Photic And Non-Photic Interactions In The Functioning Of The Circadian Clock

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Abstract

The biological clock relies on the integration of both photic and non-photic information in order to synchronize and entrain to the environment. In this thesis I examine the interaction between light and NPY, a known mediator of many non-photic stimuli. Previous studies have shown that both photic and non-photic stimuli interact *in vivo* and *in vitro*. However the precise neural pathways and cellular mechanisms utilized in such a complex interaction are as yet unknown.

In my first set of experiments I have shown that microinjections of NPY directly onto the suprachiasmatic nucleus (SCN) *in vivo*, block the phase advancing effects of light and attenuate phase delays. Furthermore, I found that a specific NPY Y1/Y5 receptor agonist was able to inhibit photic phase shifts in a similar manner to that observed with NPY.

In the following series of studies I utilized a newly developed NPY Y5 receptor antagonist. It was found that injection of the Y5 receptor antagonist during light exposure prevented NPY from inhibiting light induced phase shifts during the night. Also, the administration of a specific Y1 receptor antagonist had no effect on the ability of NPY to influence the resetting effects of light during the early and late night.

It was previously shown that phase shifts to NPY were mediated via GABAergic interneurons. However, I found that NPY did not inhibit photic phase shifts via a similar GABA dependent mechanism, as the presence of bicuculline, a $GABA_A$ antagonist did not prevent NPY from altering phase shifts to light during the night.

Finally, to determine the possible site of interaction between NPY and light a time course study was designed. It was found that microinjections of NPY up to 60 minutes post light exposure were able to attenuate photic phase advances. This was also the case when animals were presented with a novel running wheel 60 minutes after an advancing light pulse.

Overall my work has shown that NPY interacts with light during the early and late night. Furthermore, NPY utilizes the Y5 receptor subtype and a neural pathway independent of $GABA_A$ activation. Finally the present data indicates that the possible site of interaction between NPY and light lies downstream from receptor binding sites within SCN cells.

Declaration

I declare that this is my own work, carried out under the normal term of supervision.

Acknowledgements

I would like to thank Stephany Biello for her undivided supervision, encouragement and support throughout this thesis. Stephany has not only been a great mentor but also a valuable friend, she will always remain an inspiration.

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List of Tables and Figures

Preface

This thesis consists of six chapters, a general introduction followed by four experimental chapters and a final discussion chapter. Three of these experimental chapters have been published and one has been submitted for review. Following these six chapters are four appendices containing a detailed description of the surgical procedures used throughout the studies, a dose response analysis for the novel NPY Y5 receptor antagonist used in chapter 3, activity onset calculation methods and cannula placement verification techniques. The chapters reproduced here are identical to those published with a few exceptions. For all chapters the introductions have been shortened to avoid repetition. The acknowledgement and reference sections have been omitted and collated at the end of the thesis. The figures have been numbered consecutively throughout the work.

The full references for published, or submitted for review papers are as follows:

Chapter 2- Lall, G.S. and Biello, S.M. (2003) Attenuation of circadian light induced phase advances and delays by neuropeptide Y and neuropeptide Y Y1/Y5 receptor agonist. *Neurosci.* 119: 611-618.

Chapter 3- Lall, G.S. and Biello, S.M. (2003) Neuropeptide Y inhibits photic phase shifts via the Y5 receptor. *Submitted for review.*

Chapter 4- Lall, G.S. and Biello, S.M. (2003) Neuropeptide Y, GABA and circadian phase shifts to photic stimuli. *Neurosci.* 120: 915-921.

Chapter 5- Lall, G.S. and Biello, S.M. (2002) Attenuation of phase shifts to light by activity or neuropeptide Y: a time course study. *Brain Res.* 957:109-116.

The relative contribution of authors is as follows. For all papers included in this thesis, the original ideas for experiments were generated through interactions with my supervisor, Dr. Stephany M. Biello. These experiments were designed by me, but were critically evaluated by Dr. Biello. The papers were written by me but received editing from Dr. Biello.

Abbreviations

CT	Circadian Time
DD	Constant Darkness
GABA	γ-amino butyric acid
GHT	Geniculohypothalamic Tract
IGL	Intergeniculate Leaflet
LD	Light-Dark Cycle
LGN	Lateral Geniculate Leaflet
NPY	Neuropeptide Y
NMDA	N-methyl-D-aspartate
PRC	Phase Response Curve
RHT	Retinohypothalamic Tract
SCN	Suprachiasmatic Nucleus
ZT	Zeitgeber Time

Chapter 1

General Introduction

1.1 Circadian Rhythms

Throughout evolution humans have shown the ability to adapt and alter their physiological and behavioral patterns in accordance with environmental and social changes. Many physiological and behavioral rhythms exhibited by humans follow an observable circadian (close to 24 hours) pattern. Sleeping, energy metabolism, hormonal secretions are just a few examples of factors that repeat with a circadian rhythm of expression. These circadian rhythms have been primarily governed by the environmental day-night cycle. However we are able to modify these rhythms by other behavioral influences such as social activities or shift work. This displays the ability of humans to integrate both environmental and behavioral information to generate an internal physiological rhythm that allows us to perform at maximum potential and allows maximal flexibility within our ever-changing life styles. Our natural internal and external circadian rhythms not only allow us to adapt to environmental changes but also more importantly help regulate vital biological processes within specific organs, ultimately at a level of homeostatic maintenance.

1.2 The Endogenous Clock

The endogenous rhythm of the circadian clock exhibits an approximate twenty-four hour cycle in mammals. Under constant conditions of darkness many species exhibit behavioural patterns, which cycle with a circadian rhythm. The observed behavioural patterns under these conditions display the endogenous rhythm of the circadian clock, as there is no influence from environmental cues, such as day and night changes. Animals under these conditions are said to be 'free running'.

Tau (τ) or the 'free running rhythm' is a measure of the rate of change of the measurable circadian parameter relative to time, normally cycling with a period of approximately 24 hours. Changes in τ can be measured by recording biological events that exhibit a circadian pattern, for example daily activity. An actogram can be used as a graphical representation of an animal's daily activity over a number of days. Wheel running activity is widely used as a means of measuring activity. Figure 1 illustrates a schematic actogram of wheel running activity of an animal under constant conditions plotted on a 24 hour scale.

The start of activity each day provides information about the τ of an animal. An inclination to the left indicates a τ shorter than twenty-four hours i.e. onsets of activity are earlier each day, whereas an inclination to the right indicates a τ longer than twenty-four hours i.e. onsets of activity are later each day.

1.3 Entrainment.

The endogenous nature of the circadian clock may be observed in the free running situation, but out of the experimental laboratory, environmental cues govern the rhythm of the clock.

The environmental day-night cycle can be termed a zeitgeber meaning 'time giver'. If the zeitgeber is effective, then the internal clock will synchronise to it and so be entrained. The process of entrainment can be defined as the synchronisation of the biological clock to the outside world by adjusting to a twenty-four hour day.

An entrained animal will show an activity onset that is synchronised with the external zeitgeber. For example a nocturnal animal entrained to a light dark cycle will display activity when the lights go off. If a species entrains to such a stimulus then its activity pattern will remain synchronised with the given zeitgeber i.e. in nocturnal animals activity will start at lights off each day. Figure 2 shows a schematic actogram of an animal entrained to a light dark cycle. The process of entrainment involves the circadian pacemaker resetting itself to the zeitgeber each day.

1.4 The circadian time scale

In circadian biology two separate time scales are used to refer to entrained species and to those that are kept under constant conditions. Animals that are entrained to a zeitgeber are said to follow a zeitgeber time (ZT) scale, with ZT12 defined as the time of activity onset for nocturnal animals and ZT0 for diurnal species. Circadian time (CT) is assigned to animals that are kept under constant conditions and one circadian day is a measure of the time taken for the internal biological clock to complete one cycle. For example, for nocturnal animals CT12 is defined as the time of activity onset and CT0 for diurnal animals. This means that the interval from CT12 to CT24 still refers to the night, in both nocturnal and diurnal species. Most significantly, the study of subjects under constant conditions allows the observation of an individuals circadian rhythm governed by its internal clock as oppose to the external environment.

