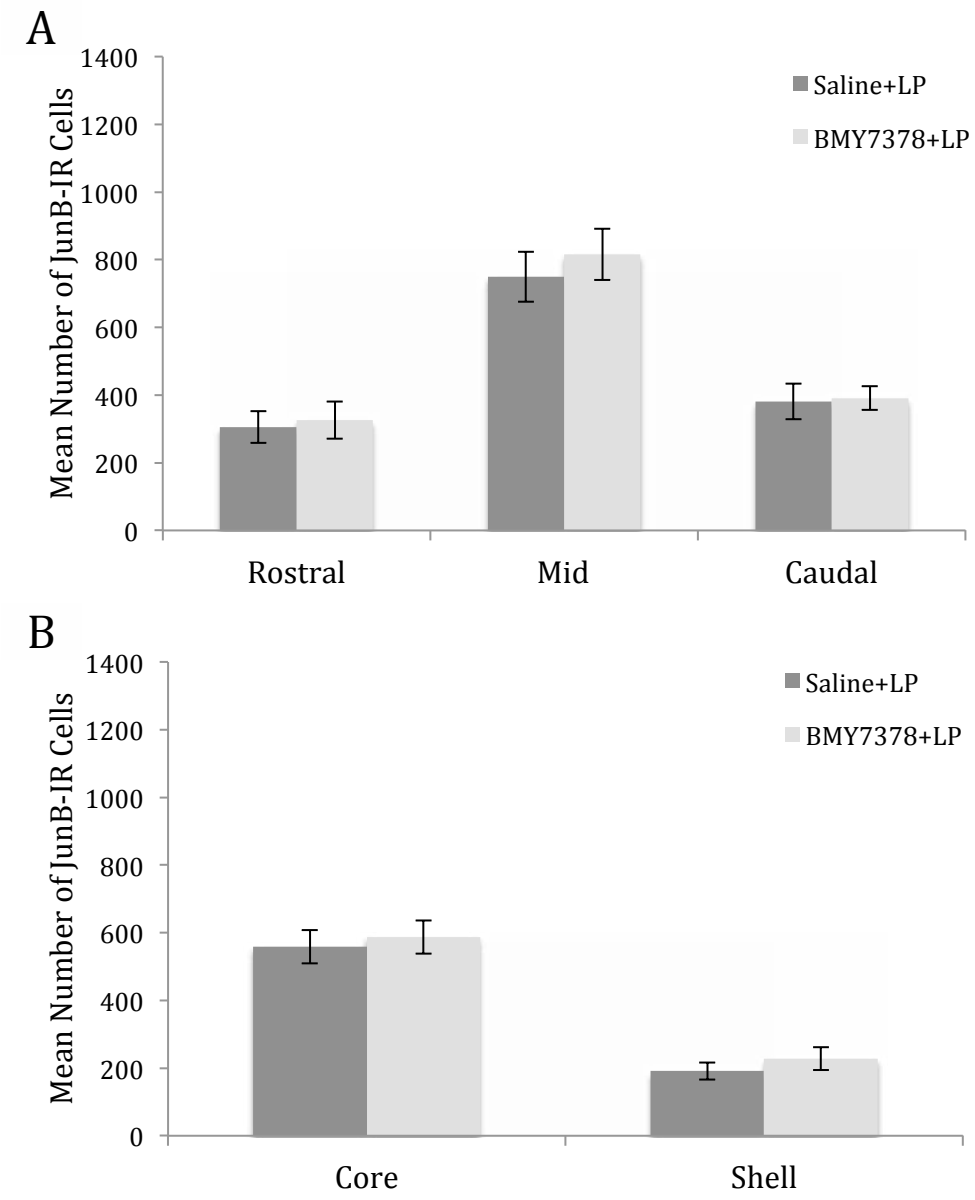

A
1400
1200
1000
800
600
400
200
0
Mean Number of JunB-IR Cells
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
1400
1200
1000
800
600
400
200
0
Mean Number of JunB-IR Cells
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.26 Photomicrographs of JunB expression 120 minutes following the start of the light exposure.

Representative SCN tissue slices indicating JunB-expressing cells at CT20 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

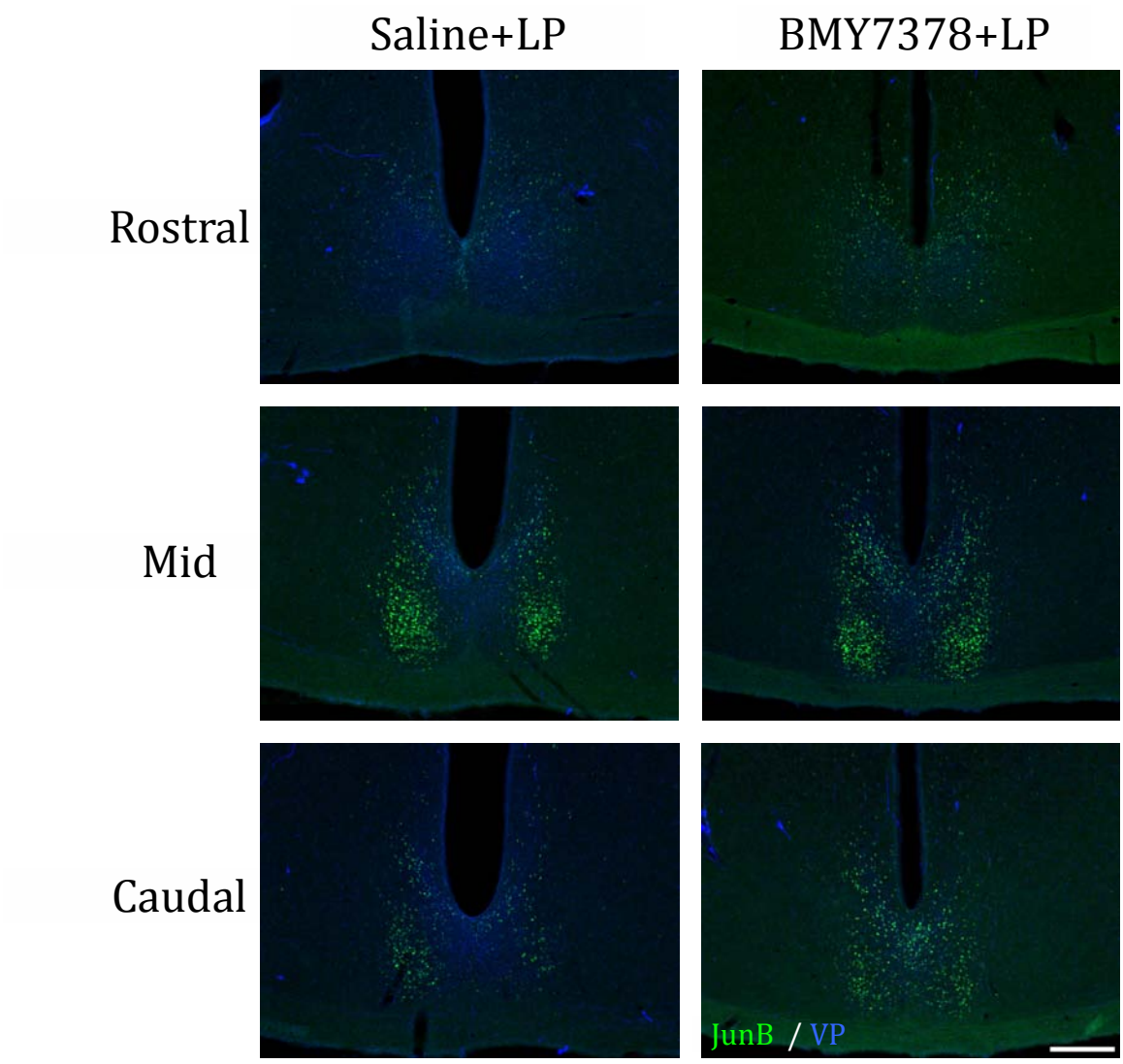

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
JunB / VP

Figure 4.27 Mean number of JunB–IR cells 120 minutes following the start of the light exposure.

Mean number of JunB-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT20.

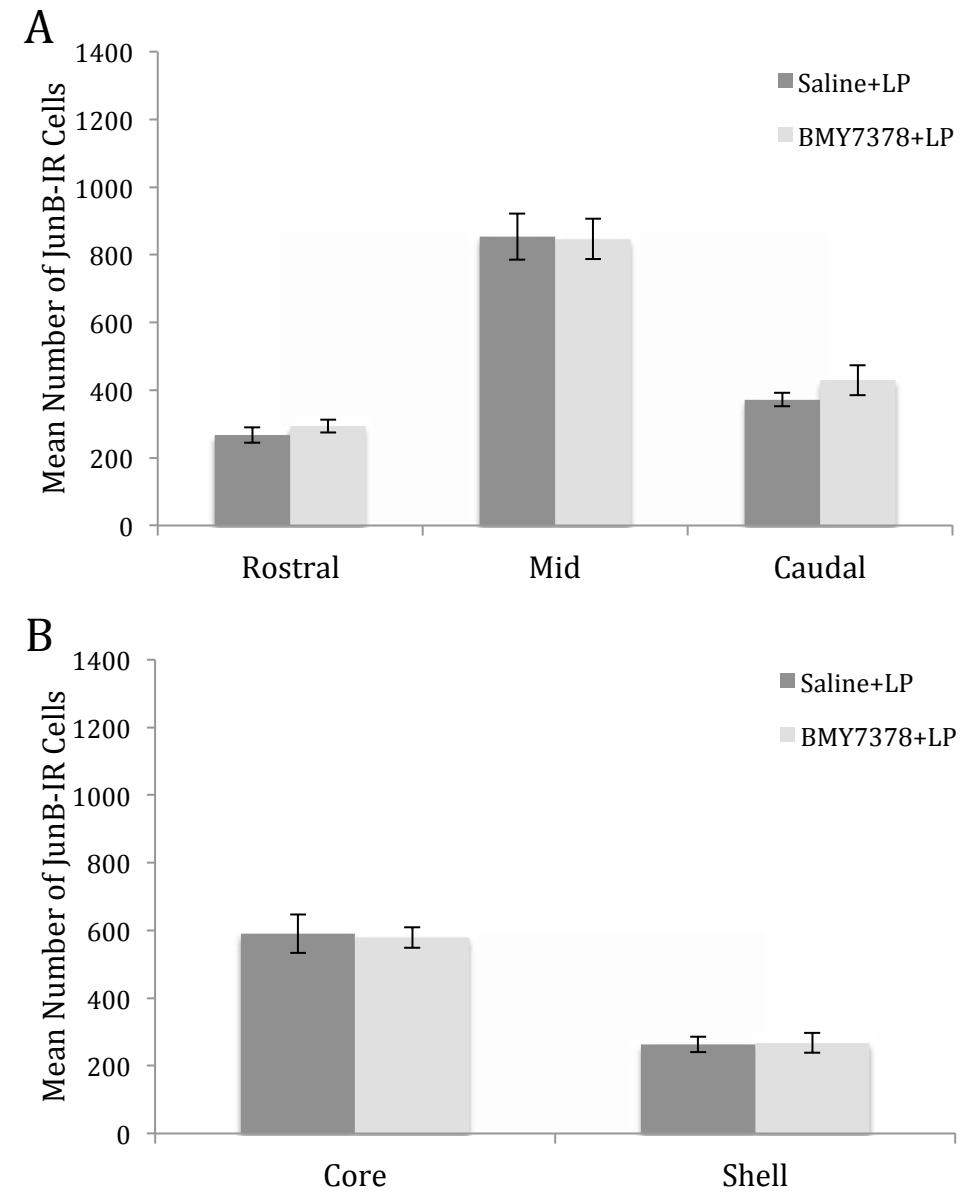

A
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.28 Photomicrographs of JunB expression 180 minutes following the start of the light exposure.

Representative SCN tissue slices indicating JunB-expressing cells at CT21 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

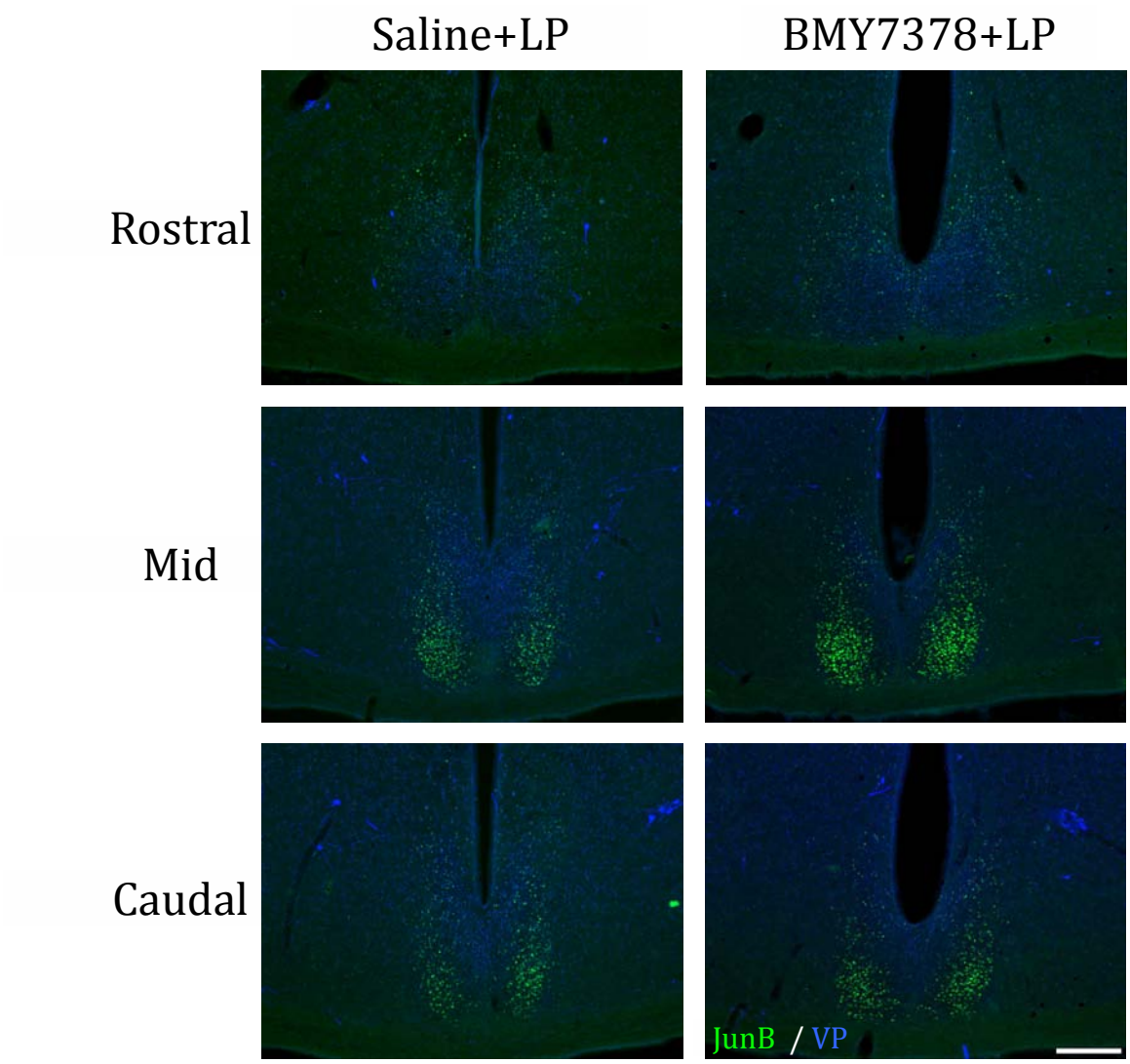

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
JunB / VP

Figure 4.29 Mean number of JunB–IR cells 180 minutes following the start of the light exposure.

Mean number of JunB-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT21.