For experimental purposes animals are either entrained to a light dark cycle thereby using a ZT time scale, or left to free run, thereby using the CT time denomination. Under the influence of a zeitgeber it is possible to observe the clock under the control of external

stimuli so allowing the study of the circadian system as it functions under the environmental day night cycle. However in order to examine the endogenous rhythm of the clock it is necessary to remove entraining stimuli such as the environmental light dark cycle therefore utilising the CT time scale.

Figure 1.

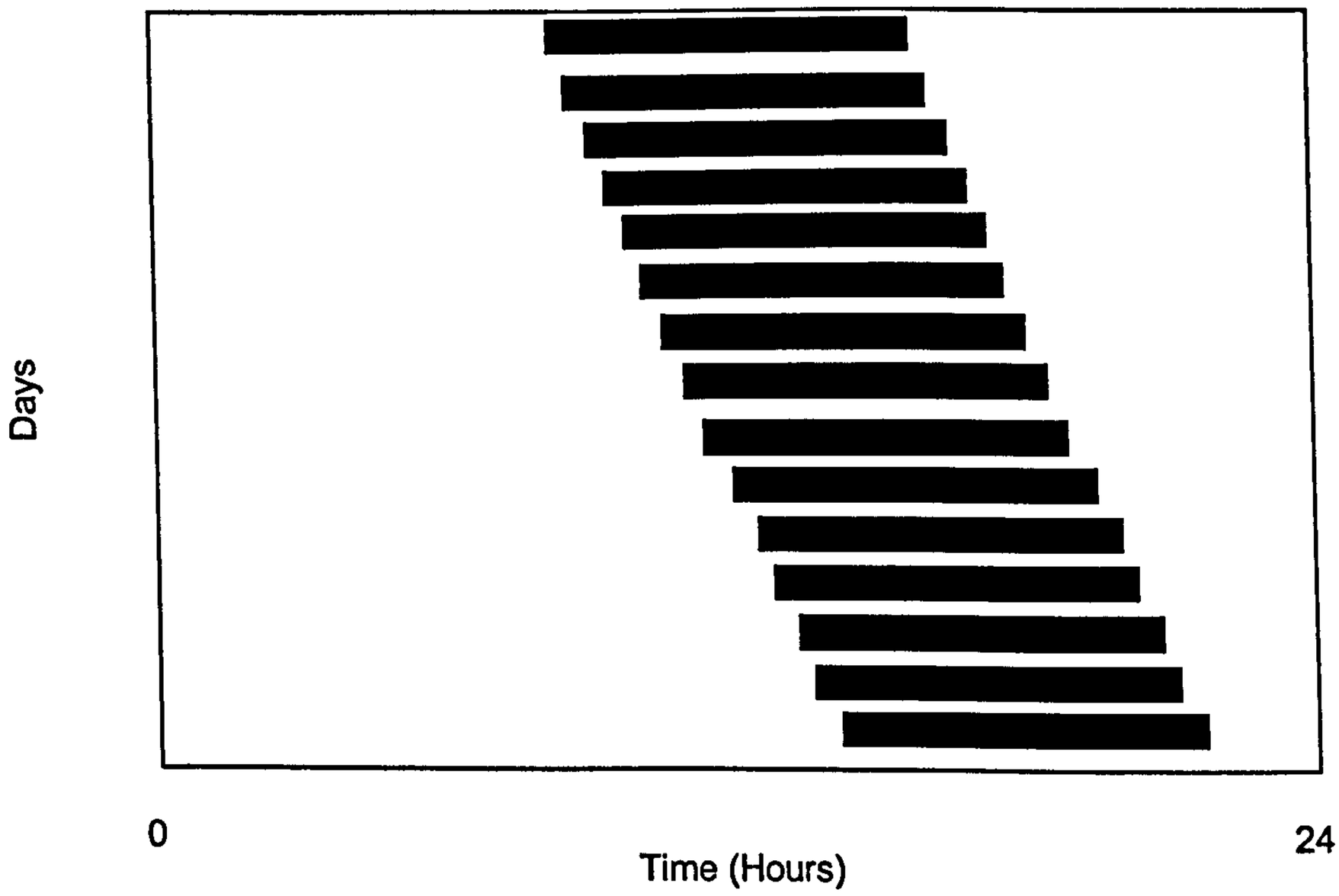


Figure 1. A schematic actogram of an animal under constant conditions. The solid black bars represent activity. Measuring the rate of change of activity onsets over a number of days allows the calculation of *tau* i.e. the natural period of the circadian clock.

Figure 2

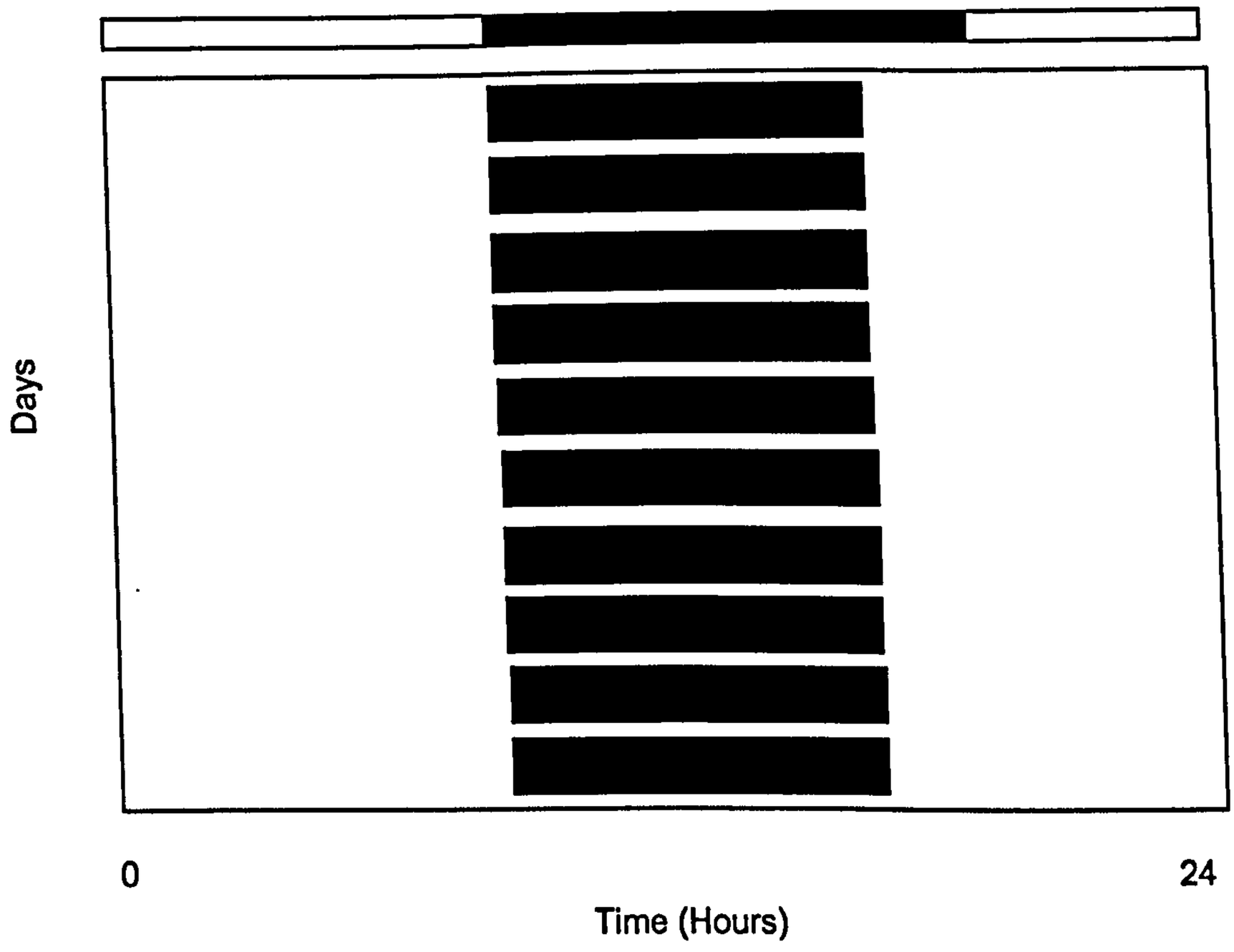


Figure 2. A schematic actogram of an animal entrained to a 24 hour light dark cycle. The open bars at the top of the actogram indicate lights on and the dark bars indicated lights off. This actogram depicts the activity pattern of a nocturnal animal; hence activity starts when light go off. This behavioural pattern is repeated each day, indicating that the animals has synchronised to the environmental light dark cycle imposed on it.

1.5 Environmental influences on the clock.

1.5.1 Photic regulation of the clock

The environmental light dark cycle provides perhaps the strongest zeitgeber for the entrainment of the circadian pacemaker. Over time light has played a key role in the development and survival of many species. Food availability and predator avoidance are just two aspects of survival that animals associate with light or darkness and so have altered their activity patterns in order to maintain a high survival rate.

Throughout evolution the biological clock has been well conserved. The effects of brief light pulses on locomotor activity in rodents maintained in constant conditions have been well studied. Pittendrigh (1976) originally found that light pulses caused consistent shifts in the phase of the activity rhythm. The magnitude and direction of the shift depends on the time (relative to the animal's clock) at which the pulses of light are presented. These responses to light are remarkably similar across species.

In mammals, light pulses presented during the "early subjective night" (the time in the animal's clock that would correspond to darkness if the animal were entrained to a light dark cycle), cause phase delays. Light pulses presented during the "late subjective night" cause phase advances, and pulses presented during most of the "subjective day" (the time in the animal's clock that would correspond to light if the animal were in entrained to a light dark cycle) results in no phase shifts (Pittendrigh and Daan, 1976). By plotting the magnitude of the phase shift and the timing of the light pulses, it is possible to construct a graph, which shows the extent of the phase shifts obtained when light pulses are presented

at given times of the animals circadian clock. Such graphs are called phase response curves (PRC). Figure 3 illustrates a schematic PRC for light in mammals.

Figure 3.

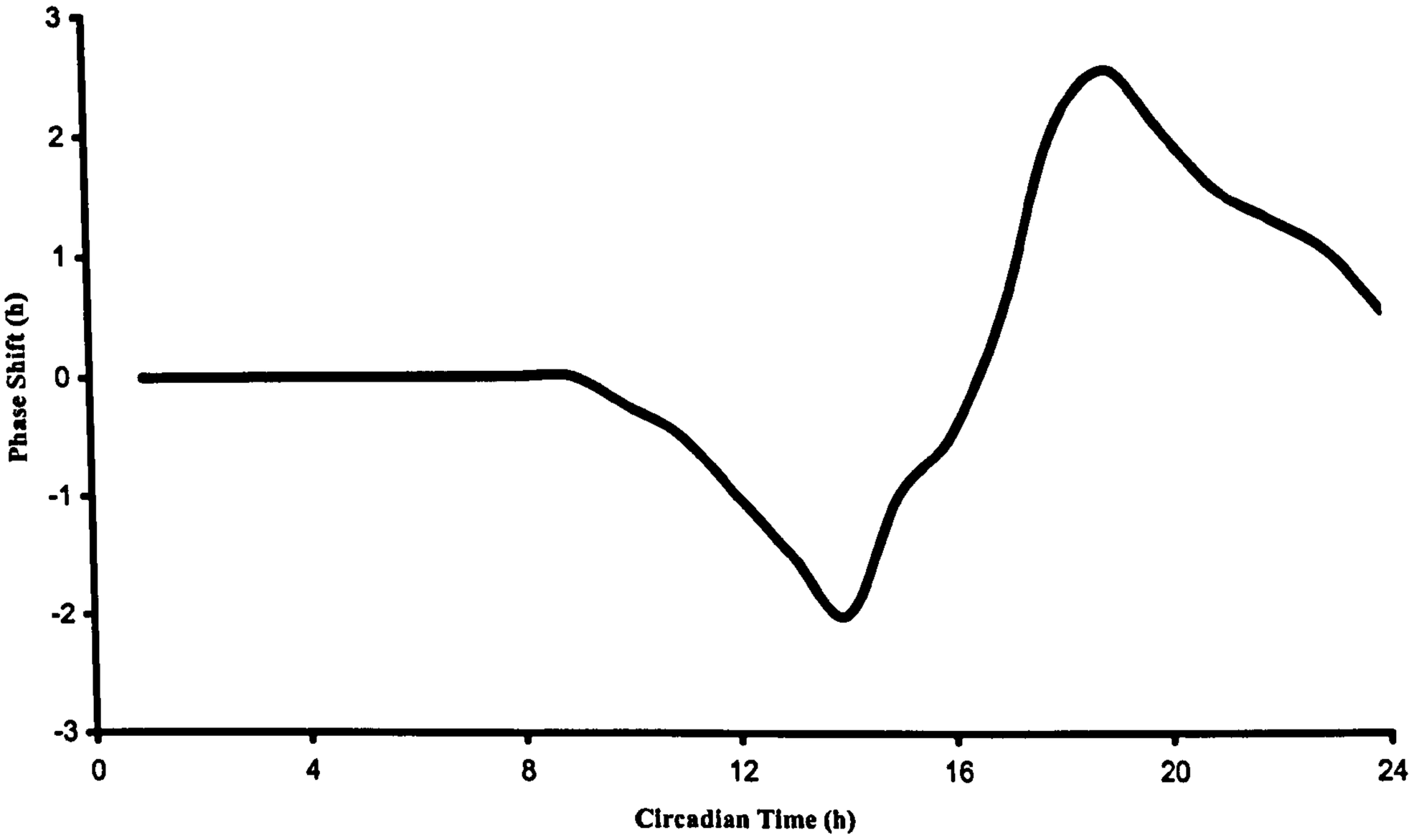


Figure 3. A schematic phase response curve for light. Light pulses given at CT2 have no effect on the clock, pulses given at CT14 cause phase delays and pulses given at CT18 result in phase advances.

1.5.2 Non-photic regulation of the clock.

Non-photic stimuli include temperature variation, social interactions, food and water availability and novel wheel exposure. Phase response curves observed to non-photic stimuli show phase advances during the subjective day and very little effect during the subjective night (Mrosovsky et al., 1989). Animals confined to novel wheels show consistent phase shift in a manner similar to the non-photic PRC (Ralph and Mrosovsky, 1992). Figure 4 illustrates a typical non-photic phase response curve. Novel wheel exposure is thought to phase shift by inducing arousal. For example hamsters that do not run in the novel wheel do not show non-photic phase shifts (Reebs and Mrosovsky, 1989). This may be because these animals are not aroused by the stimulus.

Entrainment to non-photic stimuli can be vital for the survival of a species. Times of food availability, predator avoidance and social interaction are just a few examples of non-photic stimuli that are essential for the success of a species. Throughout evolution organisms have had to integrate both photic and non-photic information for survival.

Figure 4.