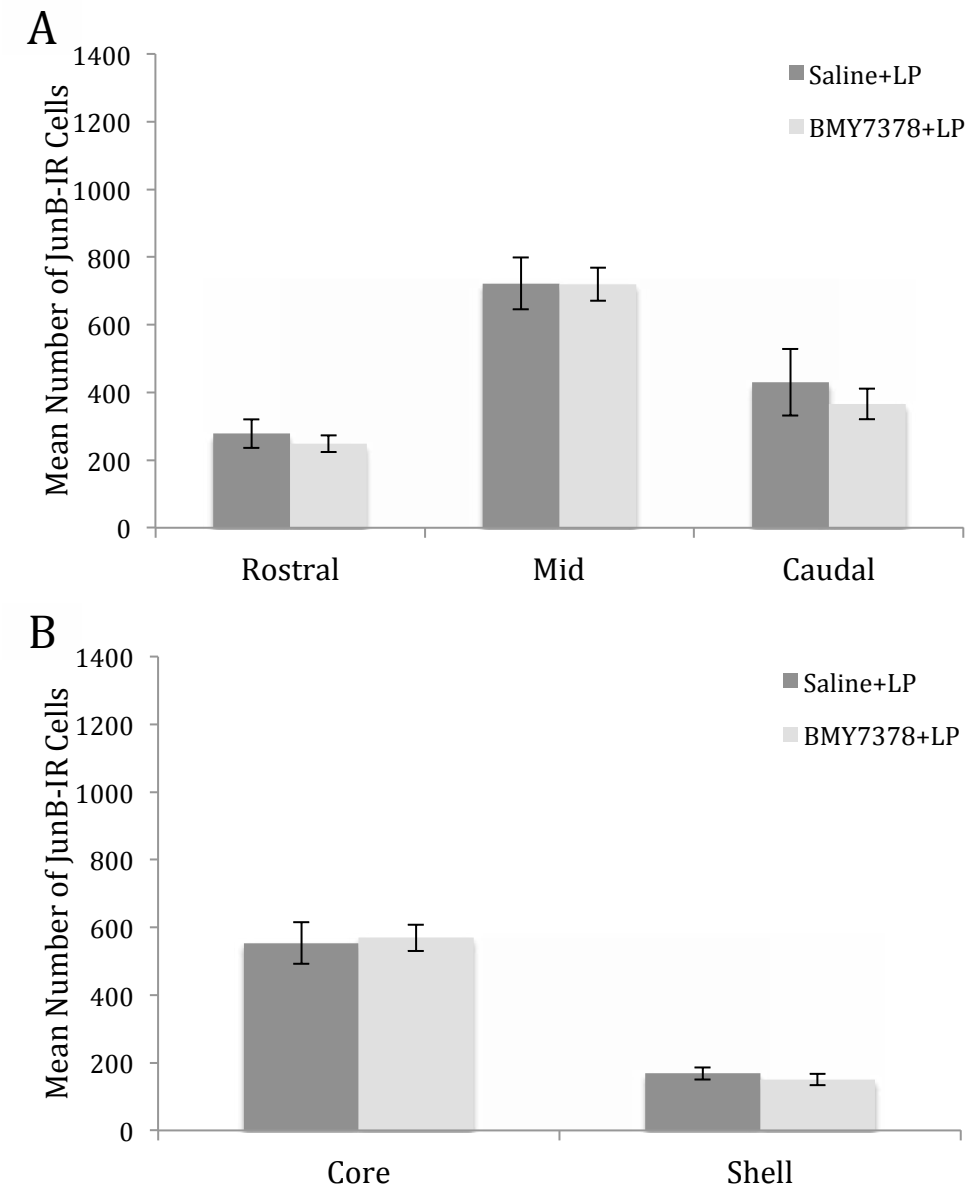

A
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
Core
Shell

Figure 4.30 Photomicrographs of JunB expression 6 hours following the start of the light exposure.

Representative SCN tissue slices indicating JunB-expressing cells at CT0 and stained using fluorescent immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

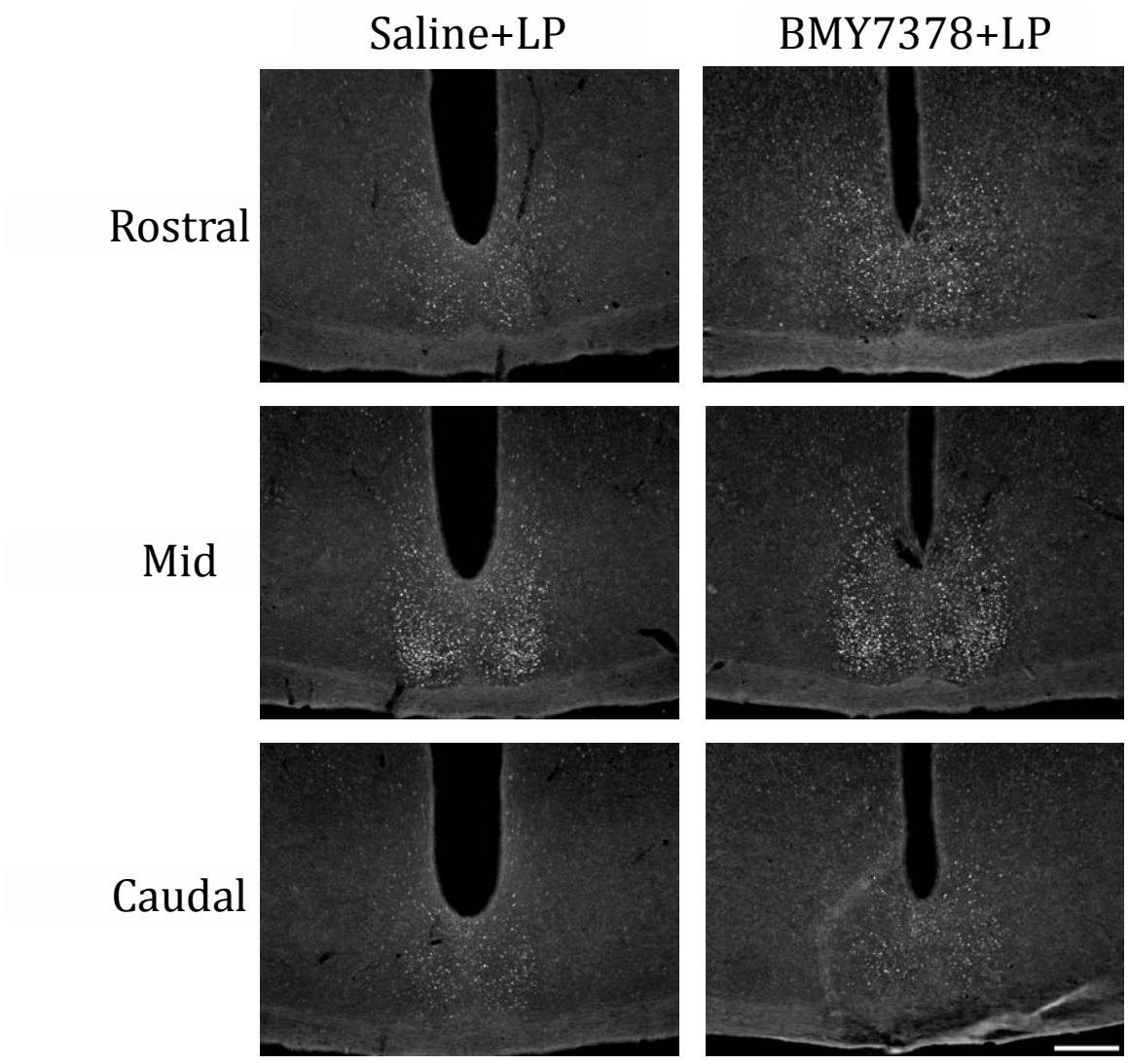

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal

Figure 4.31 Mean number of JunB–IR cells 6 hours following the start of the light exposure.

Mean number of JunB-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT0. (*), (**), and (***) indicate a statistical significance of $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

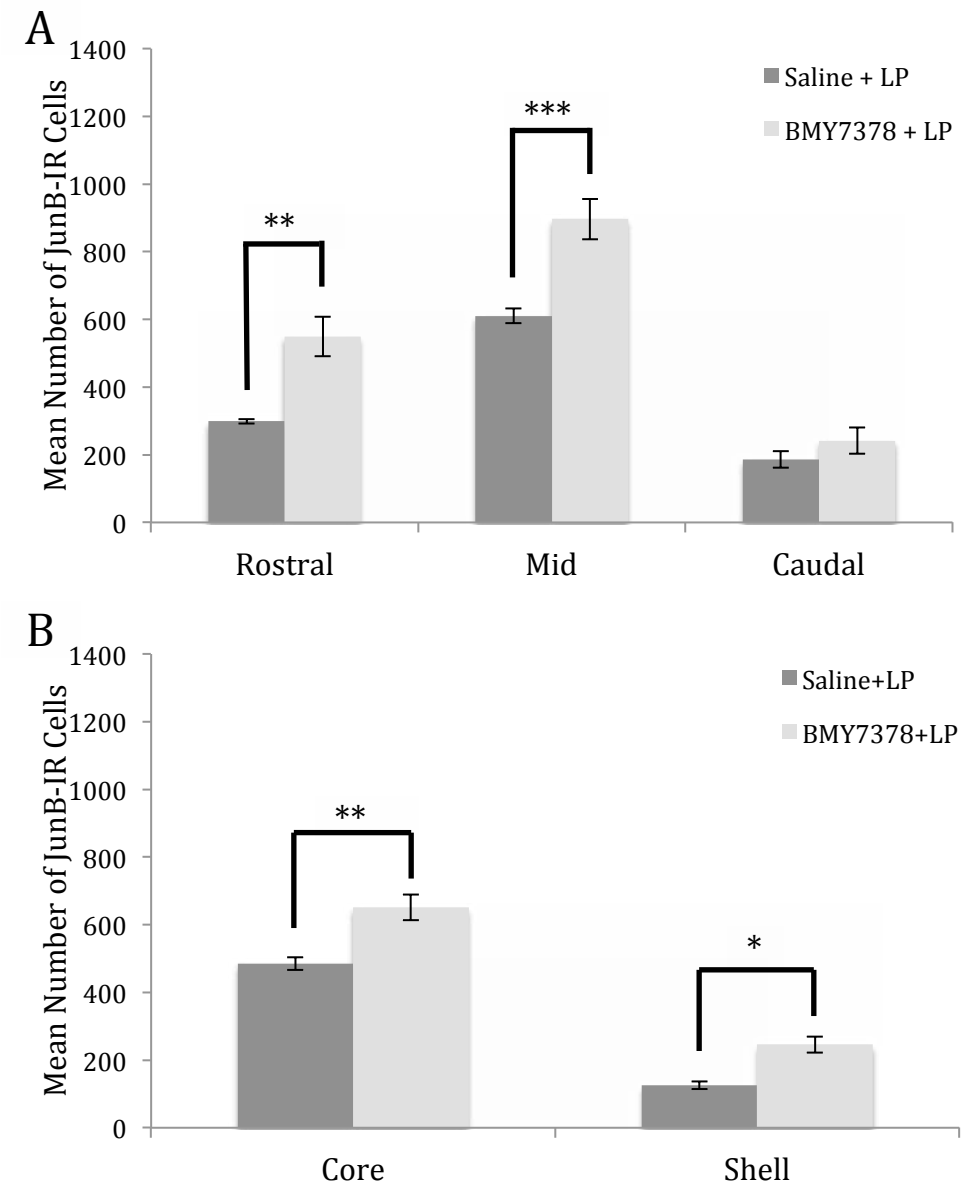

A
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline + LP
BMY7378 + LP
**
***
Rostral
Mid
Caudal
B
Mean Number of JunB-IR Cells
1400
1200
1000
800
600
400
200
0
Saline+LP
BMY7378+LP
**
*
Core
Shell

Figure 4.32 Mean number of JunB–IR cells 60, 90, 120, 180 minutes and 6 hours following the start of the light exposure.

Mean number of JunB-expressing cells (± SEM) at four times following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25.

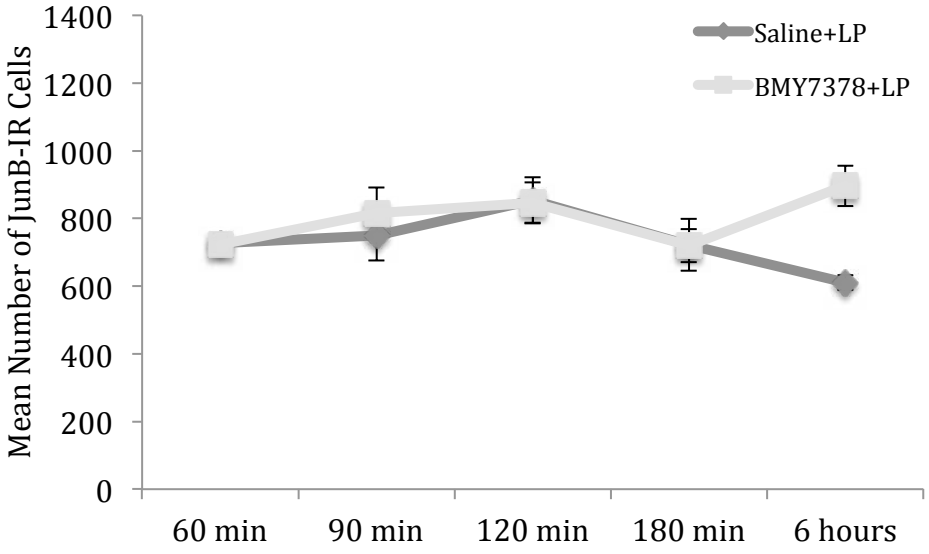

1400
1200
1000
800
600
400
200
0
Mean Number of JunB-IR Cells
Saline+LP
BMY7378+LP
60 min
90 min
120 min
180 min
6 hours

Figure 4.33 Photomicrographs of mPER1 expression 6 hours following the start of the light exposure.

Representative SCN tissue slices indicating mPER1-expressing cells at CT0 and stained using DAB immunohistochemistry following a brief light pulse in the late subjective night (CT18), pretreated at CT17.25 with an i.p. injection of either Saline or BMY7378. Scale bar = 200μm.

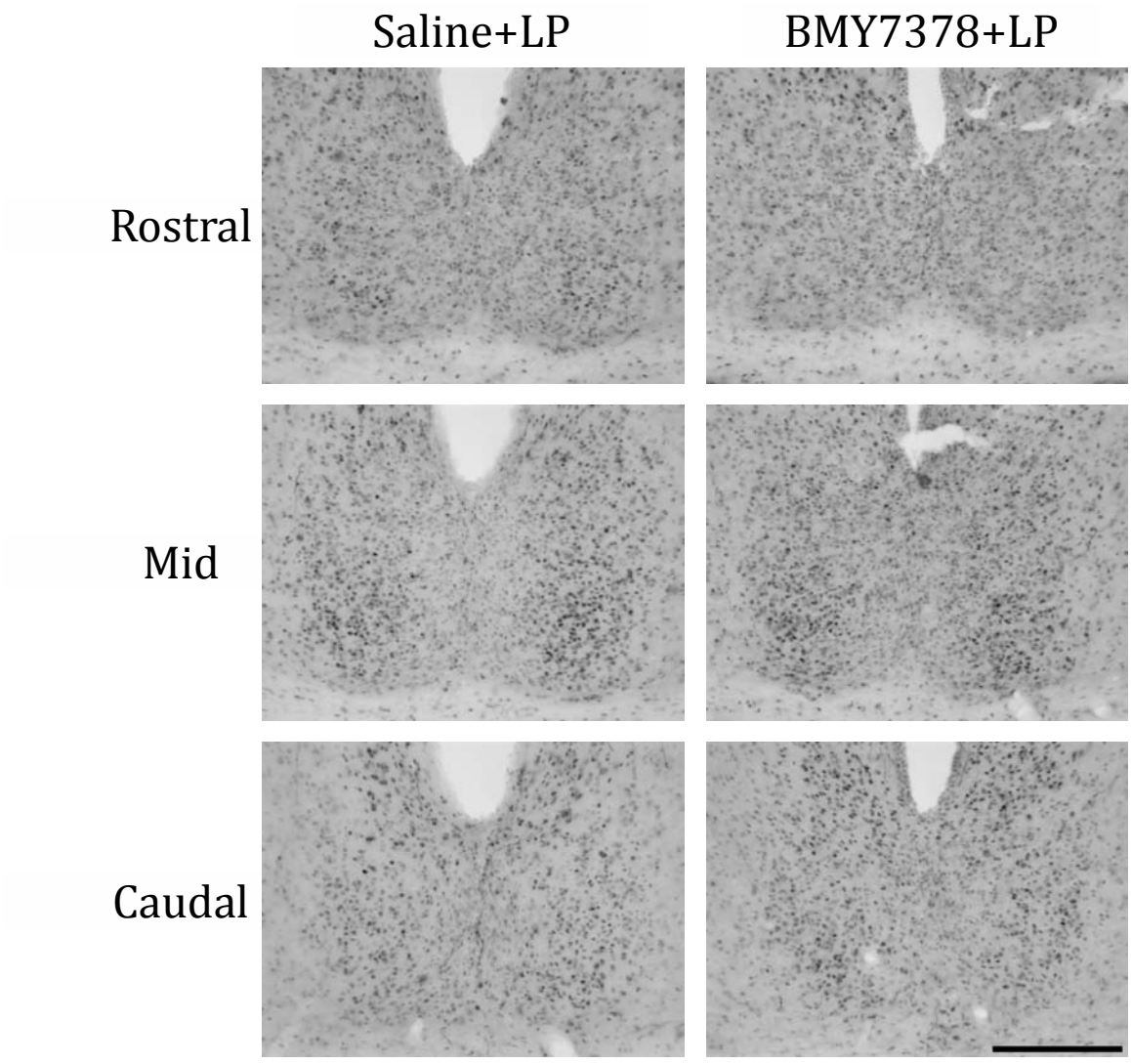
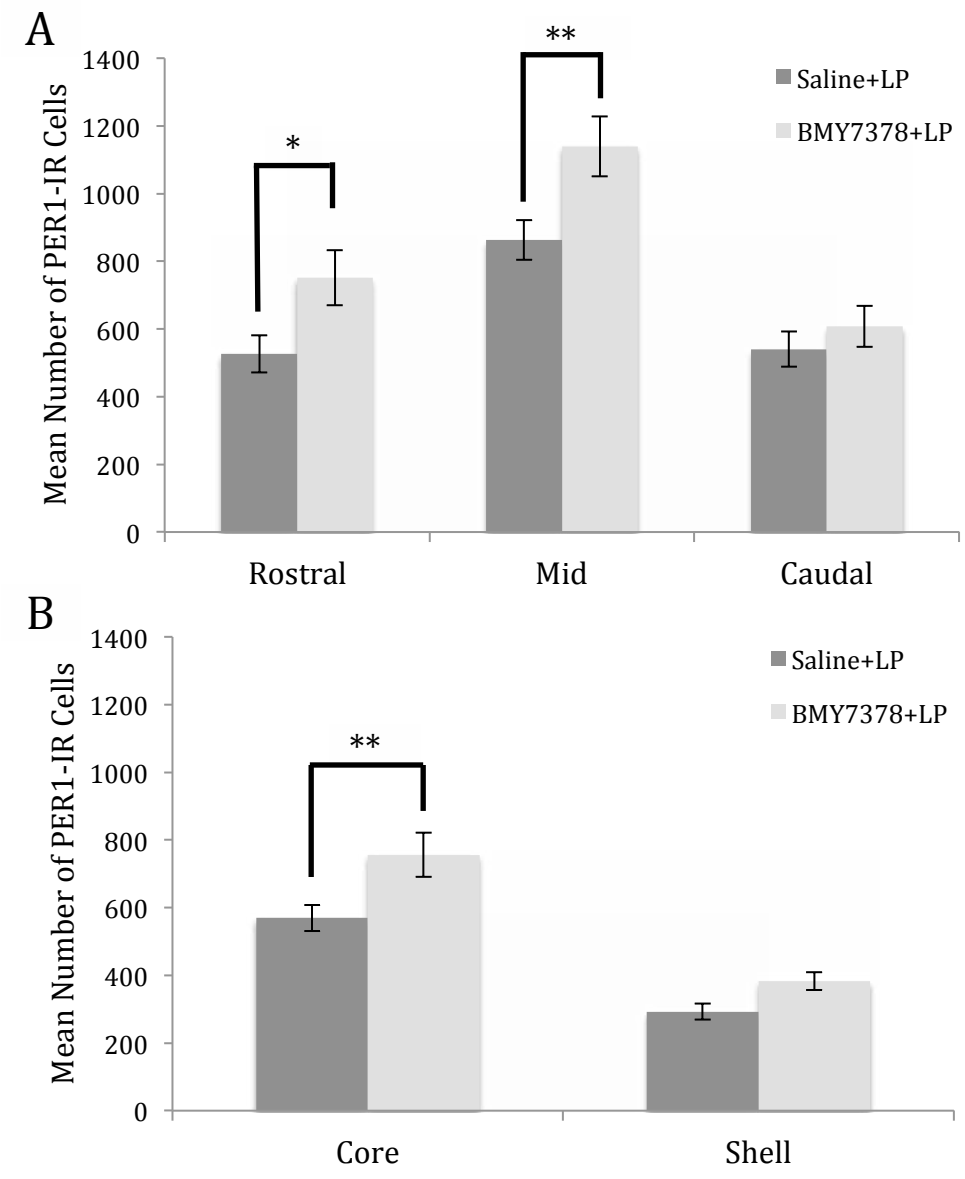

Saline+LP
BMY7378+LP
Rostral
Mid
Caudal

Figure 4.34 Mean number of mPER1–IR cells 6 hours following the start of the light exposure.