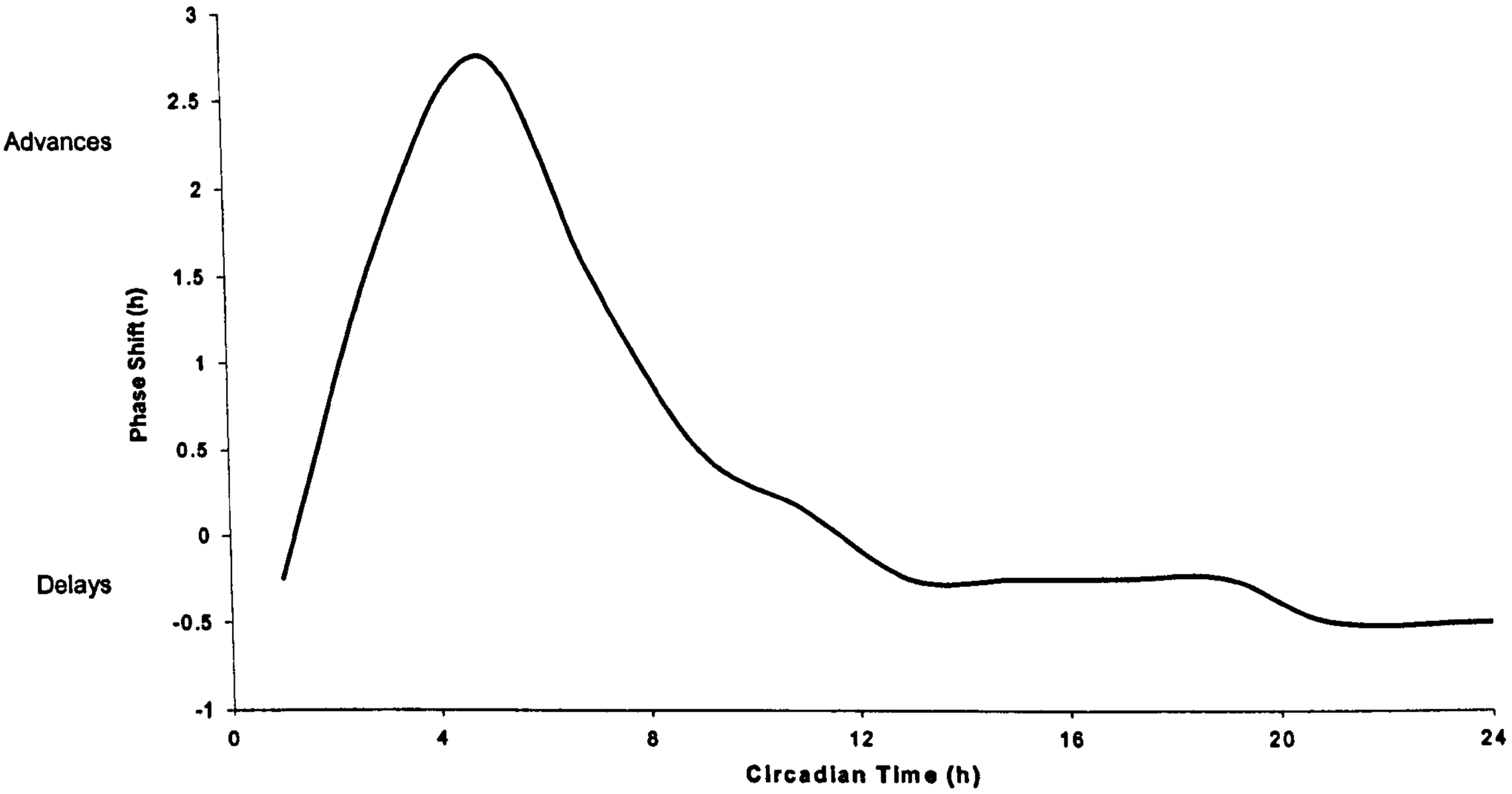


Figure 4. A schematic non-photic phase response curve. Non-photic stimuli show phase advances during the subjective day, and more variable phase delays during the subjective night.

1.6. Neuroanatomy

1.6.1. The Circadian Pacemaker.

The earliest studies of the location of the internal pacemaker were focused on the neural mechanisms. Lesion studies by Ritcher in 1967 concluded that only destruction of the hypothalamus disrupted the free running activity cycles in rats (Ritcher, 1967). This then led to further work attempting the localisation of the pacemaker within the hypothalamus. Microlesions placed within the hypothalamus combined with behavioural observations monitoring free running activity cycles identifies the central pacemaker to be located within the anterior hypothalamus, specifically in an area known as the suprachiasmatic nuclei (SCN) (Fig.5) (Moore and Eichler, 1972; Stephan and Zucker, 1972).

Lesions of the SCN disrupt such cycles as sleep-wake patterns, hormonal secretions, activity rhythms, eating and drinking patterns, reproductive and temperature cycles and corticosterone rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972; Abe et al., 1979). Further conclusive evidence using transplantation techniques showed that the SCN was the site of the circadian pacemaker. Neural grafts of SCN tissue were transplanted into hamsters with lesions of the SCN and the recipient recovered with the period of the donor (Ralph et al., 1990). The donor animals used for this study were from a mutant strain of hamsters with a very short tau.

Figure 5.

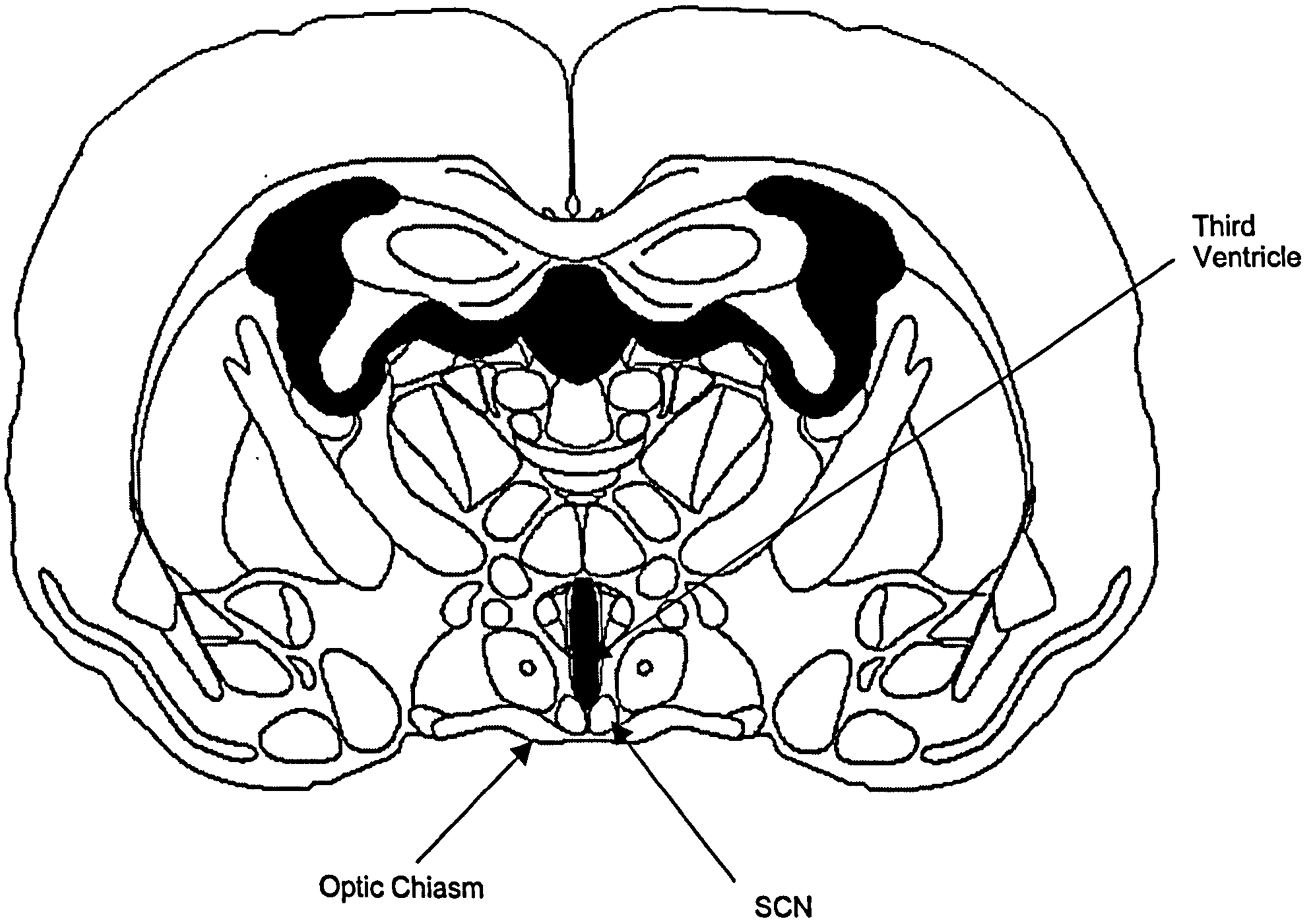


Figure 5. Illustration of a coronal section of a hamster brain. The location of the SCN is indicated just above the optic chiasm and below the third ventricle. The SCN consists of two bilaterally paired nuclei (Morrin et al, Hamster atlas 2000).

1.6.2. Major inputs of the clock.

Three major afferent pathways have been found to convey both environmental and behavioral information to the SCN. Photic signals project from the retina via the retinohypothalamic tract (RHT), directly to the SCN (Moore and Lenn, 1972). However, a second indirect route for light signals to be transmitted to the SCN also exists. Terminals from the retina also synapse onto the intergeniculate leaflet (IGL) of the lateral geniculate nucleus within the thalamus (Pickard et al., 1987), which in turn projects directly onto the SCN via the geniculohypothalamic tract (GHT) (Harrington, 1997). Non-photic information is also relayed to the SCN via two predominant routes. Firstly via the GHT originating from the IGL (Harrington, 1997) and from the raphe nuclei (Azmitia and Segal, 1978). The dorsal raphe innervates the IGL while the median raphe synapses directly onto SCN terminals. Figure 6 illustrates the various inputs of the SCN.

1.6.3. Neurotransmitters of the circadian system

Neurotransmitters utilised by the afferent pathways of the SCN act to regulate pacemaker activity and provide information about the environment, such as length of daylight. The RHT is primarily responsible for mediating photic information to the SCN. The principal neurotransmitter of this pathway is thought to be the excitatory amino-acid glutamate, which binds to glutamatergic receptors located on SCN cells (Ebling, 1996).

Numerous neurotransmitters have been implicated in mediating the effects of non-photic stimuli to the SCN. The IGL utilizes neuropeptide Y (NPY), Gamma-amino-butyric acid (GABA) and enkephalin as neurotransmitters, whilst the raphe nucleus utilizes serotonin in

conveying non-photic information to the SCN (Ajika and Ochi, 1978; Decavel and Van den Pol, 1990; Moore and Speh, 1993; Morin and Blanchard, 1995)(Fig.6).

Previous neurochemical studies have shown that a number of neuropeptide Y-immuno-reactive (NPY-ir) neurons originate from within the IGL in many species including rat, hamster and cat (Card and Moore, 1988, 1989; Covenas et al., 1990; Morin and Blanchard, 1995). A majority of NPY-ir cells also contain the neurotransmitter gamma-aminobutyric-acid (Francoisbellan et al., 1990). Neuronal fibres originating from the IGL also show immunoreactivity for serotonin, enkephalin, histamine and substance P, however not all of these afferents innervate the SCN (Agarwala et al., 1992; Morin, 1992). At present, predominate innervation from the IGL to the SCN appears to be from fibres containing NPY, GABA and enkephalins.

Figure 6.

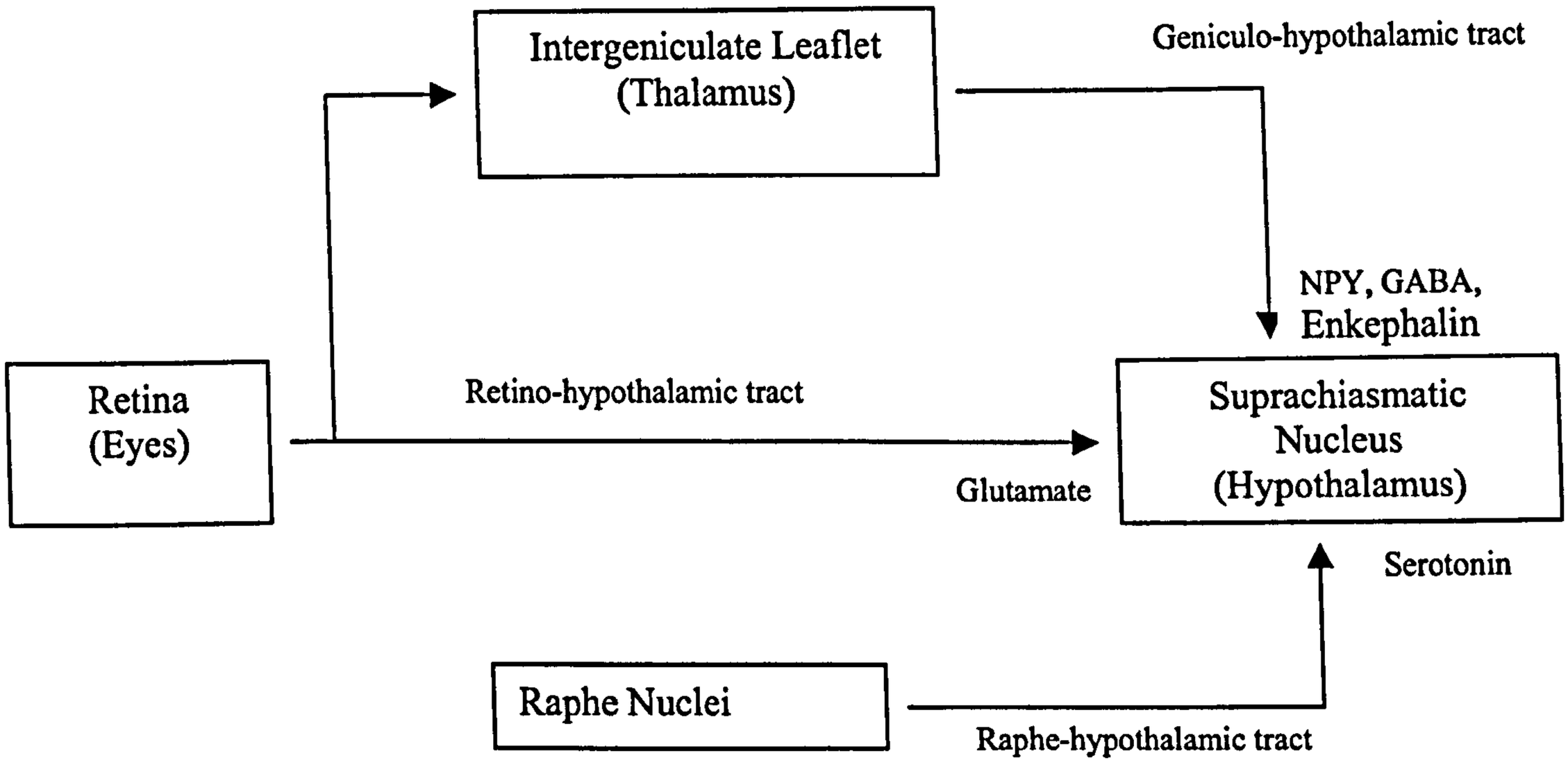


Figure 6. Illustrates the major afferent pathways that synapse onto the SCN. These inputs and their associated neurotransmitters are thought to be the major regulators of the SCN.

1.7. NPY and non-photic resetting of the circadian clock.

1.7.1. Mediation of non-photic stimuli by NPY

Non-photic stimuli have been studied in many animal models, with the hamster being among the most observed due to its robust circadian pattern of activity. In nocturnal animals non-photic stimuli reset the circadian pacemaker during the mid- subjective day, i.e. the rest phase in nocturnal rodents and have little or no effect during the subjective night, the active phase in nocturnal rodents

Behavioral arousal is amongst the most powerful of these non-photic stimuli in the hamster. One method of invoking such arousal is by placing an animal into a novel environment, such as confinement to a novel running wheel. Hamsters confined to a novel running wheel can become aroused by the new surroundings and this elevated level of excitement leads to excessive running. Those animals that run tend to phase shift to the stimulus if presented during the mid-subjective day (Reebs and Mrosovsky, 1989).

The involvement of NPY in the non-photic resetting of the circadian clock was shown using the novel wheel stimulus. Lesions of the IGL abolish the phase shifting effects of novelty-induced wheel running (Janik and Mrosovsky, 1994). Electrical stimulation of the GHT produces a PRC that resembles that of a non-photic stimulus (Rusak et al., 1989). The cannula administration of NPY onto the SCN *in vivo* produces PRC's similar to those observed with the novel wheel (Biello and Mrosovsky, 1996). Furthermore, the application of NPY to the SCN, *in vitro*, produces shifts in cellular firing rhythms comparable to non-photic stimuli (Biello et al., 1997b). Finally, antiserum raised against

NPY microinjected onto the SCN via a cranial cannula implant *in vivo*, inhibited the phase shifts to novel wheel exposure during the mid-subjective day (Biello, 1995).

1.7.2. The modulation of photic phase shifts by non-photic stimuli.

Photic and non-photic stimuli have been shown to interact with each other when presented simultaneously. Discoveries of such interactions have shown the versatility of the circadian system and how rapidly it can respond to different stimuli. Novel wheel running can attenuate light induced phase advances when administered together during the late subjective night (Ralph and Mrosovsky, 1992). The processes by which such attenuation can occur is unclear.

The RHT appears to be the sufficient for the synchronization of the circadian pacemaker to a light-dark cycle (Rusak and Zucker, 1979). However some alterations in the pattern of entrainment are seen after lesions of the IGL (Harrington and Rusak, 1986; Pickard et al., 1987; Johnson et al., 1989). Furthermore, the IGL and the SCN are innervated by collaterals from the same set of retinal neurons (Pickard, 1985). In addition, NPY containing fibres from the IGL project to the ventrolateral portion of the SCN, which is also innervated by the RHT (Card and Moore, 1988). Also, it was shown that neurons activated by stimulation of the RHT are more likely to respond to the application of NPY than those not activated (Shibata and Moore, 1988). These studies indicate that the IGL is involved in the modulation of photic responses to light within the circadian system.