Mean number of mPER1-expressing cells (± SEM) at (A) three rostral-caudal levels of the SCN and (B) in the core and shell regions of the SCN of animals following a brief light pulse at CT18, pretreated with either Saline or BMY7378 at CT17.25. Hamsters were euthanized at CT0. (*), (**), and (***) indicate a statistical significance of $p<0.05$, $p<0.01$, and $p<0.001$ respectively.


A
Mean Number of PER1-IR Cells
1400
1200
1000
800
600
400
200
0
*
**
Saline+LP
BMY7378+LP
Rostral
Mid
Caudal
B
Mean Number of PER1-IR Cells
1400
1200
1000
800
600
400
200
0
**
Saline+LP
BMY7378+LP
Core
Shell

4.4 Discussion

In this study, we show that the expression patterns of multiple biochemical cascade components and light-induced proteins, activated or expressed in the hamster SCN following exposure to light in the late subjective night, are altered following pretreatment with the 5-HT_{1A} mixed agonist/antagonist BMY7378. Pretreatment with BMY7378 led to an increase in the amount of light-induced P-ERK 60 minutes following light exposure, as well as an increase in the amount of CREM protein expressed 240 minutes following light exposure. The amount of cFos expression 180 minutes following light exposure, as well as JunB and PER1 expression 6 hours following light exposure, were all increased following pretreatment with BMY7378. However, P-ERK expression was unaffected at 15, 30, 45 and 120 minutes following light exposure, as was P-CREB expression at 120 minutes with BMY7378 pretreatment. Similarly, cFos expression at 60, 120 and 180 minutes, as well as JunB expression at 60, 90, 120 and 180 minutes following light exposure in the late subjective night were also unaffected by BMY7378 pretreatment (Figure 4.35).

The modulation of light-induced cFos has previously been studied in animals pretreated with a mixed agonist/antagonist similar to BMY7378, namely NAN-190. However, these previous studies have come with inconsistent results. One study found no change in the amount of cFos expressed in the hamster SCN following NAN-190 pretreatment (Glass *et al.*, 1994), while two other studies found significant decreases in cFos expression in the rat and mouse SCN (Recio *et al.*, 1996; Smith *et al.*, 2010). Our present findings, although using BMY7378 pretreatment instead of NAN-190, observed

cFos expression somewhat consistent with both of these previous findings. We found no change in the amount of cFos induced by light, pretreated with BMY7378, 60 minutes following light exposure, along with a trend towards decreased cFos expression at 90 minutes. We also extended previous findings by examining more of the time course of cFos expression, finding BMY7378 induces a significant increase in the amount of cFos expression 180 minutes following light exposure.

The decrease in cFos expression found previously, 60 minutes following light exposure in the rat and 90 minutes following in the mouse, is in contrast to the observation that the magnitude of light-induced cFos expression in the SCN is proportional to the size of the expected phase shift to that light pulse (Kornhauser *et al.*, 1990). This disconnect between the magnitude of phase shift and the amount of cFos expression induced by a light pulse has also been previously noted in other studies from our lab (Smith *et al.*, 2008; Sterniczuk *et al.*, 2008). Our present findings, that BMY7378 induces increased cFos expression 180 minutes following a phase shifting light pulse, could explain this disconnect previously observed. It appears that drugs such as BMY7378 may accomplish an enhancement of the phase shift magnitude by altering the time course of cFos expression, such that there is an initial decrease in expression, followed by an increase in expression that could then be what actually leads to the increased phase shift magnitude later observed in the behavioural activity rhythm.

The precise role of cFos in photic phase shifting has not yet been determined. In order to activate the AP1 promotor region on downstream genes and regulate further gene expression, cFos must dimerize with other IEG gene products to form an AP1 complex.

However, Fos-deficient mice are able to phase shift to light stimuli, albeit with a smaller magnitude than wildtype mice (Honrado *et al.*, 1996), indicating that cFos is not strictly necessary for the circadian clock to be shifted by light. In fact, it is currently still unknown exactly which combination of AP1 IEGs are involved in light-induced phase shifts. Simultaneous knockdown of both cFos and JunB using antisense oligonucleotides does block photic phase shifts (Wollnik *et al.*, 1995), further indicating that some form of an AP1 complex does appear to be necessary for photic phase shifting. The Wollnik et al. (1995) study would suggest that JunB may be the necessary AP1 complex component allowing for photic phase shifting to occur. Our current findings are consistent with this idea, such that JunB was unaffected by BMY7378 pretreatment in the first few hours following a light pulse but was increased 6 hours later.

BMY7378 and other 5-HT_{1A} mixed agonists/antagonists have been shown to enhance the magnitude of photic phase shifts when injected up to 6 hours after a light pulse (Kessler *et al.*, 2008; Lungwitz & Gannon, 2009). There are many different IEGs (Morgan & Curran, 1989) as well as the protein products of the *Period* genes that exhibit peak expression up to 6 hours following light exposure (Field *et al.*, 2000). IEGs such as fra-2, fos-B (Schwartz *et al.*, 2000), c-Jun, Jun-D, NGFI-A and NGFI-B (Rusak *et al.*, 1992) show peak expression many hours after light exposure, and thus could also be upregulated or downregulated by BMY7378 treatment. Based on our current findings, that both JunB and PER1 demonstrated increased expression 6 hours after light exposure, it would appear that these two proteins may play important roles in the underlying mechanism of photic phase shift potentiation with BMY7378 treatment.

Given the effect of BMY7378 on proteins expressed many hours following light exposure, it is possible that the early changes observed in previous experiments to cFos and P-CREB (Smith *et al.*, 2010) may not be at all related to the enhancement of photic phase shifts. It is therefore also possible that the increased activation of ERK at 60 minutes, as well as the increased expression of cFos expression at 180 minutes and CREM at 240 minutes after light exposure may be unrelated to the enhancement of phase shift magnitude with BMY7378 treatment. Although, the increased P-ERK expression at 60 minutes and increased cFos expression at 180 minutes are possible indications of an extended activation of this biochemical cascade and IEG pathways to further gene expression, such that they may remains active beyond their normal timeframes. It is therefore also possible that had the present study examined multiple time-points of CREM expression, it may have been observed that this modulator of CRE-related genes may also have had an altered time-course. This would be consistent with the observation that MKC-242, another 5-HT_{1A} agonist that also potentiates photic phase shifts, extends the normal time-course of *Period* gene expression following light exposure (Takahashi *et al.*, 2002). An extension of the activation timeframe of any genes pertinent to generating photic phase shifts, could underlie the enhancement of this process which is observed with BMY7378.

In fact, CREM modulates CRE-promotor regions by either heterodimerizing with other isoforms of itself, but also by heterodimerizing with members of the cFos and Jun families of IEGs (Lalli *et al.*, 1996). Changes is how CREM interacts with cFos or Jun IEGs would then likely alter how those proteins then dimerize and interact with their own

AP1 promotor region of the same or other genes. CREM's main repressor isoform (ICER), involved in turning off the biochemical cascades activated following an incoming light signal by dimerizing with CREM or CREB proteins to form non-activating dimers, has been shown to be increased in the rat SCN following light exposure (Molina *et al.*, 1993; Schwartz *et al.*, 2005). However, both rats and multiple strains of mice have been shown to have constitutive expression of ICER thought the day in the SCN. ICER expression has been shown to be rhythmic, even if it is always present, such that its expression peaks approximately 6 hours following the peak of P-CREB expression (Maronde *et al.*, 1999). Thus, alterations of the expression patterns or time-course of ICER could in turn alter those photoinducible genes which only begin to decline after ICER levels have begun to rise, including *fra-2* and *per2* (Schwartz *et al.*, 2005), or even JunB and PER1, which were demonstrated to be altered in the present study following BMY7378 treatment.

Although the present study found several proteins to have altered expression levels or time-courses following BMY7378 pretreatment, we still have not fully explained the molecular underpinnings of BMY7378's action on the cells of the SCN to induce enhanced photic phase shifts. By better understanding the precise underlying molecular mechanisms responsible for the modulation of the sensitivity of the mammalian circadian system to light stimuli, it may be possible to eventually develop chronobiotics which are specifically targeted for the rapid resynchronization of circadian rhythms. Effective management of even prevention of the chronic desynchronization between the internal circadian system and the external environment, such as is observed

with rotating shift work, jet lag, or circadian rhythms disorders, could play a major role in the elimination of the adverse health effects associated with such desynchrony.

Table 4.1 Summary of protein expression findings.

Summary of all proteins stained and whether they demonstrated decreased, unchanged, or increased expression levels following BMY7378 pretreatment and late subjective night light exposure.

Staining / Time Following Start of LP	↓	≈	↑
P-ERK @ 15min		✓	
P-ERK @ 30min		✓	
P-ERK @ 45min		✓	
P-ERK @ 60min			✓
P-ERK @ 120min		✓	
P-CREB @ 120min		✓	
CREM @ 240min			✓
Fos @ 60min		✓	
Fos @ 90min	trend		
Fos @ 120min		✓	
Fos @ 180min			✓
JunB @ 60min		✓	
JunB @ 90min		✓	
JunB @ 120min		✓	
JunB @ 180min		✓	
JunB @ 6hrs			✓
PER1 @ 6hrs			✓

5. Attenuation of photic phase shifts by the serotonin agonists 8-OH-DPAT and buspirone: Receptor contributions and early biochemical / molecular events

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Running Head: Photic phase shifting with serotonin agonists

Author Contributions:

Victoria Smith:
- Experimental design
- Laboratory Research: Predictions for manipulation times, light pulse manipulations, injections, phase shift calculations, perfusions, tissue collection, immunohistochemistry, microscopy, cell counting and ROD analysis
- Manuscript Preparation: Entered and organized data; ran statistical analyses, composed manuscript

Stephanie Iannatonne:
- Experiment 4: Predictions for manipulation times, light pulse manipulations, injections, phase shift calculations

Sanjay Achal:
- Experiment 1: Predictions for manipulation times, light pulse manipulations, injections, phase shift calculations

Ryan Jeffers:
- Experiment 2 & 5: Assisted with mounting some tissue onto slides

Dr. Michael Antle:
- Assisted with experimental design
- Provided laboratory, equipment and financial support necessary to carry out experiments
- Manuscript Preparation: produced figures (prepared for imminent submission to *Eur J Neurosci*), provided feedback and editing of the manuscript

Abstract

The 5-$HT_{1A/7}$ agonist 8-OH-DPAT has been shown to attenuate photic phase shifts in rodents. It is unknown if the 5-HT_{1A} partial agonist buspirone uses a comparable mechanism, or if it is more similar to the 5-HT_{1A} agonists such as MKC-242 which potentiate photic phase shifts. Both 8-OH-DPAT and buspirone are known to act through the 5-HT_{1A} receptor, however they also bind to several other receptors, making it difficult to ascertain which receptor is responsible for the photic attenuation observed. Also uncertain are the intracellular changes in the suprachiasmatic nucleus associated with such attenuation. Here we examine the role of the 5-HT_{1A} receptor, in the behavioural response to photic stimuli following pretreatment with 8-OH-DPAT and buspirone, as well as the molecular underpinnings in terms of biochemical cascade activation and gene expression following buspirone pretreatment. Administration of 8-OH-DPAT or buspirone to wildtype mice significantly attenuated late subjective night photic phase shifts, while mice lacking the 5-HT_{1A} receptor (knockouts) exhibited potentiated photic phase shifts when pretreated with either drug. In wildtype mice, the attenuated phase shifts were accompanied by increased cFos expression in the SCN, whereas the potentiated phase shifts in knockouts was accompanied by increased ERK and CREB phosphorylation, as well as decreased cFos expression. Hamsters also exhibited significantly attenuated late subjective night photic phase shifts, which were accompanied by decreased activation of the ERK and CREB biochemical cascades. It was also shown that the normal non-photic phase advance following buspirone administration at CT6 was abolished with the lack of the 5-HT_{1A} receptors in the

knockouts. These findings suggest that the 5-HT_{1A} receptor plays a critical role in the attenuation effect observed with 8-OH-DPAT and buspirone, and that buspirone may attenuate photic phase shifts at least partly by altering the activity of some genes and biochemical pathways involved in coupling the light signal to the output of the circadian clock.

Key Words: Serotonin, 8-OH-DPAT, Photic phase shift, 5-HT_{1A} receptor, P-ERK, P-CREB, cFos, JunB, Mice, Hamsters

5.1 Introduction

Circadian rhythms, regulated by the master circadian pacemaker located in the suprachiasmatic nuclei (SCN; Moore & Eichler, 1972; Stephan & Zucker, 1972), are entrained to the daily environmental cycles by light information conveyed from the retina to the SCN via the retinohypothalamic tract (RHT; Daan & Pittendrigh, 1976; Rusak & Zucker, 1979; Mintz & Albers, 1997; Mintz *et al.*, 1999). Appropriately timed light exposure during a nocturnal rodent's subjective night induces a predictable shift in the timing of activity onset (Gillette & Mitchell, 2002). Such photic phase shifts are accompanied by the induced expression of numerous genes within the cells of the SCN, including immediate-early genes (IEGs), along with the activation of second messenger signaling pathways (Guido *et al.*, 1996a; von Gall *et al.*, 1998; Guido *et al.*, 1999b; Butcher *et al.*, 2003; Dziema *et al.*, 2003).

Serotonin (5-HT) has been shown to modulate the effects of light on the circadian clock, by way of the dense innervation from the midbrain raphe nuclei into the SCN, which serves to dampen the excitatory signals sent to the cells of the SCN following light exposure (Meyer-Bernstein & Morin, 1996; Mistlberger, 2000). When administered systemically to hamsters prior to phase shifting light exposure in the late subjective night, the 5-HT agonist 8-OH-DPAT attenuates the magnitude of the elicited phase advance and suppresses light-induced IEG expression (Selim *et al.*, 1993; Glass *et al.*, 1994; Rea *et al.*, 1994; Mintz *et al.*, 1997; Moriya *et al.*, 1998; Antle *et al.*, 2003). However, the 5-HT_{1A} receptor agonist MKC-242 has been shown to potentiate the effects of phase shifting light exposure in the subjective night, in both mice and hamsters, and also

prolonged the light-induced expression of *mPer1* and *mPer2* in the SCN of mice (Moriya *et al.*, 1998; Takahashi *et al.*, 2002). Similarly, the 5-HT_{1A} receptor partial agonist BMY7378, proposed to function at least partially by acting as an agonist at the somatodendritic autoreceptors in the median raphe, has also been shown to potentiate photic phase shifts in the subjective night (Rea *et al.*, 1995; Gannon, 2003; Smith *et al.*, 2010).

The 5-HT_{1A} partial agonist buspirone is a commercially available drug, originally developed as a pharmacotherapy for generalized anxiety disorder, however its anxiolytic properties are now thought to be useful in various other neurological and psychiatric disorders (Loane & Politis, 2012). Buspirone has been shown to induce a phase advance of the wheel-running activity rhythm of hamsters when administered systemically 4 hours prior to activity onset (Tominaga *et al.*, 1992). This effect has also been reported with other 5-HT agonists, including 8-OH-DPAT, when administered systemically or directly to the raphe nuclei in the middle of the subjective day (Mintz *et al.*, 1997; Antle *et al.*, 2003; Horikawa & Shibata, 2004). The use of transgenic mice lacking either the 5-HT_{1A} or 5-HT_7 receptor indicates that each of these receptor subtypes appears to be required for the phase shift induced by 8-OH-DPAT in the mid-subjective day (Gardani & Biello, 2008; Smith *et al.*, 2008).