Hamsters with complete lesions of the GHT show significant decreases in phase advance shifts produced by light pulses (Harrington and Rusak, 1986). However, Harrington and

Rusak (1986) found no effect of GHT lesions on phase delays to light, while Pickard et al (1987) reported larger phase delays with light in GHT lesioned animals. Furthermore animals with LGN lesions show significant retardation in the re-entrainment to a new light-dark cycle (Harrington, 1997). Hamsters with IGL lesions have a free running period in constant light similar to that in constant darkness (Harrington and Rusak, 1988), while intact hamsters show lengthened free running periods in constant light.

Microinjections of antiserum raised against NPY, via indwelling intracranial cannula's aimed directly at the SCN *in vivo*, results in the potentiation of light induced phase advances in the hamster (Biello, 1995). Furthermore NPY administration prior to light exposure during the early night attenuate the photic phase shift at this time, however phase delays to light are unaffected by NPY injections (Weber and Rea, 1997).

Taken together this evidence indicates the involvement of the GHT and NPY in the modulation of photic phase shifts. In order for such regulation to occur both photic and non-photic pathways must interact. Currently, the precise mechanism and site of interaction is unknown.

1.8 Neuropeptide Y receptors

Recent cloning and pharmacological studies have identified six major NPY receptors; Y1, Y2, Y3, Y4, Y5 and Y6. This family of NPY Y receptors has been pharmacologically defined by its ability to bind NPY, neuropeptide YY (PYY), pancreatic polypeptide (PP) and synthetic derivatives of these compounds (Wahlestedt and Reis, 1993; Gehlert, 1994; Chen and van den Pol, 1996).

Ligand binding studies using autoradiology have aided in the characterization of these NPY Y receptors. The binding profiles and rank order of binding potencies are as follows: Y1 binds NPY, PYY,[Leu^{31}, Pro^{34}]NPY and COOH-terminal fragments of NPY (Herzog et al., 1992; Larhammar et al., 1992), Y2 binds NPY, PYY and COOH- terminal fragments of NPY; Y3 binds NPY and PYY (Gerald et al., 1995; Rose et al., 1995); Y4 binds PP, NPY, PYY (Bard et al., 1995; Lundell et al., 1995; Yan et al., 1996) and the Y5 binds NPY, PYY, [Leu^{31}, Pro^{34}]NPY, NPY$^{2-36}$, human PP and COOH- terminal fragments of NPY (Gerald et al., 1996), while the Y6 receptor binds NPY, PYY, [Leu^{31}, Pro^{34}]NPY, NPY$^{2-36}$, NPY$^{13-36}$ and human PP (Weinberg et al., 1996). However, the Y6 gene is present in most species, but encodes foe a non-functional receptor (Weinberg et al., 1996).

Anatomical location studies (*in situ* hybridization histochemnical techniques) show that the topographical distribution of NPY Y receptor expression in the central nervous system is sparse. The Y1 and the Y2 receptors have been located in the paraventricular nucleus (PVN), arcuate nucleus (Arc) and the lateroanterior hypothalamic nucleus. The Y4 receptor was only identified in the PVN. The Y5 receptor was localized to the Arc. However it was found that both the Y1 and Y5 receptors were present in the SCN (Larsen

and Kristensen, 1998). Within the SCN the topographical distribution and expression of the Y1 and Y5 receptors is predominantly restricted to the ventrolateral portion of the SCN, where the majority of NPY immunoresponsive nerve fibres are also found (Card and Moore, 1982; Mikkelsen, 1990). This region of the SCN also receives afferent nerve fibres from the visual system both via direct and indirect routes generating from the GHT (Moore and Lenn, 1972; Swanson et al., 1974; Card and Moore, 1989; Mikkelsen, 1990).

Neuropeptide Y Y2 receptor expression was observed within the vicinity of the SCN but not in the nucleus itself (Gustafson et al., 1997). These studies are consistent with in vivo behavioural work showing the presynaptic role of the Y2 receptor in the mediation of NPY phase shifts (Huhman et al., 1996) and with the post synaptic role of the Y1/Y5 receptors in the inhibition of photic resting (Lall and Biello, 2003b). At present little is known about the topography of the Y3 receptor.

Very little is known regarding the associated intracellular signal transduction cascades for the NPY Y receptors. At present it is know that all NPY Y receptors characterized are G-protein coupled (Balasubramaniam, 1997). The NPY Y1 receptor has been associated with the activation of phospholipase C via a G-protein linked mechanism (Parker et al., 1998). Also the NPY Y5 receptor was found to cause the inhibition of cyclic AMP accumulation, suggesting that activation of this receptor influences second messenger pathways (Gerald et al., 1996).

The majority of the pharmacological characterization of all NPY Y receptors has been conducted using specific agonists. Examples of NPY agonists would include NPY, PP,

PYY, [Leu^{31}, Pro^{34}]NPY and NPY$^{2-36}$. All these agonists bind to various NPY Y receptors with varying potencies. Recently, many behavioural studies have relied on the development of specific NPY Y receptor antagonists. The commercial availability of NPY Y receptor antagonists has been extremely limited. However, private laboratories and independent pharmaceutical companies have developed a few antagonists for some of the NPY receptor subtypes. Currently Boerhinger pharmaceuticals have developed two Y1 receptor antagonist (BIB03304 and BIBP3226) and one Y2 receptor antagonist (BIIBE0246) (Doods et al., 1999; Dumont et al., 2000). Also a novel Y5 antagonist has recently been developed by Johnson and Johnson pharmaceuticals (RWJ57926). It is likely that antagonists for the various other NPY Y receptors are probably under development.

1.9 Molecular genetics of the clock

1.9.1 The molecular loop

The first 'clock' gene to be discovered was identified in the *Drosophila* and was called the *Drosophila* Period (*Per*) gene (Konopka and Benzer, 1971). Analysis of the kinetics of this gene's expression led to the generation of a simple molecular model (Hardin et al., 1990) which was found to be the basis of all the genetic models used to study circadian rhythms in the majority of species. This model consists of gene transcription and consequent translation steps leading to the formation of a protein. The protein produced then directly or indirectly inhibits its own transcription and the cycle restarts following protein degradation. This process can take place with an approximate 24 hour oscillation of RNA and protein, as is observed for many clock genes and their protein products. This forms the basis of a molecular loop that has a simple negative feedback system with several components, each of which depends on the previous component in the loop; for example, the rate of protein synthesis depends on RNA levels transcribed. The initial feedback model for the *Drosophila* involved just one gene and its two components, RNA and protein (Hardin et al., 1990). Recent advances in genetic research has led to the identification of many more components, however they all form part of the same transcription-translation feedback loop, functioning as activators, inhibitors or kinases.

The discovery and development of the mammalian circadian molecular mechanism came, initially, from the study of mutants. The first mutant to be studied was the tau mutant hamster. The tau mutant hamster possesses a clock that 'runs' a lot faster than that observed in the wild type (Ralph and Menaker, 1988). Large-scale mutant screening in the mouse aided the discovery of the CLOCK (Clk) gene, which has now been cloned and

found to encode for transcription factor (Gekakis et al., 1998). Period genes were later identified, in mammals, using available gene sequences (Albrecht et al., 1997) and various homolog searches (Tei et al., 1997). These results showed that many species shared the same clock genes. It was found that the 'core' of the mammalian molecular loop consisted of three Per genes (*Per1, Per2* and *Per3*), two cryptochrome (*Cry1* and *Cry2*) genes, a Clk gene and a gene called BMAL1 (Brain, Muscle And Liver protein) (Reppert and Weaver, 2001). Cell transfection experiments and genetics have allowed the understanding of how these genes and their products work. Results from experiments using these techniques have showed that *Per* and *Cry* expression is activated by a Clk-BMAL1 dimer, which binds to a specific promotor sequence, the 'E-box', CACGTG, while *Per* and *Cry* proteins dimerise and inhibit their own transcription by influencing Clk-BMAL1 activation (Gekakis et al., 1998; Hogenesch et al., 1998). In order for the dimerisation of the *Per* protein to occur, *Per* must first be phosphorylated. Period phosphorylation is mediated by Casein kinase 1e (Ck1e) (Reppert and Weaver, 2001). Once phosphorylated *Per* can freely bind to *Cry* to form stable heterodimers. Within the Per-Cry dimer, *Cry* provides the repressor function (Kume et al., 1999), while the role of *Per* is not entirely clear in the mechanism of the negative feedback loop (figure 7). A positive effect was found for the *Per2* protein on BMAL1 levels (Shearman et al., 2000).