In addition to the uncertainty of the effect that the 5-HT partial agonist buspirone has on the phase shifts induced by light exposure in the subjective night, the precise receptor mediating the attenuation of photic phase shifts following 5-HT agonist administration, with either 8-OH-DPAT or buspirone, has yet to be elucidated. It is

equally uncertain as to what the cellular and molecular underpinnings are of the attenuation of photic phase shifts in either hamsters or mice following the administration of buspirone.

5.2 Methods

5.2.1 Animals and housing

A total of 38 adult male mice were used, consisting of 19 5-HT_{1A} receptor knockout (KO) mice and 19 5-HT_{1A} receptor wildtype (WT) mice, as well as a total of 12 adult male Syrian hamsters (*Mesocricetus auratus,* 80-90g upon arrival; Charles River, Kingston, NY). These experiments were the only studies in which these animals were use. The knockout and wildtype mice were generated from a breeding colony established in our lab from mice originally developed and generously donated by Dr. Thomas Shenk (Princeton University, Princeton, NJ), provided by Dr. Miklos Toth (Cornell University Medical College, New York, NY) and bred on the C57BL/6J background. For information regarding the generation of the 5-HT_{1A} receptor KO mice see Parks et al. (1998). Animals from our breeding colony were genotyped using a previously described protocol (see Smith *et al.*, 2008). Briefly, a pair of KO primers (amplifying a 400 base-pair product that included a portion of the neomycin cassette that replaced the start codon of the 5-HT_{1A} receptor gene) as well as a pair of WT primers (amplifying a 238 base-pair product that included a portion of the 5-HT_{1A} receptor gene replaced by the neomycin cassette in the KO animals) were used to identify the presence or absence of each allele of the 5-HT_{1A} receptor gene (KO up primer: CTT TAC GGT ATC GCC GCT CCC GAT TC; KO down primer: TGC AGG ATG GAC GAA GTG CAG CAC A; WT up primer:

AGT GCA GGC AGG CAT GGA TAT GTT; WT down primer: CCG ATG AGA TAG TTG GCA ACA TTC TGA).

Mice were individually housed in Nalgene Type L clear polycarbonate cages (Nalg Nunc International, Rochester, NY), equipped with a stainless steel running wheel (diameter of 24.2 cm). Hamsters were individual housed in polycarbonate cages (20 x 45 x 22 cm) equipped with running wheels (diameter 14 cm). Animals were maintained in 0 lux constant darkness (DD) for the duration of all studies, and were housed in a temperature- (21 ± 1°C) and humidity-controlled room, with *ad libitum* access to food and water. Cages were changed approximately every 14 days (7-10 days prior to, and following a manipulation). All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary, and adhered to the Canadian Council on Animal Care guidelines for the ethical use of animals in research.

5.2.2 Drugs and reagents

The 5-$HT_{1A/7}$ receptor agonist (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT) and 5-HT_{1A} receptor partial agonist buspirone were obtained from Sigma-Aldrigh Canada, Ltd. (Oakville, ON). Both drugs were dissolved in physiological saline. Injections were administered intraperitoneally (i.p.), with the dose of 8-OH-DPAT being 5mg/kg (based on the dose previously used by Smith et al. (2008) and the dose of buspirone being 30mg/kg (based on the dose used previously used by Tominaga et al. (1992). Vehicle control injections (physiological saline) were comparable by volume based on weight (0.1mL/30g mouse or 0.1mL/100g hamster). All injections

were performed in constant darkness with the aid of night-vision goggles (BG15Alista, Richmond Hill, ON).

5.2.3 Activity rhythms

Wheel-running activity was continuously monitored using magnetic switches mounted on the running wheel, and connected to a computer running the Clocklab data collection software package (Coulbourn Instruments, Allentown, PA). Actograms (graphical representation of wheel-running behavior) were generated and analyzed by Clocklab analysis software. Mice and hamsters were allowed to freerun in constant darkness (DD) for at least 10 days prior to the start of manipulations. Onset of running wheel activity for manipulation day was predicted using a regression line fit to the activity onsets for the 7-10 days prior to the testing day.

5.2.4 Experiment 1: Photic phase shifts following 8-OH-DPAT and buspirone pretreatment in mice

Experiment 1 examined the effects of 8-OH-DPAT and buspirone on the behavioural phase shifts of activity rhythm induced by brief exposure to dim light. Mice were housed in DD (0 lux) for the duration of the experiment. The dim light pulse was administered using a light-sealed box with a light bulb partially covered in aluminum foil such that the light intensity at cage level was measured at 40 lux. Mice (n = 26, 13 KO and 13 WT) were exposed to a pretreatment of an i.p. injection of either 8-OH-DPAT, buspirone or vehicle control 45 minutes prior to a 15-minute light pulse at two different phases in their circadian activity rhythm, either 4 hours or 10 hours after their predicted activity onset (i.e., either at circadian time (CT) 16 or 22, where activity onset is

designated to be CT12 by convention). Animals received an injection of one of the drugs and vehicle control on separate occasions in a counterbalanced fashion.

Following the manipulations, actograms were analyzed using the automatic line-fitting function included in the Clocklab software, creating one line of best fit for the activity onsets of the 10 days prior to and including the manipulation day, and another line of best fit for the 10 days following the light pulse (excluding the first 3 onsets to avoid the effects of transient cycles). The phase shift in the behavioral activity rhythm was calculated based on the horizontal difference between the predicted onsets from the two regression lines on the day following the manipulation.

5.2.5 Experiment 2: Molecular mechanisms underlying photic phase shifts in mice pretreated with buspirone

Experiment 2 examined the effects of buspirone followed by a brief exposure to light (40 lux at cage level) on the phosphorylation of ERK and CREB proteins, and the levels of cFos and JunB proteins. Mice (n = 38, 19 KO and 19 WT) were randomly assigned to a treatment group (buspirone or vehicle control) and for the expression of either the phosphorylated form of ERK and CREB (P-ERK and P-CREB, respectively; n = 12, 6 KO and 6 WT) or the expression of cFos and JunB (n = 26, 13 KO and 13 WT).

For experiments involving the phosphorylation of ERK and CREB, animals were euthanized with an i.p. injection of sodium pentobarbital (240mg/mL, 0.3mL per mouse), 15 minutes after the start of the light pulse (i.e., at CT22.25). For experiments examining the expression of cFos and JunB, animals were euthanized 90 minutes after the start of the light pulse (i.e., at CT23.5). The animal's head was wrapped in aluminum foil to

prevent the retinas from being exposed to light prior to and during the perfusion. Animals were then perfused using only 50 mL of buffered 4% paraformaldehyde (ERK, CREB), or perfused using approximately 50 mL of phosphate buffered saline (PBS) followed by 50 mL of buffered 4% paraformaldehyde (cFos, *JunB*). Brains were extracted and postfixed in 4% paraformaldehyde overnight, and subsequently cryoprotected in a 20% sucrose solution for 24 hours. Brains were sliced through the anterior hypothalamus using a cryostat, with alternate 35 μm sections through the SCN being collected for immunohistochemistry (IHC).

5.2.6 Experiment 3: Non-photic phase shifts to the serotonin partial agonist buspirone

Experiment 3 examined the behavioural phase shifts of activity rhythm induced by buspirone administration in the middle of the subjective day. Mice were housed in DD (0 lux) for the duration of the experiment. Mice (n = 12, 6 KO and 6 WT) were exposed to an i.p. injection of either buspirone or vehicle control 6 hours prior to their predicted activity onset (i.e., CT6). Animals received an injection of the drug and vehicle control on separate occasions in a counterbalanced fashion. Following the manipulations, actograms were analyzed as described previously.

5.2.7 Experiment 4: Photic phase shifts following buspirone pretreatment in hamsters

Experiment 4 examined the effects of buspirone on the behavioural phase shifts of activity rhythm induced by brief exposure to dim light. Hamsters were housed in DD (0 lux) for the duration of the experiment. The 40 lux dim light pulse was administered as

described previously. Hamsters (n = 12) were exposed to a pretreatment of an i.p. injection of either buspirone or vehicle control 45 minutes prior to a 15-minute light pulse at two different phases in their circadian activity rhythm, either 1 hour or 6 hours after their predicted activity onset (i.e., either at CT13 or CT18). Animals received an injection of the drug and vehicle control on separate occasions in a counterbalanced fashion.

5.2.8 Experiment 5: Molecular mechanisms underlying photic phase shifts in hamsters pretreated with buspirone

Experiment 5 examined the effects of buspirone followed by a brief exposure to light (40 lux at cage level) on the phosphorylation of ERK and CREB proteins. Hamsters (n = 12) were randomly assigned to a treatment group (buspirone or vehicle control). Animals were euthanized with an i.p. injection of sodium pentobarbital (240mg/mL, 0.7mL per hamster), 15 minutes after the start of the light pulse (i.e., at CT18.25). The animal's head was wrapped in aluminum foil to prevent the retinas from being exposed to light prior to and during the perfusion. Animals were then perfused using 50 mL of buffered 4% paraformaldehyde. Brains were extracted and postfixed in 4% paraformaldehyde overnight, and subsequently cryoprotected in a 20% sucrose solution for 24 hours. Brains were sliced through the anterior hypothalamus using a cryostat, with alternate 35 μm sections through the SCN being collected for immunohistochemistry (IHC).

5.2.9 Diaminobenzidine (DAB) immunohistochemistry

Diaminobenzidine IHC staining was completed using a previously described method from our lab (see Antle *et al.*, 2008). Brain sections were exposed to a series of

treatments at room temperature on a shaker tray unless otherwise indicated. Sections were rinsed for 30 min in 0.5% H_2O_2 in PBSx to inactivate endogenous peroxidases, followed by three 10 min PBSx rinses, a 90 min incubation in 1% normal goat serum (for P-ERK and P-CREB) or 1% normal horse serum (for cFos) in PBSx, and a 2-day incubation at 4°C in the primary antibody for P-ERK (rabbit anti-Phospho-p44/42 MAP Kinase (Thr202/Tyr204; 1:600; (#9101) Cell Signaling Technology, Inc., Danvers, MA), P-CREB (rabbit anti-Phospho CREB; 1:250; (#9198) Cell Signaling Technology, Inc.) or cFos (goat anti-cFos; 1:20,000; (Sc 52-G) Santa Cruz Biotechnology, Santa Cruz, CA) in 1% normal goat or horse serum. These antibodies have been shown by the manufacturer to be highly selective for their respective antigens. Subsequently, sections were exposed to three 10 min PBSx rinses, a 1 h incubation in the secondary antibody biotinylated goat anti-rabbit (for P-ERK and P-CREB; 1:200; Vector Laboratories, Burlingame, CA) or horse anti-goat (for cFos; 1:200; Vector Laboratories, Burlingame, CA), three 10 min PBSx rinses, a 1 h incubation in ABC (Vectastain Elite kit, Vector Laboratories, Burlingame, CA), and three 10 min PBSx rinses. Slices were then developed for approximately 5 min in 0.05% DAB and 0.02% NiCl in 25mL of 0.1M Tris buffer activated with 80μL of 30% H_2O_2. Finally, slices were mounted on gelatin-coated slides, air-dried, dehydrated through an alcohol series, cleared with xylenes, and coverslipped with Permount.

5.2.10 Fluorescent single-label immunohistochemistry

Sections were labeled for JunB (mouse anti-JunB; 1:2,000; (sc-8051) Santa Cruz Biotechnology, Santa Cruz, CA). IHC staining was completed using a previously

described method (see Smith *et al.*, 2008). Briefly, brain sections were exposed to three 10-min washes in 0.1M PBS, followed by a 1 h incubation in a blocking buffer (2% normal donkey serum, in PBSx), after which the tissue was incubated for 48 h at 4°C in the primary antibody diluted in blocking buffer. Tissue was then washed using six 10-min rinses in 0.1% PBS with 0.3% Triton X-100 (PBSx), and left overnight in 0.1% PBSx. Finally, the brain sections were washed with another six 10-min 0.1% PBSx rinses, incubated for 1 h in the secondary antibody AlexaFluor 488 donkey anti-mouse (1:200; (#715-545-150) Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and exposed to three final 10-min washes in 0.1M PBS. Sections were then mounted on gelatin-coated slides, air dried, dehydrated in a series of alcohol rinses, cleared with xylenes, and coverslipped with Krystalon.

5.2.11 Quantification of biochemical cascade components and protein expression

Images of the immunofluorescently-labeled tissue were captured via an Olympus BX51 microscope equipped with filters for the cyanine dye. Bilateral counts of JunB-immunoreactive (JunB-IR) cells were performed using computerized image analysis software (ImagePro Plus 7.0.0.591; Media Cybernetics, Inc., Bethesda, MD), and were manually counted by the researcher who was blind to the treatment conditions. Images of the DAB-labeled tissue were captured using the bright field setting of the same microscope. Bilateral counts of P-CREB-labeled and cFos-labeled cells were performed using the same techniques as used above. Bilateral calculations of the relative optical density (ROD) of P-ERK expression were performed using computerized image analysis software (ImageJ 1.42q; National Institutes of Health, Bethesda, MD). The grey level of

the SCN was expressed as a ratio over the grey level of adjacent non-SCN tissue in the same frame. Cell counts and ROD were calculated at the mid-caudal level of the SCN (Experiment 2) and at three levels of the SCN centered around the mid-caudal level of the SCN (Experiment 5). The sections approximately 140μm anterior and posterior to the mid-caudal level were included in the analysis.

5.2.12 Statistical analysis

For experiment 1, a mixed two-way analysis of variance (ANOVA) was used to determine if there was a significant difference in the magnitude of the photic phase shifts between the genotypes in each of the three pretreatment conditions (8-OH-DPAT, buspirone or vehicle control) for each of the two time points separately (CT16 and CT22). For experiment 2, the light-induced expression of P-ERK, P-CREB, cFos, and JunB were each analyzed using a two-way ANOVA to determine the effect of buspirone pretreatment compared to vehicle control between the two genotypes. For experiment 3, a two-way repeated measures ANOVA was used to determine if there was a statistical difference in the magnitude of the phase shifts induced by buspirone compared to vehicle control between the two genotypes. For experiment 4, a paired t-test was used to determine if there was a significant difference in the magnitude of the photic phase shifts in each of the two pretreatment conditions (buspirone or vehicle control) at each of the two time points separately (CT13 and CT18). For experiment 5, the light-induced expression of P-ERK and P-CREB were each analyzed using a mixed two-way ANOVA to determine the effect of buspirone pretreatment compared to vehicle control at each of the three rostral-caudal levels. Statistical significance was set at $p<0.05$ for all tests, using

SigmaPlot Version 11.2.0.5 (Systat Software, Inc.; San Jose, CA). All significant ANOVAs were followed up by Student-Newman-Keuls post hoc multiple comparisons. All means are reported as ± standard error of the mean.

5.3 Results

5.3.1 Experiment 1: Photic phase shifts following 8-OH-DPAT and buspirone pretreatment in mice

As expected, brief light exposure at CT 16 produced phase delays, while light exposure at CT 22 produced phase advances, following all pretreatments. While significantly smaller phase shifts to light exposure at CT16 were observed in KO animals following all pretreatments (main effect of genotype: $F_{(1,22)} = 8.316$, $p < 0.01$), there was no significant effect of either 8-OH-DPAT or buspirone on the magnitude of phase shift elicited at CT16 (main effect of treatment: $F_{(2,22)} = 3.076$, $p = 0.066$; Figure 5.1). However, there was no significant interaction between genotype and injection pretreatment ($F_{(2,22)} = 0.320$, $p = 0.729$). Following light exposure at CT22, however, both 8-OH-DPAT and buspirone pretreatment significantly attenuated the magnitude of the phase shift induced in WT animals, but significantly potentiated the phase shift induced in KO animals (Genotype x Treatment interaction: $F_{(2,22)} = 20.090$, $p < 0.001$; Figure 5.2). Specifically, wildtype animals pretreated with 8-OH-DPAT demonstrated significantly smaller photic phase shifts than those receiving a pretreatment of saline vehicle ($p < 0.01$), as did wildtype animals receiving a pretreatment of buspirone compared to saline ($p < 0.05$). There was no significant difference between the phase shifts elicited in wildtype animals by 8-OH-DPAT and buspirone ($P = 0.361$). In contrast,

knockout animals demonstrated significantly potentiated photic phase shifts following 8-OH-DPAT pretreatment ($P < 0.05$) or buspirone pretreatment ($P < 0.01$) when compared to saline control.

Figure 5.1 Mean phase shifts in WT and KO mice with 8-OH-DPAT and buspirone with light exposure at CT16.

(A) Mean phase shifts (±SEM) of wildtype and knockout animals treated with a light pulse at CT16, pretreated with saline (control), 8-OH-DPAT (5 mg/kg) or buspirone (30 mg/kg) at CT15.25. The negative numbers indicate a phase delay of the activity rhythm. (B-E) Representative actograms of running wheel activity for (B,D) a wildtype and (C,E) a knockout animal receiving a 15-minute 40 lux light pulse at CT16 pretreated with an i.p. injection of (B,C) saline and 8-OH-DPAT or (D,E) saline and buspirone at CT15.25. Each horizontal line of an actogram represents a single day, with each subsequent day being plotted directly below the previous day. Daily activity is represented by black vertical marks in each row, with the height of the vertical marks being proportional to the amount of activity.

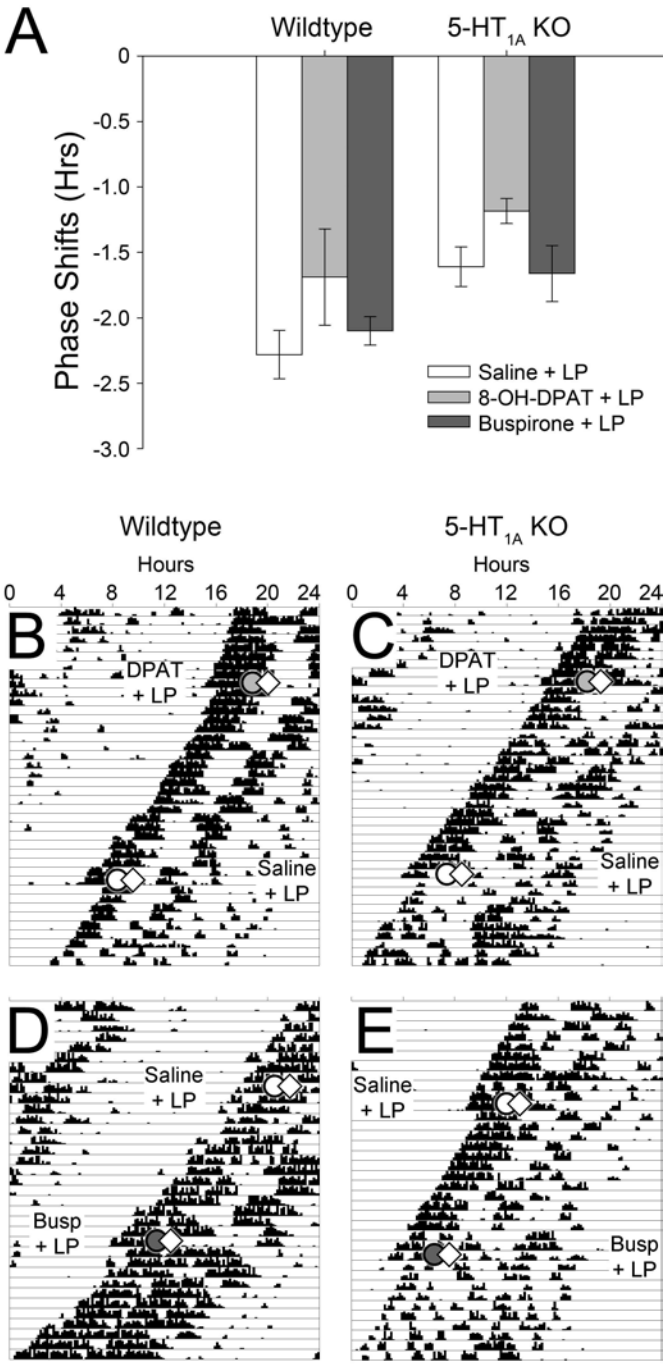
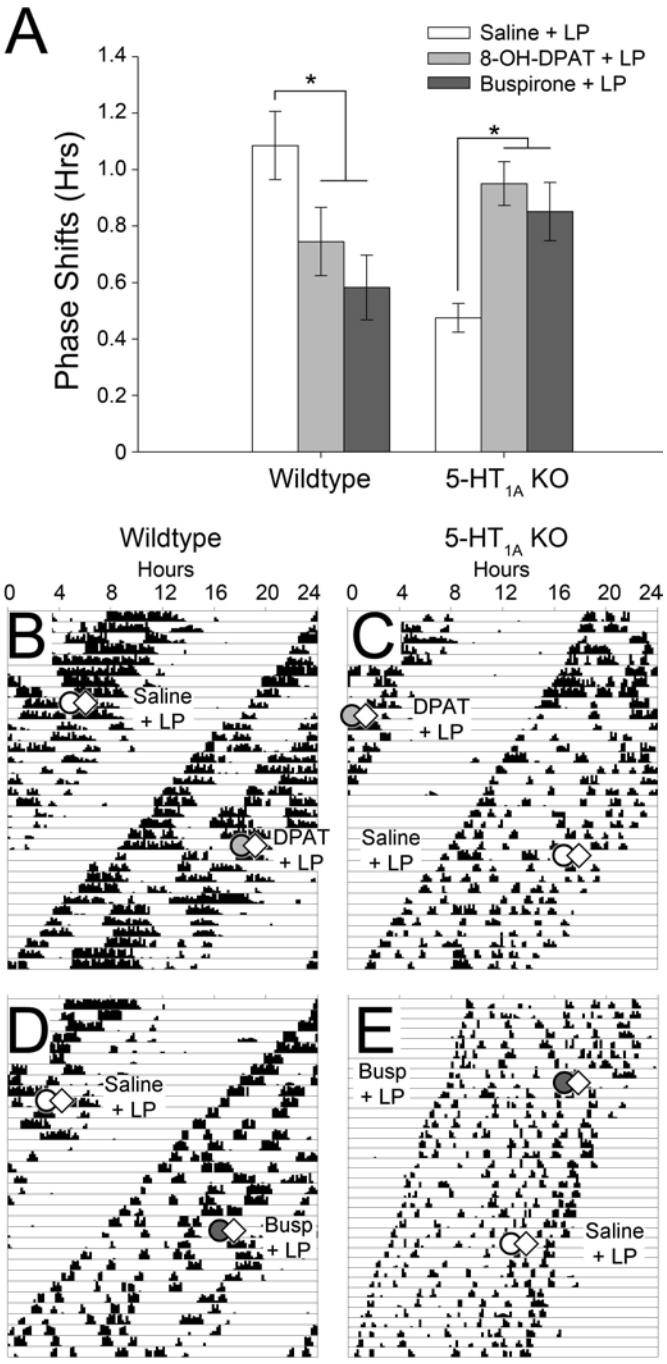

A
Wildtype
5-HT1A KO
Phase Shifts (Hrs)
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Saline + LP
8-OH-DPAT + LP
Buspirone + LP
Wildtype
5-HT1A KO
Hours
0 4 8 12 16 20 24
B
DPAT + LP
Saline + LP
C
DPAT + LP
Saline + LP
D
Saline + LP
Busp + LP
E
Saline + LP
Busp + LP

Figure 5.2 Mean phase shifts in WT and KO mice with 8-OH-DPAT and buspirone with light exposure at CT22.

(A) Mean phase shifts (±SEM) of wildtype and knockout animals treated with a light pulse at CT22, pretreated with saline (control), 8-OH-DPAT (5 mg/kg) or buspirone (30 mg/kg) at CT21.25. The positive numbers indicate a phase advance of the activity rhythm. (B-E) Representative actograms of running wheel activity for (B,D) a wildtype and (C,E) a knockout animal receiving a 15-minute 40 lux light pulse at CT22 pretreated with an i.p. injection of (B,C) saline and 8-OH-DPAT or (D,E) saline and buspirone at CT21.25. (*) Indicates statistical significance between control and drug treatments within a genotype to be $p < 0.05$.


A
Saline + LP
8-OH-DPAT + LP
Buspirone + LP
Phase Shifts (Hrs)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
Wildtype
5-HT1A KO
Wildtype
5-HT1A KO
Hours
0 4 8 12 16 20 24
B
Saline + LP
DPAT + LP
C
DPAT + LP
Saline + LP
D
Saline + LP
Busp + LP
E
Busp + LP
Saline + LP

5.3.2 Experiment 2: Molecular mechanisms underlying photic phase shifts in mice pretreated with buspirone

Late night light exposure led to high levels of P-ERK, P-CREB, cFos and JunB expression at the mid-caudal level of the SCN of both saline and buspirone pretreated mice. Although there was a significant interaction between genotype and treatment ($F_{(1,8)} = 6.262$, $p < 0.05$; Figure 5.3a,b), there was no significant effect of buspirone on the phosphorylation of ERK in either WT ($p = 0.099$) or KO mice ($p = 0.134$). There was, however, significantly more immunoreactivity for P-ERK observed in KO animals than WT animals that were pretreated with buspirone ($p < 0.05$). There was a non-significant trend towards higher levels of P-CREB in the SCN of animals pretreated with buspirone ($F_{(1,8)} = 3.966$, $p = 0.082$; Figure 5.3c,d). When examined separately, there was no effect of treatment on the P-CREB expression in WT mice ($t_{(4)} = 0.138$, $p = 0.897$), while there was a significant potentiation in the amount of P-CREB in KO mice following buspirone pretreatment ($t_{(4)} = 11.355$, $p < 0.001$).

Buspirone pretreatment also had a significant effect on the number of cFos-immunoreactive cells in the SCN following late night light exposure ($F_{(1,20)} = 4.937$, $p < 0.05$; Figure 5.4a,b). Specifically, buspirone pretreatment significantly increased the number of Fos-expressing cells in WT mice ($p < 0.01$) compared to saline pretreatment, whereas there was no effect of treatment in KO mice ($p = 0.934$). There were also significantly more cFos-immunoreactive cells in WT mice than KO mice following phase shifting light exposure in the late subjective night pretreated with buspirone ($p <0.01$). There was also a non-significant trend towards lower levels of JunB expression in the

SCN of animals pretreated with buspirone ($F_{(1,21)} = 2.523$, $p = 0.127$; Figure 5.4c,d), such that there was no effect of treatment in WT mice ($p = 0.711$) and a trend towards fewer JunB-immunoreactive cells in KO mice treated with buspirone compared to saline treatment ($p = 0.095$).

Figure 5.3 Photomicrographs of P-ERK and P-CREB expression at 15 minutes following the start of the light exposure, mean P-ERK ROD and mean number of P-CREB-IR cells.

Representative SCN tissue slices from wildtype and knockout animals indicating cells expressing the phosphorylated form of ERK (P-ERK; A) and CREB (P-CREB; C) at CT22.25 stained using DAB immunohistochemistry following a 15-min 40 lux light pulse at CT22, pretreated with an i.p. injection of either saline or buspirone at CT21.25. (B,D) Mean relative optical density (±SEM) of P-ERK (B) and mean number of P-CREB-immunoreactive (IR) nuclei (±SEM) in the SCN of animals euthanized 15 min following a light pulse at CT22 and pretreated with either saline or buspirone. (*) Indicates statistical significance between treatments in knockout animals to be $p < 0.05$. Scale bar = 200μm.

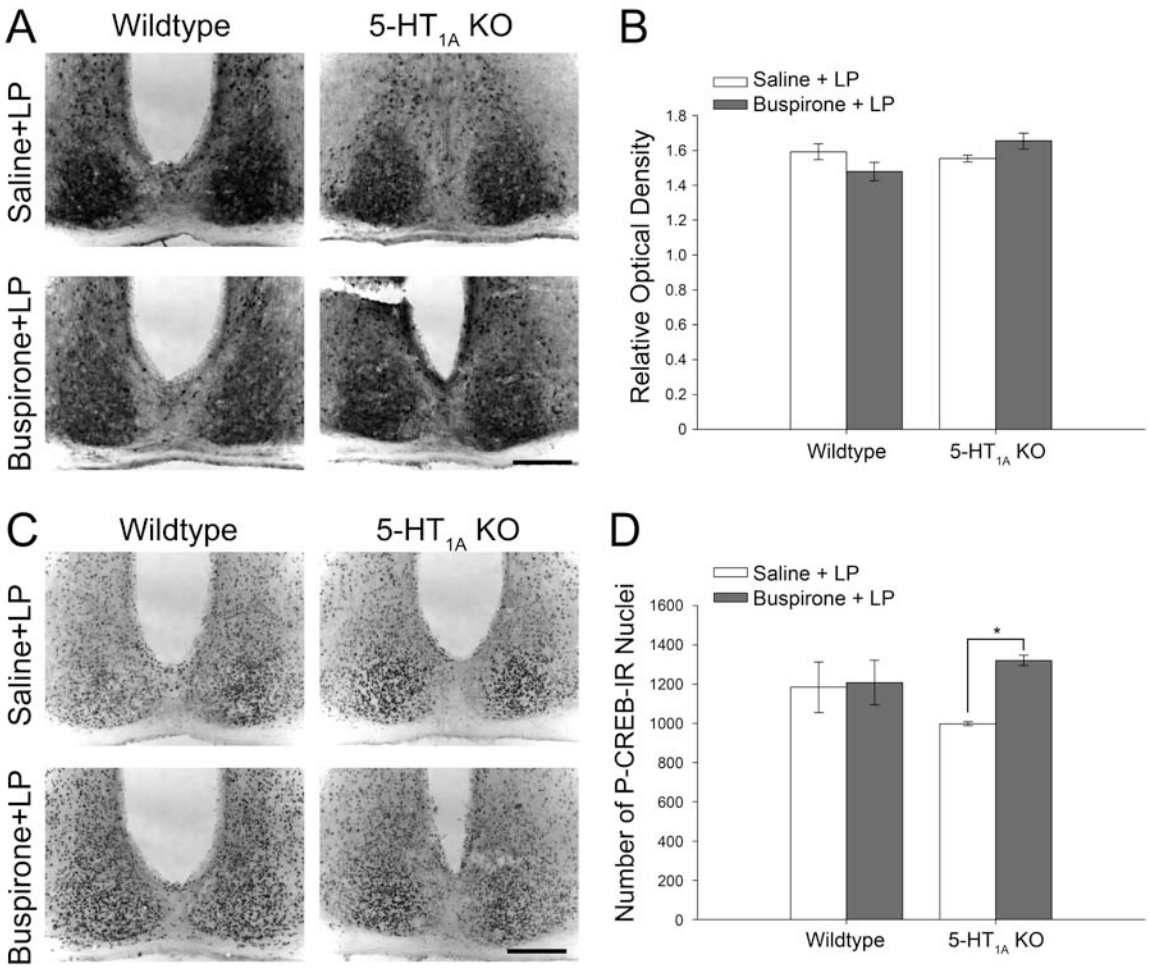
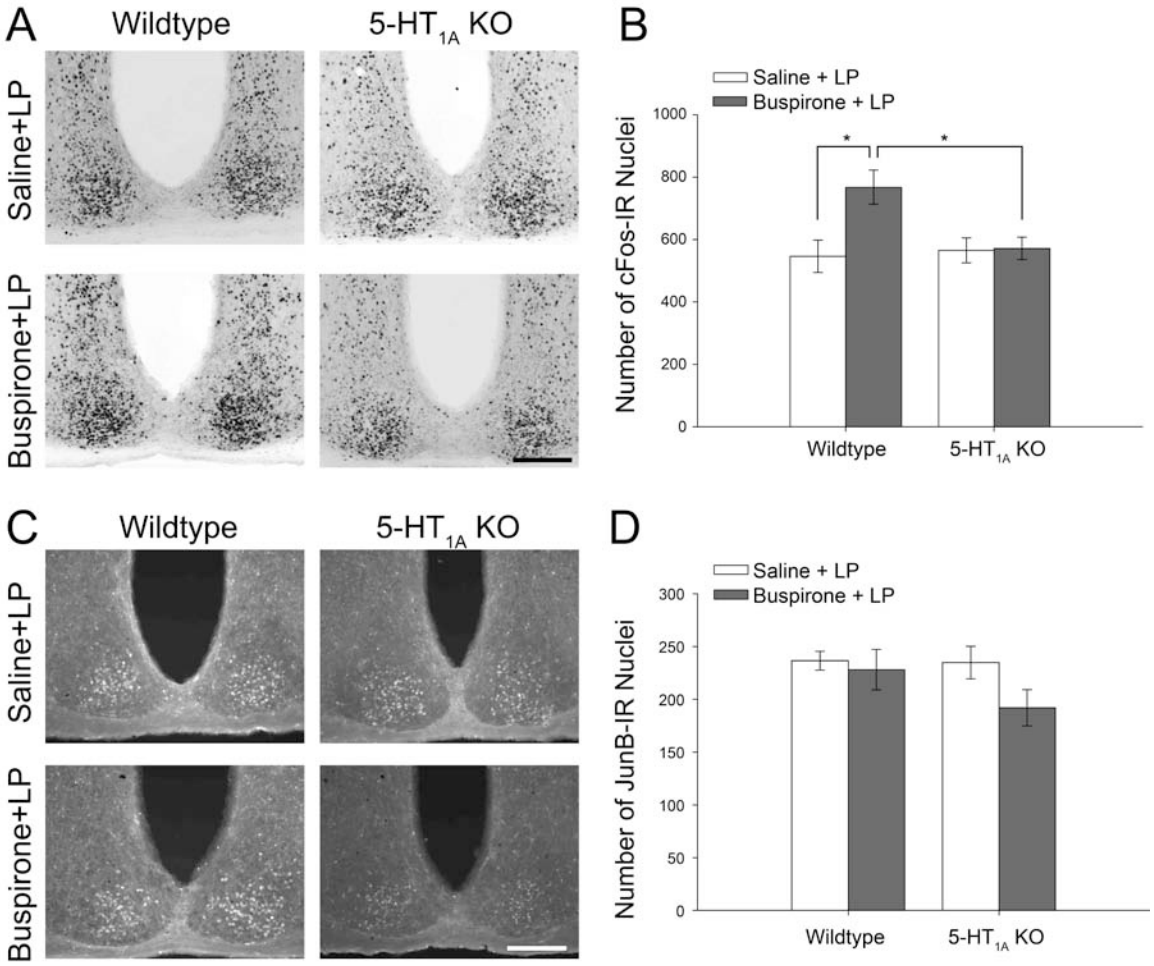

A
Wildtype
5-HT1A KO
Saline+LP
Buspirone+LP
B
Saline + LP
Buspirone + LP
Relative Optical Density
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Wildtype
5-HT1A KO
C
Wildtype
5-HT1A KO
Saline+LP
Buspirone+LP
D
Saline + LP
Buspirone + LP
Number of P-CREB-IR Nuclei
1600
1400
1200
1000
800
600
400
200
0
*
Wildtype
5-HT1A KO

Figure 5.4 Photomicrographs of cFos and JunB expression at 90 minutes following the start of the light exposure and the mean number of cFos-IR and JunB-IR cells.