Within this autoregulatory loop it has been found that the three *Per* and two *Cry* homologs can mix and match to form different complexes (Kume et al., 1999). Furthermore, new components have recently been discovered such as Rev-ErbB_{α}, which functions as a nuclear receptor that regulates the effects of BMAL1 (Preitner et al., 2002). The

generation of this molecular loop provides the internal 'time keeping' mechanism within SCN cells.

Under constant conditions both Per and Cry display an endogenous rhythm (Reppert and Weaver, 2001). These rhythms have been localized to specific regional area within the SCN. In rats, peak expression of both *Per1* and *Per2* RNA under constant conditions is greatest in the dorsomedial SCN; however endogenous rythmicity of *Per1* and *Per2* is also seen in the ventral SCN of the rat, particularly at the rostal level (Dardente et al., 2002). In mice, endogenously rhythmic *Per1* and *Per2* is also topographically organized and is present in rostal, dorsomedial and ventromedial portions of the SCN, but is absent from the central and ventrolateral regions of the mid and caudal SCN (LeSauter et al., 2003).

Figure 7

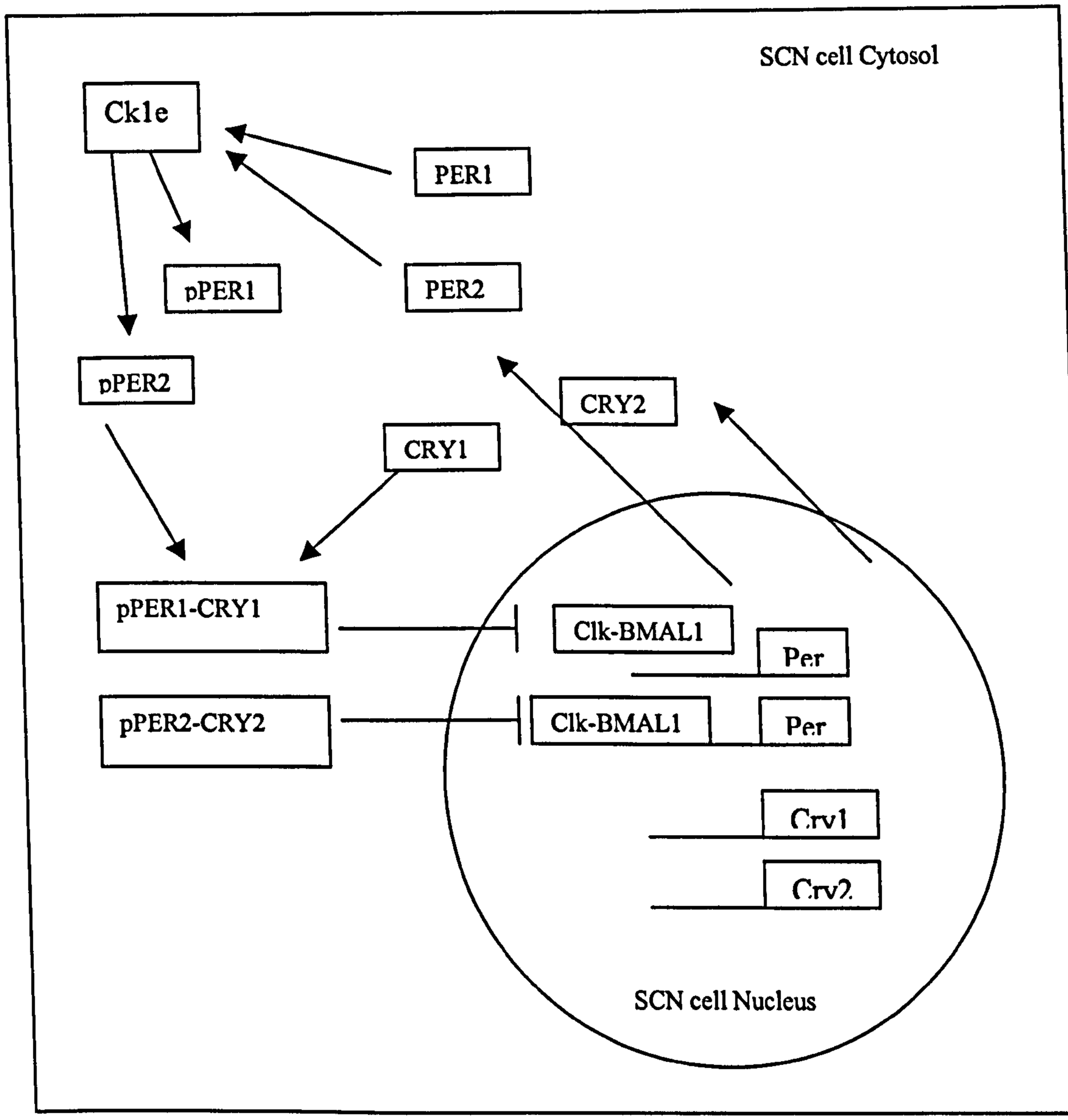


Figure 7. A simplified diagrammatic representation of the molecular clock in a mammalian SCN cell. CLOCK-BMAL1 (Clk-BMAL1) induces Period gene (*Per1* and *Per2*) expression. Cryptochrome genes (*Cry1* and *Cry2*) are also expressed. Within the SCN cell cytosol Per and Cry proteins are produced. Per proteins are phosphorylated (pPer1 and pPer2) by Casein Kinase 1e (Ck1e). pPer1 and pPer2 form dimers with Cry1 and Cry2, which then negatively feedback into the nucleus to inhibit *Per* transcription.

1.9.2 Photic phase shifts at the molecular level

Behavioural shifts in circadian rhythms observed with brief pulses of light during the subjective night occur via altering gene expression within the cells of the SCN. The expression of *Per1* and *Per2* can be induced by light pulses presented during the night (Albrecht et al., 1997; Shigeyoshi et al., 1997; Yan et al., 1999). Levels of *Per1* and *Per2* RNA rapidly increase after 15 to 30 minutes of light exposure (Shearman et al., 1997; Shigeyoshi et al., 1997; Yan et al., 1999). It has been shown that the degree of induction of *Per1* expression is dependent on the phase of the clock. Also *Per1* expression is induced at time points when light phase shifts the clock. Furthermore, the degree of *Per* induction is directly related to the size of the phase shift (van Esseveldt et al., 2000). Finally *in vivo* photic and glutamate induced phase shifts can be blocked by intracerebroventricular application of *Per1* RNA antisense oligonucliotides (Akiyama et al., 1999), further indicating the critical role of the period genes in the response to photic stimuli.

Photic phase shifts have been shown to increase Per transcription at times when levels of *Per* transcription are low. In the endogenous rhythm of the SCN *Per* transcription is initiated by the binding of the Clk-BMAL1 dimer to the promotor site of the *Per* genes. However, during the early and late night photic pulses initiate *Per* transcription via the phosphorylation of Ca^{2+}/cyclic AMP response element binding proteins (CREB) which lead to CRE-mediated transcription activation (Ginty et al., 1993; Ding et al., 1997; Obrietan et al., 1999). This mechanism in the induction of *Per* transcription allows the rapid increase in Per protein synthesis, ultimately leading to a photic resetting response.

During the early night, when light pulses cause phase delays in behavioural rhythms, light-induced *Per1* is expressed within the ventrolateral and central SCN in both rats and mice (Shigeyoshi et al., 1997; Yan and Okamura, 2002). However, the localization of *Per2* expression is not clear. Initially it was shown that *Per2* was expressed predominantly in the ventrolateral and central SCN following light pulses during the early night (Yan and Okamura, 2002), but also in this study *Per2* expression was seen in the dorsomedial SCN. Therefore, the regional specificity of *Per2* expression during a photic pulse is not absolute.