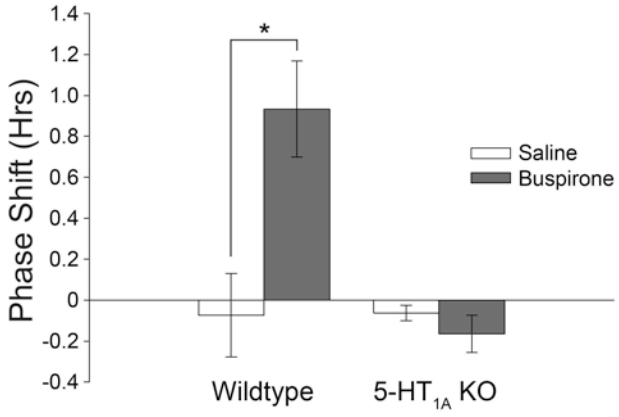
Representative SCN tissue slices from wildtype and knockout animals indicating cells expressing the cFos (A) and JunB (C) at CT23. 5 stained using DAB immunohistochemistry (A) or single-label fluorescent immunohistochemistry (C) following a 15-min 40 lux light pulse at CT22, pretreated with an i.p. injection of either saline or buspirone at CT21.25. (B,D) Mean number of cFos-immunoreactive (IR;B) and JunB-IR (D) nuclei (±SEM) in the SCN of animals euthanized 90 min following a light pulse at CT22 and pretreated with either saline or buspirone. (*) Indicates statistical significance between treatments in a genotype or between genotypes on a treatment to be $p < 0.05$. Scale bar = 200μm.


A
Wildtype
5-HT1A KO
Saline+LP
Buspirone+LP
B
Saline + LP
Buspirone + LP
Number of cFos-IR Nuclei
1000
800
600
400
200
0
*
*
Wildtype
5-HT1A KO
C
Wildtype
5-HT1A KO
Saline+LP
Buspirone+LP
D
Saline + LP
Buspirone + LP
Number of JunB-IR Nuclei
300
250
200
150
100
50
0
Wildtype
5-HT1A KO

5.3.3 Experiment 3: Non-photic phase shifts to the serotonin partial agonist buspirone

The magnitude of phase shifts induced by the 5-HT_{1A} partial agonist buspirone and its vehicle control (saline) at CT6, resulted in a significant interaction between genotype and injection ($F_{(1,9)} = 9.526$, $p < 0.05$; Figure 5.5). Specifically, WT mice exhibited significantly larger phase advances when treated with buspirone compared to vehicle control ($p < 0.01$). Whereas, KO mice injected with buspirone demonstrated not just smaller phase advances, but actually reversed their phase shift to a delay, which was larger but not significantly different from the very small phase delay observed with the injection of saline ($p = 0.710$).

Figure 5.5 Mean phase shifts of WT and KO mice with saline and buspirone at CT6. Mean phase shifts (±SEM) of wildtype and knockout animals treated with either saline or buspirone at CT6. (*) Indicates statistical significance between treatments in a genotype to be $p < 0.05$.

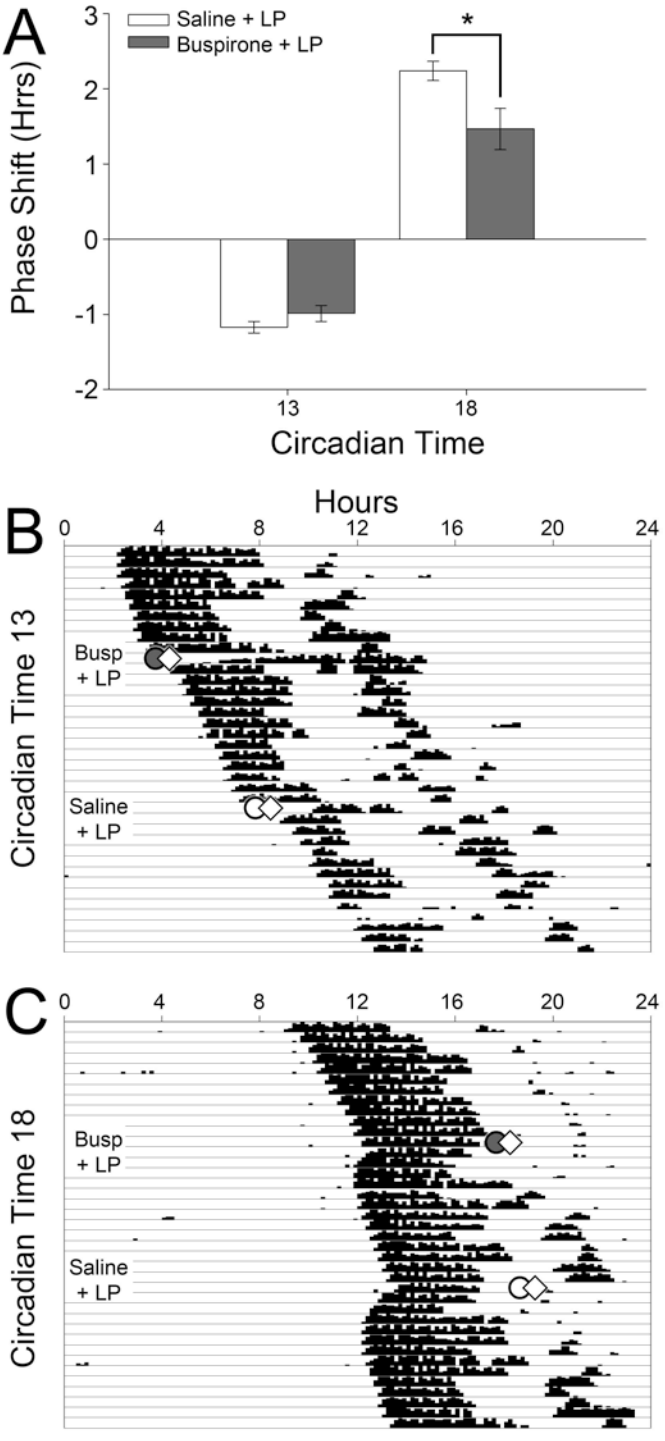

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
Phase Shift (Hrs)
*
Saline
Buspirone
Wildtype
5-HT1A KO

5.3.4 Experiment 4: Photic phase shifts following buspirone pretreatment in hamsters

Buspirone pretreatment had no significant effect on the magnitude of photic phase shifts induced by light exposure at CT13 ($t_{(20)} = 1.384$, $p = 0.182$; Figure 5.6). However, phase shifts, induced by light exposure at CT18, were significantly smaller following buspirone pretreatment than following saline control pretreatment ($t_{(20)} = 2.565$, $p < 0.05$).

Figure 5.6 Mean photic phase shifts and actograms of hamsters pretreated with saline and buspirone.

(A) Mean phase shifts (±SEM) of hamsters treated with a light pulse at CT13 and CT18, pretreated with saline (control) or buspirone (30 mg/kg) at CT12.25 or CT17.25. A positive value indicates a phase advance of the activity rhythm, while a negative value indicates a phase delay. (B,C) Representative actograms of running wheel activity for a hamster receiving a 15-min 40 lux light pulse at either (B) CT13 or (C) CT18, pretreated by an i.p. injection of saline or buspirone. (*) Indicates statistical significance between treatments to be $p < 0.05$.

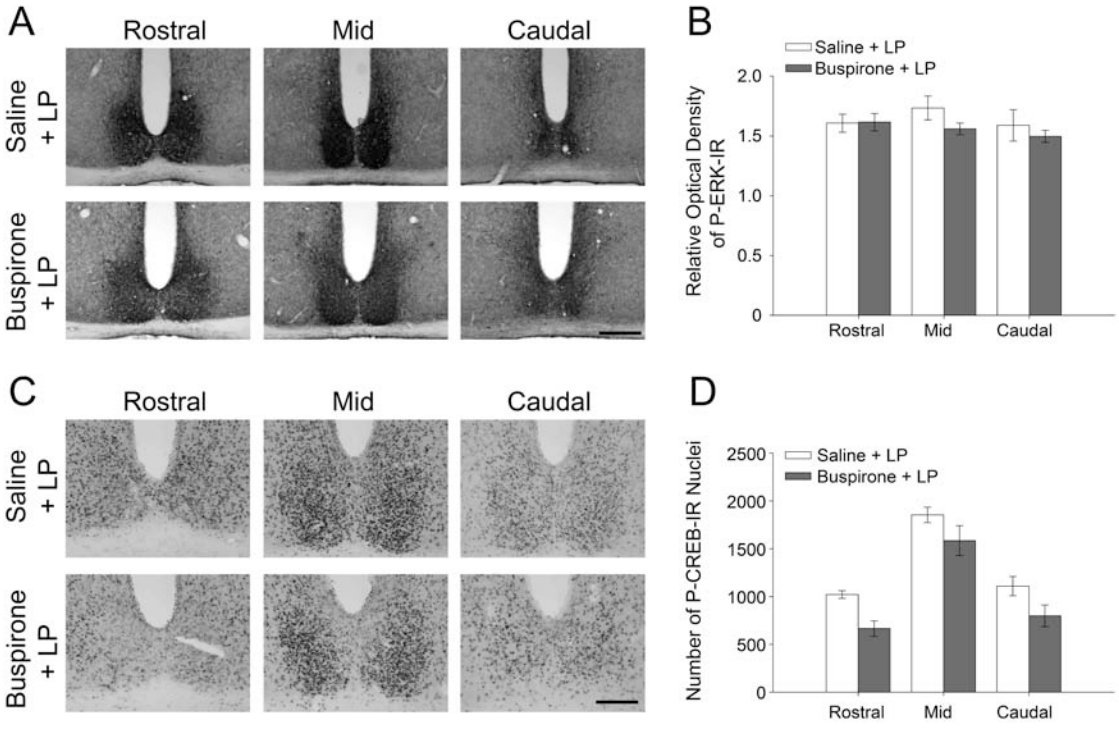

A
Saline + LP
Buspirone + LP
*
Phase Shift (Hrrs)
3
2
1
0
-1
-2
13
18
Circadian Time
B
Hours
0
4
8
12
16
20
24
Circadian Time 13
Busp
+ LP
Saline
+ LP
C
0
4
8
12
16
20
24
Circadian Time 18
Busp
+ LP
Saline
+ LP

5.3.5 Experiment 5: Molecular mechanisms underlying photic phase shifts in hamsters pretreated with buspirone

Light exposure at CT18 led to high levels of both P-ERK and P-CREB expression in the SCN of hamsters pretreated with both saline and buspirone. As expected, there was a significant change in the amount of P-ERK expression across the three SCN levels examined (main effect of SCN level: $F_{(2,20)} = 3.656$, $p < 0.05$; Figure 5.7a,b). Specifically, there was significantly less P-ERK expression at the caudal level when compared to the mid level ($p < 0.05$), whereas the rostral level was no different in terms of expression when compared to either the mid ($p = 0.381$) or the caudal ($p = 0.178$) levels. There was no significant effect of treatment ($F_{(1,20)} = 0.595$, $p = 0.458$), such that buspirone pretreatment had no effect on the amount of P-ERK immunoreactivity compared to saline pretreatment at any of the three SCN levels examine. There was also a significant change in the amount of P-CREB expression across the three levels of the SCN examined (Main effect of Level: $F_{(2,20)} = 90.627$, $p < 0.001$; Figure 5.7c,d), with significantly higher expression at the mid level than either the rostral ($p < 0.001$) or caudal ($p < 0.001$) levels. There was also significantly fewer P-CREB immunoreactive cells in hamsters pretreated with buspirone than saline (Main effect of treatment: $F_{(1,20)} = 6.989$, $p < 0.05$). Specifically, buspirone resulted in significantly fewer cells expressing P-CREB at the rostral ($p <0.05$) and caudal ($p < 0.05$) levels, while there was a non-significant trend at the mid level ($p = 0.075$).

Figure 5.7 Photomicrographs of P-ERK and P-CREB expression in the SCN, mean P-ERK ROD and mean number of P-CREB-IR cells.

Representative SCN tissue slices from hamsters at three rostral-caudal levels indicating cells expressing the phosphorylated form of ERK (P-ERK; A) and CREB (P-CREB; C) at CT18.25 stained using DAB immunohistochemistry following a 15-min 40 lux light pulse at CT18, pretreated with an i.p. injection of either saline or buspirone at CT17.25. (B,D) Mean relative optical density (±SEM) of P-ERK (B) and mean number of P-CREB-immunoreactive (IR) nuclei (±SEM) in the SCN of animals euthanized 15 min following a light pulse at CT18 and pretreated with either saline or buspirone. For (A) scale bar = 300μm, for (C) scale bar = 150 μm.


A
Rostral
Mid
Caudal
Saline + LP
Buspirone + LP
B
Saline + LP
Buspirone + LP
Relative Optical Density of P-ERK-IR
2.0
1.5
1.0
0.5
0
Rostral
Mid
Caudal
C
Rostral
Mid
Caudal
Saline + LP
Buspirone + LP
D
Saline + LP
Buspirone + LP
Number of P-CREB-IR Nuclei
2500
2000
1500
1000
500
0
Rostral
Mid
Caudal

5.4 Discussion

In this study, we show that mice exhibit attenuated photic phase advances when pretreated with the 5-HT_{1A} agonists 8-OH-DPAT or buspirone. However, the elimination of the 5-HT_{1A} receptors in the knockout animals not only eliminated this attenuation of photic phase advances, but actually led to a significant potentiation of the response. The attenuated photic phase advance following buspirone pretreatment in wildtype mice was accompanied by increased cFos activation in the SCN, but unaltered expression of P-ERK, P-CREB and JunB. In contrast, the potentiated photic phase advance resulting from buspirone pretreatment in knockout mice was accompanied by increased CREB activation and a trend towards decreased JunB expression in the SCN when compared to saline pretreatment, as well as increased P-ERK expression and decreased cFos expression as compared to the expression observed in wildtype mice pretreated with buspirone. The lack of 5-HT_{1A} receptors also eliminated the non-photic phase advance normally observed following the administration of buspirone in the middle of the subjective day. We show that hamsters also exhibit attenuated photic phase advances when pretreated with buspirone, and this attenuation is accompanied by decreased P-ERK and P-CREB expression in portions of the SCN.

Buspirone is considered to be an anxiolytic drug, which was initially developed as an antipsychotic drug acting on dopamine D_2 receptors. However, it was soon discovered that it was ineffective for the targeted psychotic symptoms (Loane & Politis, 2012). Instead, it was found to bear resemblance to benzodiazepines, proving to be clinically effective in the treatment of generalized anxiety disorder. It is suggested that the main

neuropharmacological effects of buspirone are mediated by the 5-HT_{1A} receptor, and that acting as a partial agonist it has an inhibitory effect on neurotransmission by binding to the 5-HT_{1A} receptor and dislocating the inhibitory G-proteins which serve to inhibit adenylate cyclase and prevent the conversion of ATP to cAMP along with preventing the initiation of other second messenger signaling mechanisms, all of which inhibits neuronal depolarization (Loane & Politis, 2012). In fact, it is thought to be a full agonist for presynaptic 5-HT_{1A} receptors in the raphe nuclei where it inhibits 5-HT neuronal firing as well as the synthesis and use of 5-HT. However, buspirone has also been shown to possess weak affinity for the 5-HT_2 receptors and preferentially blocks presynaptic dopamine D_2 autoreceptors as opposed to postsynaptic D_1 and D_2 receptors (with a 15-fold weaker affinity for D_2 receptor than for 5-HT_{1A} receptors), and even increases noradrenergic levels at some doses due to an affinity for both α_1- and α_2-adrenergic receptors (Rimele *et al.*, 1987; Loane & Politis, 2012).

Evidence from both hamsters and mice suggests that 5-HT inhibits the activation of both retinal terminals in the SCN (Rea & Pickard, 2000) and the retinorecipient neurons within the SCN (Ying & Rusak, 1994), producing an overall inhibition of the excitatory effects of light on the circadian clock. This serotonergic modulation of photic phase shifting is thought to be mediated by 5-HT_{1B} receptors on RHT terminals (Pickard *et al.*, 1996; Pickard & Rea, 1997b; Pickard *et al.*, 1999; Rea & Pickard, 2000), 5-$HT_{1A, 1B, \text{and } 7}$ receptors on the cells of the SCN (Ying & Rusak, 1994; 1997; Rea & Pickard, 2000; Belenky & Pickard, 2001), as well as 5-HT_{1A} autoreceptors in the raphe nuclei (Verge *et al.*, 1986; Sotelo *et al.*, 1990). There is good evidence that the SCN region also

contains α_1-adrenoreceptors (Weiland & Wise, 1987), however there do not appear to be any D_2 receptors in the SCN (Wamsley *et al.*, 1989; Weiner *et al.*, 1991). Taken together, this complement of receptors in the SCN region leads us to believe that it is likely that buspirone and 8-OH-DPAT are both exerting their influence on photic phase shifts though the 5-HT_{1A} receptors, either acting directly in the SCN or by decreasing 5-HT output from the raphe by activating somatodendritic autoreceptors. The observation that the attenuation of photic phase advances did not occur in the 5-HT_{1A} knockout animals pretreated with either 8-OH-DPAT or buspirone in the present study indicates this is likely to be the case.

However, the finding that either 8-OH-DPAT or buspirone pretreatment significantly potentiated photic phase advances in the 5-HT_{1A} knockout animals suggests that 8-OH-DPAT and buspirone must also bind to some other receptors involved in the circadian system, which would result in the potentiation of the phase shifting effects of light. One possible candidate would be the α_1-adrenoreceptors, since both 8-OH-DPAT and buspirone do have a weak affinity for α_1-adrenoreceptors (Centurion *et al.*, 2006), and these receptors are found in the SCN (Weiland & Wise, 1987). However, the role of norepinephrine in the regulation of circadian rhythms has not been extensively examined thus far (Dwyer & Rosenwasser, 2000). It is known that norepinephrine predominately innervates the intrinsically rhythmic cells of the SCN (Cagampang *et al.*, 1994) and thus may affect oscillator cells directly as opposed to modulating the responsiveness of the retinorecipient cells, which is a possible mechanism for the potentiated shifts observed in the knockout animals.

Another candidate receptor would be the 5-HT_7 receptors, for which buspirone has been demonstrated to have a low binding affinity (Lovenberg *et al.*, 1993). It has been previously demonstrated that both the 5-HT_{1A} (Smith *et al.*, 2008) and 5-HT_7 (Gardani & Biello, 2008) receptors are both necessary for 8-OH-DPAT to induce a phase advance at CT6, such that 8-OH-DPAT was unable to induce non-photic phase shifts in either type of receptor knockout mice. Since the present study demonstrated that buspirone was also unable to induce non-photic phase shifts in the 5-HT_{1A} knockout animals, it is possible that buspirone and 8-OH-DPAT work through the same, or at least similar mechanisms. In order to more fully understand the specific receptor contributions of each of these receptor subtypes to the effects of 8-OH-DPAT and buspirone, these drugs could be administered to 5-HT_{1A} knockouts in conjunction with selective antagonists for the various receptors to which it is known or suspected to bind. Similarly, 8-OH-DPAT and buspirone could also be tested in mice bearing knockouts of other receptors of interest. However, both of these methods are complicated by the possible alterations to receptor populations and densities in various brain regions in any knockout model examined. Thus, it would also be useful to examine wildtype animals pretreated with selective agonists or antagonists for these other receptors prior 8-OH-DPAT or buspirone treatment.

The present study demonstrated quite a lot of variability in the effects that buspirone had on biochemical cascade components and immediate-early gene expression following late night light exposure, and the pattern exhibited differed between wildtype and knockout mice, as well as hamsters. Although the role of cFos in photic phase

shifting has yet to be determine, cFos-deficient mice are still able to phase shift to light to some degree (Honrado *et al.*, 1996), indicating that cFos is not entirely necessary for the circadian clock to shift to light exposure. However, dimers of cFos and other immediate-early gene products such as JunB form AP1 complexes that regulate further gene expression. It is not yet known which genes in the SCN are regulated by AP1 following phase shifting light exposure, however simultaneous knockdown of both cFos and JunB using antisense oligonucleotides has been shown to block phase shifts (Wollnik *et al.*, 1995), suggesting that a functioning AP1 complex is necessary even though cFos may not be a necessary AP1 dimer component. Thus it is not surprising that different AP1 components could be involved in the different effects found on cFos and JunB expression between wildtype and knockout mice in the present study. Our findings indicate that an attenuated phase shift in wildtype animals is accompanied by increased cFos activation, while a potentiated phase shift in knockouts is accompanied by increased CREB activation, decreased cFos and a trend towards decreased JunB expression. There are also several other immediate-early genes, including fra-2, fos-B (Schwartz *et al.*, 2000), c-Jun, Jun-D, NGFI-A and NGFI-B (Rusak *et al.*, 1992) which may also be upregulated or downregulated in conjunction with the effects we observed with cFos and JunB. Effects on these other immediate-early genes may actually better explain the behavioural phase shift effects we observed, considering our results were somewhat unexplanatory as to what is occurring at the molecular level underlying the phase shifts we observed.

ERK and CREB are both components of the biochemical cascades that couple incoming photic information to subsequent gene expression in the SCN (von Gall *et al.*,

1998; Butcher *et al.*, 2003; Dunlap *et al.*, 2004). The phosphorylation of these biochemical cascade components occurs extremely rapidly and in a phase-dependent manner, occurring within minutes but only when light is able to phase shift the circadian activity rhythm (Ginty *et al.*, 1993; Ding *et al.*, 1997; Obrietan *et al.*, 1998). Disruption of these biochemical pathways has been shown to disrupt both photic phase shifts and the concomitant light-induced gene expression (Butcher *et al.*, 2002; Coogan & Piggins, 2003; Dziema *et al.*, 2003; Tischkau *et al.*, 2003; Gamble *et al.*, 2007). Buspirone appears to have very limited effects upon the ERK pathway, as buspirone pretreatment had no real effect on the phosphorylation of ERK in either genotype of mice, nor in hamsters. Buspirone pretreatment did however affect the CREB pathway in hamsters, such that attenuated photic phase shifts in hamsters were accompanied by decreased CREB activation, which would lead to reduced activity at the CRE promotor region of several light-induced genes, thereby decreasing their subsequent transcription (Dunlap *et al.*, 2004) which could be related to the eventual attenuation of the shift in the behavioural activity rhythms observed. Similarly, the increased CREB activation observed in the knockout mice following buspirone pretreatment could be linked to the ultimate potentiated response observed in their photic phase shifts.

Serotonin has been proposed to inhibit photic responses in the SCN by way of two complementary mechanisms. First, serotonin inhibits neurotransmitter release from retinal terminals through the 5-HT_{1B} receptors (Pickard *et al.*, 1999). Second, serotonin also activates 5-$HT_{1A/7}$ receptors on the cells of the SCN (Rea *et al.*, 1994; Ying & Rusak, 1994; Antle *et al.*, 2003). These two mechanisms would result in an overall

inhibitory effect on the cells of the SCN, which would be expected to result in an attenuation of the photic phase shift, as well as light-induced biochemical cascade activation and immediate-early gene expression. These mechanisms would explain the attenuated phase shifts observed in the hamsters and wildtype mice, as well as the decreased level of activation of the ERK and CREB biochemical cascades that was observed in hamsters. The lack of the ability of the second mechanism to function properly without the postsynaptic 5-HT_{1A} receptors in the knockout mice would be expected to result in decreased serotonergic inhibition and thus could result in a potentiated photic phase shift, as was observed with the knockout mice in the present study.

However, 5-HT agonists, including 8-OH-DPAT, have also been shown to function when administered directly to the raphe nuclei, and thus have been proposed to function by way of activating 5-HT_{1A} autoreceptors in the raphe nuclei (Mintz *et al.*, 1997), thereby resulting in a disinhibition of the cells of the SCN due to decreased serotonin release from the cells originating in the raphe nuclei. In fact, the serotonergic fibers connecting the raphe nuclei to the SCN have been shown to be pertinent to the non-photic phase shifting abilities of 8-OH-DPAT, such that selectively lesioning these fibers results in a greatly diminished effect of 8-OH-DPAT (Schuhler *et al.*, 1998). Although the disinhibition at the SCN due to autoreceptor activation in the raphe nuclei would be expected to lead to a potentiation of photic phase shifts, which is the opposite of what was observed in the hamsters and wildtype mice in this study, it would also be expected to result in an increased activation of light-induced immediate-early gene expression such

as was observed with cFos in the wildtype mice. Similarly, a lack of the 5-HT_{1A} autoreceptors in the raphe nuclei of knockout mice would be expected to result in a lack of this disinhibition and thus increased serotonin release onto the SCN, which would be expected to lead to the type of decreased cFos and JunB immediate-early gene expression that was observed in the knockout mice in the present study.

Together, these somewhat contradictory results and proposed mechanisms indicate that 8-OH-DPAT and buspirone likely attenuate photic phase shifts through a more complicated mechanism, which could involve altered input to the SCN from disinhibited afferent regions such as the intergeniculate leaflet (IGL). In fact, 8-OH-DPAT injected directly into the IGL has been shown to induce phase advances of the activity rhythm (Challet *et al.*, 1998) comparable to those when the drug is delivered systemically or directly to the raphe nuclei, again indicating the complexity of the mechanism of action of this class of drugs. It is also quite likely that 8-OH-DPAT and buspirone administered systemically may be functioning by binding to multiple receptor classes simultaneously in multiple brain regions, including α_1-adrenoreceptors, 5-HT_{1A} and possibly even 5-HT_7 receptors in the SCN, as well as 5-HT_{1A} receptors in both the raphe nuclei and the IGL.

The fact that some 5-HT_{1A} agonists or partial agonists, including MKC-242 and BMY7378, have been shown to significantly potentiate photic phase shifts (Moriya *et al.*, 1998; Gannon, 2003), it was an exciting possibility that a drug like buspirone that is already commercially available could function by way of a similar mechanism. Had this been the case, then it would have been possible to simply add a new clinical use for

buspirone to help alleviate the adverse health effects of a mismatch between one's internal clock time and the external time of day, as occurs with transcontinental travel and rotating shift work. Although the results of the present study indicate that buspirone functions like traditional 5-HT agonist, attenuating the effects of light on the circadian system, it is still very important to better understand what effects this commercially available drug has on the circadian system. By understanding the precise pharmacology and underlying molecular mechanisms responsible for the modulation of the circadian system's sensitivity to the effects of light with 5-HT agonists such as buspirone, it may be possible to better deal with or adjust for the possible circadian-related side effects in patients currently taking buspirone for general anxiety disorder or depression. Plus, an increased understanding of how these drugs function within the circadian system may also allow for the eventual development of chronobiotics specifically targeted for the rapid resynchronization of circadian rhythms, thus more effectively managing or even preventing the adverse health effects associated with the desynchrony between the circadian system and the external environment observed in circadian rhythm disorders, jet lag, and shift work.

6. General discussion

6.1 Summary of major findings

The current studies first examined the location of action and molecular underpinnings of the responses of the circadian clock to light stimuli following administration of a 5-HT_{1A} receptor mixed agonist/antagonist or a 5-HT_{1A} receptor partial agonist. By injecting the 5-HT_{1A} mixed agonist/antagonist BMY7378 directly into specific brain regions, we were able to determine which brain structure appears to be mediating the enhanced photic phase shifts observed. Intracerebral injections into the SCN demonstrated a complete lack of phase shift potentiation normally observed with systemic application of BMY7378. However, BMY7378 injected directly into the MRN produced a potentiation effect which was similar in percent magnitude increase to that observed with systemic administration. We also demonstrated that the serotonergic fibers directly connecting the MRN to the SCN are not necessary for the potentiation effect following systemic administration. These results suggest that BMY7378 functions by binding to 5-HT_{1A} autoreceptors in the median raphe nucleus, not by binding to receptors within the suprachiasmatic nuclei. Further, they indicate that BMY7378 does not alter the phase of the circadian activity rhythm by affecting the serotonergic signal along the direct pathway connecting the raphe nuclei to the SCN.

The second set of experiments focused on the underlying *Period* gene expression that could give insight into the molecular mechanism by which BMY7378 induces enhanced photic phase shifts. BMY7378 was found to have no potentiation effect on the light-induced phase shifts observed in mice, as well as no significant phase shifting effect

on its own, unlike what has been observed in hamsters. This result was contrary to our predictions since we previously found NAN-190 to be as effective in mice as in hamsters in terms of its potentiation abilities (Smith *et al.*, 2010). We also observed no significant effect of BMY7378 on the expression of *Period1* or *Period2* mRNA in the SCN of mice 90 minutes following light exposure. This lack of effect on clock gene expression was not unexpected at this time point, as NAN-190 also had no effect on the *Period* gene expression of mice (Smith *et al.*, 2010).

We further examined the molecular underpinnings of photic potentiation with BMY7378 in the hamster, by looking at the activation of multiple biochemical cascade components, the expression of multiple immediate-early gene protein products, and the protein product of the *Period1* gene, at different time points following the light exposure. Both the phosphorylated form of ERK (a component of one of the second-messenger signaling pathways coupling photic input to clock gene expression) and cFos (the protein product of the immediate-early gene *c-fos*) were shown to have increased expression with BMY7378 pretreatment at a time point beyond when their expression would have been expected to have begun to taper off following light exposure (Coogan & Piggins, 2003). P-ERK expression normally peaks around 30 minutes following light exposure and has dropped dramatically by 45 minutes, but with BMY7378 pretreatment, P-ERK expression was found to still be elevated at 60 minutes after the light exposure. Similarly, cFos expression generally peaks around 90 minutes following light exposure and drops substantially by 120-180 minutes (Morgan & Curran, 1989), whereas it was found to still be elevated 180 minutes with BMY7378 pretreatment. Similarly, with BMY7378

pretreatment, JunB (the protein product of the immediate-early gene *JunB*) as well as PER1 (the protein product of the *Period1* gene), both of which are normally activated or remain active many hours following light exposure (Morgan & Curran, 1989; Field *et al.*, 2000), were both found to have elevated expression levels 6 hours after light exposure. Finally, CREM (another component of one of the second-messenger signaling pathways, which can either activate or repress further gene expression depending on the specific isoform; Lalli *et al.*, 1996; Sassone-Corsi, 1998; De Cesare *et al.*, 1999) was also found to be elevated at 240 minutes following light exposure. If the particular isoform being expressed were an activator of further gene expression, its increased expression could indicate a possible mechanism by which downstream clock expression in the SCN could be altered in order to produce the potentiated phase shift observed following BMY7378 treatment. Both increased expression as well as seemingly extended action of these different biochemical cascade pathways and gene expression are all indications of possible underlying mechanisms by which the behavioural output of the circadian clock could be altered to induce the enhanced phase shifts we observe with BMY7378.

The final set of experiments focused on the effect that the 5-HT_{1A} receptor partial agonist buspirone has on photic phase shifts of the circadian activity rhythms of both mice and hamsters. BMY7378 has been considered by some to also be a partial agonist, thus we sought to determine if buspirone would have a similar potentiation effect as is seen with BMY7378. Since buspirone is a commercially available drug used for depression and general anxiety disorder, had it behaved in a manner equivalent to BMY7378 then it would have been a possible chronobiotic to help alleviate the adverse

effects related to chronic desynchronization between the timing of the internal circadian system and the external environment. Buspirone was found to attenuate photic phase shifts in the late subjective night, in both wildtype mice as well as hamsters. However, mice lacking the 5-HT_{1A} receptors exhibited an enhanced phase shift to light at this time of the night. This effect observed with buspirone was very similar to what was also observed with the 5-$HT_{1A/7}$ receptor agonist 8-OH-DPAT.

We also examined possible molecular mechanisms underlying the attenuated shifts observed in wildtype mice and hamsters, as well as the enhanced shifts seen in 5-HT_{1A} receptor knockout mice. Hamsters were found to have an overall decrease in CREB activation with buspirone 15 minutes after light exposure, with no change in ERK activation, underlying the attenuated photic phase shifts in the late subjective night. While wildtype mice showed no change in either ERK or CREB activation, but did demonstrate increased cFos expression at 90 minutes which underly their attenuated shifts. Conversely, underlying the enhanced photic phase shifts in the late subjective night with 5-HT_{1A} receptor knockout mice, increased CREB activation was observed, along with a lack of the increase in cFos expression that was observed in the wildtype mice. Together, these results indicate that buspirone generally behaves more like the 5-$HT_{1A/7}$ agonist 8-OH-DPAT on the circadian system than like the 5-HT_{1A} mixed agonist/antagonist BMY7378. Further, they indicate that although the attenuation effect on photic phase shifts appears to be through binding at the 5-HT_{1A} receptor, an enhancement was uncovered when the 5-HT_{1A} receptor is removed and the effect of binding at some other receptor can be observed.

6.2 Overall conclusions

Several different conclusions can be drawn from the present findings regarding the underlying mechanisms by which BMY7378 potentiates photic phase shifts in the late subjective night. In general, 5-HT is considered to play an inhibitory role on the effects of light, and of the several 5-HT receptors found in SCN, a postsynaptic mechanism involving the 5-HT_{1A} receptor has been proposed to be one way by which serotonin modulates photic information (Pickard & Rea, 1997a). Serotonergic agonists are able to reduce the firing rate in retinorecipient cells of the SCN (Miller & Fuller, 1990; Ying & Rusak, 1994), an effect that appeared to be mediated by a receptor with properties similar to that of the 5-HT_{1A} subtype (Ying & Rusak, 1994). However, the potentiation of photic effects with BMY7378 indicates an overall antagonism of the normal effects that serotonin would have on the retinorecipient cells, such that a reduced amount of inhibition leads to a potentiated response. One of our major findings from the current studies, that BMY7378 injected into the SCN had no potentiation effect but injection into the median raphe nucleus did elicit a potentiated response, is in line with this idea of overall disinhibition at the SCN.

It has previously been suggested that systemically administered BMY7378 and other similar 5-HT_{1A} mixed agonists/antagonists function by a combined effect as an antagonist postsynaptically in the SCN and as an agonist at the autoreceptors in the raphe nuclei (Gannon, 2003). Our findings indicate that this hypothesized mechanism is not entirely correct. We have shown that the potentiation effect observed with BMY7378 does indeed involve receptor binding in the median raphe nucleus, but does not appear to

involve binding directly in the SCN. It was originally suspected that it would be an agonistic activity at the 5-HT_{1A} autoreceptors in the raphe nuclei which would result in a decrease in the amount of serotonin released onto the SCN, by way of the direct connection between these two brain structures, that would be the likely route of action for BMY7378. In fact, the agonistic action of NAN-190 at presynaptic somatodendritic 5-HT_{1A} autoreceptors has been shown to lead to decreased release of 5-HT at the SCN (Dudley *et al.*, 1998). However, we also demonstrate in present studies that the direct serotonergic connection from the median raphe nucleus to the SCN is not necessary to achieve photic potentiation with BMY7378. Thus, although BMY7378 binding in the median raphe may also produce a decreased release of serotonin onto the SCN, our results indicate that this effect is not necessary to produce the potentiation effect.

It is likely that the potentiation effect observed is the result of reduced serotonin release onto the IGL, which in turn alters its effect on the cells of the SCN through this indirect pathway. Recent evidence from our lab corroborates the importance of this indirect pathway in the phase shift potentiation effects with BMY7378 pretreatment. A radiofrequency lesion to the IGL, completely destroying the structure and therefore removing any ability to relay information either from its retinal inputs or from its serotonergic raphe nuclei inputs, completely eliminates the photic potentiation effect normally observed with systemic administration of BMY7378 (Jeffers, 2013). Similarly, removing only the serotonergic fibers connecting the raphe nuclei to the IGL, but leaving the structure itself along with all its other inputs fully intact, also eliminates the normal photic potentiation effect of BMY7378 (Jeffers, 2013). Together with the present finding,

that direct injection of BMY7378 into the median raphe potentiates photic phase shifts, the aforementioned findings demonstrating that the IGL is necessary indicate that the indirect pathway leading from the raphe nuclei to the SCN by way of the IGL is pertinent to the ability of BMY7378 to produce its effect on the circadian system. These results do not however inform us as to whether the IGL is sufficient, or if it is merely necessary, for phase shift potentiation with BMY7378 (Figure 6.1).

Previous studies indicated that the potentiation effect of mixed agonists/antagonists such as BMY7378 and NAN-190 were accompanied by a decrease in the amount of light-induced *c-fos* expression in the SCN 60 to 90 minutes following the photic stimulus (Recio *et al.*, 1996; Smith *et al.*, 2010). The inhibitory effects of serotonergic agonists that lead to an attenuation of light-induced phase shifts, not a potentiation, also inhibit light-induced *c-fos* expression in the SCN (Weber et al., 1998). In fact, it has been observed previously that the magnitude of light-induced cFos in the SCN is generally proportional to the size of the expected phase shift to a similar intensity and duration of light pulse (Kornhauser *et al.*, 1990). These seemingly contradictory results, that BMY7378 and NAN-190 behave more like antagonists by potentiating photic phase shifts but behave more like agonists by attenuating gene expression, have yet to be fully reconciled. Although we only observed a trend towards an attenuation of cFos at 90 minutes following the light exposure in the current studies, we also extended previous findings by examining a time point longer after the light exposure (180 minutes following the start of light exposure), which indicates an increased duration of immediate-early gene activation in the SCN. It is still unknown the precise role that cFos plays in photic

phase shifting, and it is not known which genes in the SCN are regulated by AP1 complexes nor is it known the precise dimer combinations of *c-fos* and *JunB* immediate-early gene protein products that are pertinent for AP1 promotor site gene activation resulting from phase shifting light exposure. This apparent extended activation of cFos (increased expression 180 minutes after light exposure), along with the increased JunB expression we observed with BMY7378 pretreatment, could all indicate an alteration to the AP1 complex combinations and/or duration of AP1 promotor site activation for downstream genes, which could underlie the phase shift potentiation effect observed.

The increased P-ERK expression observed at 60 minutes following light exposure in the present studies, indicating an extended biochemical cascade activation, could also be part of the mechanism by which immediate-early gene activation is extended. Similarly, increased CREM expression at 240 minutes following light exposure could also underlie extended IEG activation. All of the above-mentioned biochemical cascade alterations and IEG expression changes could play a role in the observed increased PER1 expression in the SCN, which could ultimately result in the potentiated photic phase shifts observed with BMY7378 administration. However, it is important to note that with all the different proteins and timings examined, only JunB expression at 6 hours following the exposure to light was found to be altered in the shell region of the SCN, the region which contains the bulk of the intrinsically rhythmic cells expressing the autoregulatory transcription/translation feedback loop that appears to be quite central to the overt circadian rhythms in behaviour. The vast majority of the responses we observed in terms of gene expression were found in the non-rhythmic retinorecipient core region of

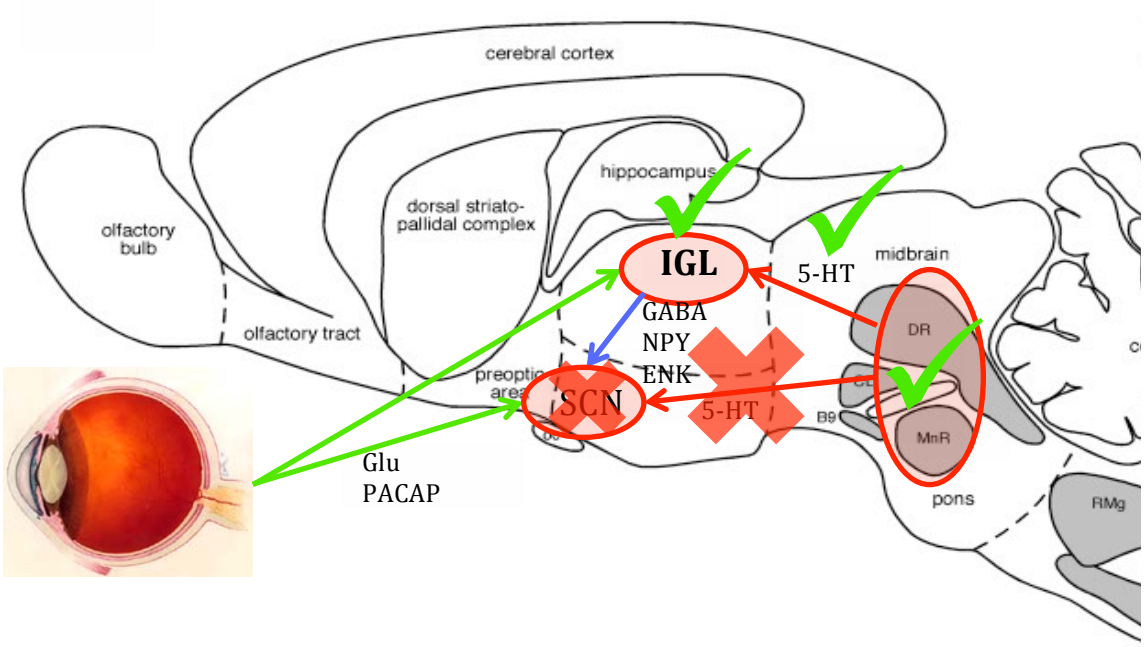
the SCN, and not in the rhythmic cells of the shell where the expression of clock genes must be ultimately adjusted in order to induce a phase shift. Thus, it is evident that we still have not uncovered the complete mechanism by which BMY7378 actually alters the magnitude of the photic phase shifts elicited.

Finally, our previous studies indicated the pertinence of the 5-HT_{1A} receptor subtype in the potentiation effects of NAN-190, such that the potentiation effect was absent in mice lacking this receptor subtype (Smith *et al.*, 2010). However, our present study in which buspirone attenuated photic shifts, but which uncovered a potentiation effect when the 5-HT_{1A} receptor was missing, indicates this specific potentiation effect must be mediated by one of the many other receptor types to which this drug also binds. In fact, different patterns of biochemical cascade activation and protein expression were observed between the wildtype and the 5-HT_{1A} receptor knockout mice, such that increased P-CREB expression accompanied phase shifts potentiated by buspirone in 5-HT_{1A} knockout mice along with a lack of the increase in cFos expression which was observed with the attenuated response observed in the wildtypes. Whereas, NAN-190 was shown to potentiate photic phase shifts by decreasing both P-CREB and cFos expression (Smith *et al.*, 2010). These potentially contradictory results indicate the possibility of different mechanisms and/or different receptor binding profiles underlying the attenuated and potentiated shifts observed with these drugs. It is also possible that binding at the same receptors, but in different locations within the brain, could result in the different effects we have observed with these different types of potentiated photic responses. All of these findings indicate that there is still much research to be done in

order to fully understand how mixed agonists/antagonists such as BMY7378 function to induce phase shift potentiation, although the present findings do provide many possible avenues for investigating this phenomenon further.

Figure 6.1 Summary of findings for location of action of BMY7378.

A schematic representation of the major afferent pathways leading into the SCN, and which structures/pathways are necessary or sufficient to produce the photic phase shift potentiation effect observed with BMY7378. The suprachiasmatic nucleus (SCN) is not sufficient to induce potentiation of photic phase shifts when BMY7378 is injected directly into the structure. Injecting BMY7378 directly into the raphe nuclei is sufficient to induce photic phase shift potentiation. The direct serotonergic input from the raphe nuclei to the SCN is not necessary for phase shift potentiation with BMY7378. The intergeniculate leaflet (IGL) is necessary for BMY7378 to induce photic phase shift potentiation. The serotonergic projection from the raphe nuclei to the IGL is also necessary to induce photic phase shift potentiation with BMY7378. Therefore, it appears that BMY7378 binds to receptors in the raphe nuclei, and then sends altered phase shifting information indirectly to the SCN, via the IGL.


cerebral cortex
hippocampus
dorsal striato-
pallidal complex
olfactory
bulb
olfactory tract
IGL
5-HT
midbrain
GABA
NPY
ENK
DR
SCN
5-HT
B9
MnR
Glu
PACAP
pons
RMg

6.3 Limitations and future directions

6.3.1 Location of Action of BMY7378

Our examination of the location of action of BMY7378 indicated that the median raphe nucleus is sufficient to induce photic phase shift potentiation with BMY7378 pretreatment and that the SCN is not sufficient to produce this potentiation effect. However, we do not yet know if either of these structures are strictly necessary for this type of phase shift potentiation. Obviously the SCN is necessary in terms of it being required for the expression of any type of overt circadian rhythmicity for the entire organism, however whether any specific aspect of the SCN or its functioning is directly necessary for the induction of the potentiation effect of BMY7378 has yet to be determined. Further, the serotonergic connections from the raphe nuclei to the SCN were shown not to be necessary. Conversely, the serotonergic connections from the raphe nuclei to the IGL, as well as the IGL itself, have both been shown to be necessary for BMY7378 to potentiate photic phase shifts. Therefore, further experiments are required to determine if the raphe nuclei are necessary (by lesioning the raphe nuclei), or if the IGL is sufficient (by injecting BMY7378 directly into the IGL) for BMY7378 to induce its phase shift potentiation.

6.3.2 Behaviour and *Period* gene expression in mice with BMY7378

It was unexpected that BMY7378 was found to have no effect on the magnitude of photic phase shifts induced in mice. We had previously shown NAN-190 to be comparably effective in mice (Smith *et al.*, 2010) as it is in hamsters (Rea *et al.*, 1995). A dose of 5mg/kg of NAN-190 was found to be maximally effective in hamsters (Rea *et al.*,

1995), while a dose 2.5mg/kg produced a similar mean magnitude of phase shift, although with increased variability. The smaller dose of 2.5mg/kg was found to be quite effective in mice (Smith *et al.*, 2010), producing a comparable percent increase in phase shift magnitude. Although a dose of 5mg/kg of BMY7378 has been shown to be maximally effective in hamsters, a higher dose was less effective despite still significantly potentiating the magnitude of phase shift elicited (Gannon, 2003). Therefore, it is possible that the lack of potentiation effect we observed in mice could be the result of a dose that is higher than maximal and is on the declining side of a bell curve shaped dose response curve. Thus, it would be important to perform a more complete dose response curve for BMY7378 in the mouse model. Perhaps a dose of 2.5mg/kg, as we used previously for NAN-190 (Smith *et al.*, 2010), would have produced an entirely different result.

Second, as a result of our lack of observable potentiation effect in normal mice, we were not able to determine the role of the 5-HT_{1A} receptor in the ability of BMY7378 to produce its potentiation effect. Had BMY7378 been shown to effectively potentiate photic phase shifts in normal mice, for example with a different dose, we could have examined the role of the 5-HT_{1A} receptor in this effect through the use of mice lacking that receptor subtype as we had previously done with NAN-190 (Smith *et al.*, 2010). It would also be useful to examine the involvement of other serotonergic as well as non-serotonergic receptors for which BMY7378 also has binding affinities, particularly 5-HT_{1B} and α_1-adrenergic receptors (Millan *et al.*, 1994) both of which are presumed to be involved in circadian rhythmicity and are known to be located in the SCN region

(Weiland & Wise, 1987; Pickard *et al.*, 1996; Pickard & Rea, 1997b; Pickard *et al.*, 1999; Rea & Pickard, 2000).

Third, we only examined one time point of *Period* gene expression in mice. A study using MKC-242, a 5-HT agonist which is able to induce potentiated photic phase shifts in mice, found both *mPer1* and *mPer2* to be elevated at a time point at which levels would normally have already returned to basal levels, indicating an extended activation of these important clock genes (Takahashi *et al.*, 2002). Thus, our results could have been more informative had we examined time points longer after the light exposure. It would also be pertinent to examine the expression of both *Per1* and *Per2* in hamsters, both when they normally peak around 90 minutes after light exposure as well as at time points further after the light exposure. We did attempt to examine *haPer1* and *haPer2*, however our hamster *in situ* hybridization probes did not allow for successful staining of those cells in the SCN expressing the *Period* genes.

6.3.3 Biochemical cascade activation and immediate-early gene expression

We were able to observe several different biochemical cascade components and immediate-early gene protein products that were altered following BMY7378 pretreatment. However, we did not examine a time course for some of the proteins we studied. The time point we selected for CREM expression was based on the approximate peak of its expression following light exposure, and although we did observe increased expression at that time point it would be important to examine later time points to determine if its time course is also altered. Similarly, JunB and PER1 expression at 6 hours following light exposure were both found to be increased following BMY7378

pretreatment. Yet, examination of further time points could also be informative as to the underlying mechanism of the phase shift potentiation observed. As previously discussed, MKC-242 leads to an extended activation of *Period* genes underlying potentiated photic phase shifts, thus it is possible that BMY7378 could cause extended activation of *Period* genes and therefore also extended activation of the protein products of those genes.

Another possible limitation for a few of the experiments in Chapter 4 is the small sample size for a number of the different groups. Although we were able to find effects on the expression of some proteins with 3 animals per group, there were also a few types of proteins whose expression demonstrated a trend towards an effect. It is possible that a larger sample size for P-CREB at 120 minutes and cFos at 90 minutes following light exposure could have resulted in a significant effect. However, the fact that we found significant effects with the same small samples sizes for other proteins in this study indicates that our effect sizes (if they are comparable for all proteins examined, which is currently unknown) appear to be large enough to find significant differences with the sample sizes used.

Finally, even though we did observe altered expression of some proteins in the SCN, either in terms of their magnitude of expression or in terms of extended time courses, it is still unknown what role these changes might play in the process of potentiated phase shifts. Thus, it is pertinent to further examine the role that each of the proteins we found to be altered actually plays in the phase shift potentiation effect observed with BMY7378 pretreatment. For example, we could attempt to disrupt the expression or activation of each of these altered proteins one at a time along with the

administration of BMY7378 in order to more completely examine the role of each of these biochemical cascade components and immediate-early genes. It has previously been demonstrated that simultaneous knockdown of both cFos and JunB using antisense oligonucleotides blocks phase shifts (Wollnik *et al.*, 1995), whereas cFos-deficient mice are still able to phase shift to light, albeit to a lesser degree (Honrado *et al.*, 1996). Thus, we could extend these phase shifting results by examining the effect of BMY7378 along with genetic removal of a gene, the knockdown of a specific gene or combination of genes through antisense oligonucleotide application, or even a disruption of the phosphorylation process necessary for many of the biochemical cascade components to activate further gene expression in the SCN.

6.3.4 Buspirone and photic phase shifts

In order to examine the role of the 5-HT_{1A} receptor, this study made use of animals genetically engineered to lack that receptor. This method can be extremely useful in fully eliminating the effects of a specific receptor subtype instead of simply trying to selectively block that receptor, especially since it can be difficult to find truly selective antagonists for the vast majority of receptor types. However, there are issues involved with genetically modified strains of mice. The most major concern with genetically modified mouse models involves compensation effects. Because genetically modified mice, such as our 5-HT_{1A} receptor knockout mice, lack the receptor subtype throughout all of development it is possible that there are concomitant effects on other receptor subtypes (up-regulation or down-regulation) or neurotransmitter systems that could allow for compensatory mechanisms. A possible solution to this problem is to use inducible

knockout models, in which a specific gene can be turned off after the animals has reached adulthood and allowing for normal development, instead of traditional genetic knockout models.

A final experiment which could prove useful would be an attempt to mimic the phase shift potentiation induced by buspirone in the 5-HT_{1A} receptor knockout mice, but in a normal mouse using a 5-HT_{1A} antagonist to first block the receptor. Buspirone is already a commercially available drug, and thus if it were possible to induce potentiated photic phase shifts by combining an appropriate antagonist (if there were one approved for use in humans) with buspirone then it might be possible to formulate a truly useful chronobiotic to help alleviate the adverse effects of circadian rhythm desynchronization.

6.4 Clinical importance

The development of a chronobiotic which could rapidly and extensively reset the circadian system would be extremely useful in today's fast-paced society. Numerous health consequences are associated with constant desynchronization of the circadian system from the external environmental cycle in those individuals who are subjected to shift work schedules or frequent intercontinental travel (Rajaratnam & Arendt, 2001; Knutsson, 2003; Dunlap *et al.*, 2004; van Mark *et al.*, 2006). Unfortunately it can be difficult to avoid such desynchronization with the 24-hour nature of our society, and the majority of the commonly used means by which individuals attempt to reset the circadian clock are only marginally effective. Thus, the development of an oral chronobiotic that could alleviate or even prevent the negative side effects of chronic circadian rhythm desynchronization, by rapidly realigning the endogenous circadian system with the

external environment, would be invaluable for a large portion of the population. In order to accomplish this, it is critical to fully understand the location of action and molecular mechanisms underlying the potentiation of photic phase shifts induced by drugs such as BMY7378.

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