These molecular studies clearly show the key components involved in the molecular resetting effects of photic stimuli. Future studies are likely to identify the precise role of these molecular genes in the mediation of phase delays and advances to light.

1.9.3 Non-photic phase shifts at the molecular level

Currently the majority of molecular research looking at the resetting effects of various stimuli has been focused on photic stimuli i.e. light and glutamate induced phase shifts. At present there is minimal data available for the influences of non-photic stimuli on the molecular mechanism within the SCN.

Non-photic resetting has been shown to influence Period expression. Confinement of hamsters to a novel running wheel during the day results in non-photic phase shifts of the clock (Mrosovsky, 1996). Such shifts have been linked to the down regulation of *Per1* and *Per2* mRNA levels (Maywood et al., 1999). However, little is known about other molecular influences of non-photic stimuli, but it is clear that the period genes are the targets for both photic and non-photic resetting cues.

The Present Study

As reviewed, the biological clock is responsible for generating and regulating a circadian rhythm and is located in the SCN. Information regarding the environmental light-dark cycle is conveyed to the SCN via the RHT, which consists of a direct connection from the retina to the clock. A secondary indirect pathway from the retina to the SCN via the IGL is also present. This pathway is likely to be responsible for conveying both photic and non-photic information to the SCN.

From the evidence cited, it seems that NPY projections from the IGL to the SCN plays a role in the modulation of light induced resetting of the circadian clock. This thesis contains a series of experiments, which attempt to identify the mechanism utilized by NPY in its interaction with light during the early and late subjective night.

My hypothesis is that NPY modulates the phase shifting effects of light during the early and late subjective night.

Chapter 2

Microinjections of NPY during the late subjective night have been shown to inhibit phase advances to light, *in vivo* (Weber and Rea, 1997). However, in the same study phase delays to light were unaffected by NPY administration. These results differed from those found in *in vitro* studies recently published by Yannielli and Harrington (2000). In these experiments, phase delays to light were blocked by NPY application to the SCN slice preparation. Because of these apparent discrepancies in the literature it was first necessary

for me to examine this interaction *in vivo* myself. I designed my experiments to closely mimic the drug administration times of those preformed *in vitro* by other groups.

Furthermore, I also start to examine the possible site of interaction between NPY and light. I aim to investigate the possible NPY receptor subtype utilized by NPY in its interaction with light. It is known that phase shifts to NPY during the mid-subjective day are mediated via the Y2 receptor subtype (Huhman et al., 1996; Biello et al., 1997b). It is thought that either or both, the Y1 and Y5 receptor subtypes, are involved in the inhibition of photic phase shifts. In these experiments I used a specific Y1/Y5 receptor agonist to determine if these receptors were involved in the complex interaction between NPY and light.

Chapter 3

Having identified that NPY interacts with photic phase shifts via either or both the Y1 and Y5 receptor subtypes, I wanted to further clarify and pinpoint the specific receptor utilized by NPY in this interaction. Previously this was difficult due to the commercial unavailability of a specific Y5 receptor antagonist. However, we were very fortunate to obtain samples of a specific Y5 antagonist from Johnson and Johnson pharmaceuticals. In this experiment I used specific Y1 and Y5 receptor antagonists to identify the NPY receptor involved in the interaction between NPY and light.

Chapter 4

It was found that the NPY Y5 receptor subtype was utilized by NPY in the inhibition of light induced phase shifts. Phase advances to NPY have been shown to involve a

GABAergic mechanism indicating NPY shifts are mediated via the $GABA_A$ receptor (Huhman et al., 1995).

Here I examine if NPY utilizes a similar $GABA_A$-dependent pathway in the inhibition of photic phase shifts.

Chapter 5

My previous experiments have shown that NPY interacts with light via the activation of the Y5 receptor subtype and through a pathway independent of $GABA_A$ receptor activation. Taken together it is likely that the site of interaction lies within SCN cells, downstream from receptor binding sites.

In these experiments I examine the limitations of the inhibitory actions of NPY on light induced phase advances. I use NPY microinjections, which are administered in a time course manner post light exposure. Furthermore, I also run experiments using a classic non-photic stimulus, the novel running wheel. In these studies animals receive this behavioral stimulus instead of NPY microinjections in the same time dependent manner as NPY administration.

An important long-term goal of research on biological rhythms is to aid the understanding and treatment of circadian related disorders in humans. Both photic and non-photic cues play a vital role in our everyday lives. The integration of these stimuli allows our physiological processes to function in such a way that they all act to regulate and maintain an internal homeostatic environment.

Chapter 2

Attenuation of circadian light induced phase advances and delays by neuropeptide Y and a neuropeptide Y Y1/Y5 receptor agonist.

Interactions between light and NPY were investigated during the early (two hours after activity onset) and late (six hours after activity onset) night in male Syrian hamsters. Neuropeptide Y microinjections into the region of the SCN significantly attenuated light induced phase delay, during the early subjective night. Phase advances to light were completely inhibited by the administration of NPY during the late night.

The precise mechanism by which NPY attenuates or blocks photic phase shifts is unclear. The NPY Y1/Y5 receptor agonist, [Leu^{31}, Pro^{34}]NPY, was administered via cannula microinjections following light exposure during the early and late night. [Leu^{31}, Pro^{34}]NPY significantly attenuated phase delays to light during the early night and blocked phase advances during the late night, in a manner similar to NPY.

These results show the ability of NPY to attenuate phase shifts to light during the early night and block light induced phase advances during the late night. Furthermore, this study implicates the involvement of the NPY Y1/Y5 receptors in the complex interaction of photic and non-photic stimuli during the night.

2.1 Introduction

Injections of NPY have been shown to induce phase shifts similar to those observed with non-photic stimuli (Biello et al., 1994) and subsequent light exposure can attenuate these shifts (Biello and Mrosovsky, 1995). Neuropeptide Y phase shifts, *in vitro*, can be blocked by the administration of glutamate (Biello et al., 1997a), the major neurotransmitter thought to be involved in the mediation of photic information to the SCN (Ebling, 1996).

Phase shifts to light during the late phase of the subjective night (the active phase in nocturnal rodents) can be attenuated by NPY (Weber and Rea, 1997) or potentiated by antiserum to NPY (Biello, 1995), *in vivo*. A recent *in vivo-in vitro* model has provided further evidence for the role of NPY in the modulation of photic influences to the SCN (Yannielli and Harrington, 2000). In this novel paradigm, light pulses were presented *in vivo* followed by NPY administration *in vitro*.

The current study was designed to examine the effects of NPY administration on photic phase shifts during the subjective night, *in vivo*. More significantly the involvement of the NPY Y1 and/or NPY Y5 receptors were investigated in the mediation of the attenuating effects of NPY on light *in vivo*, during the early and late night.

2.2 Experimental procedures

2.2.1 Subjects

Experimental procedures were carried out under licence by the Home Office (UK) in accordance with the Animals (Scientific Procedures) Act (1986). Adult male Syrian hamsters (40 days old, Harlan Sprague-Dawley, Oxon, UK) were housed individually in plastic cages fitted with 16 cm running wheels connected to a computer that summed wheel revolutions in 10 minute activity bins (Dataquest Pro-Data software, Data Sciences Inc., Roseville, MN,USA). Food and water were available *ad libitum*. Animals (n=22) were maintained under a light-dark (LD) cycle of 14 hours light and 10 hours of darkness and then transferred to constant conditions (22 ± 2°C; 14-18 lux dim red light) 10 days before treatment. Animals were re-entrained by this method following a maximum of three treatments before continuing with further experimental conditions. All efforts were made to minimize the number of animals used and their suffering.

2.2.2 Surgery

See appendix I for a detailed description of the surgical procedure.

Hamsters were anaesthetised with halothane gas throughout the length of the surgery. Animals received cannula guides, which were implanted to a depth of 5.1mm below the skull surface and fixed to the skull with fine machine screws and dental cement. Cannula guides were stereotaxically aimed at the third ventricle, just above the SCN (co-ordinates relative to bregma: AP+0.6mm, ML 0.1mm, upper incisor bar –2 mm). After recovery from surgery (1 week under LD 14:10h), animals were transferred into cages fitted with running wheels and maintained for 7-10 days under LD 14:10h before being placed into constant conditions. Porcine NPY (Calbiochem, UK) and the Y1/Y5 agonist [Leu^{31},

Pro^{34}]NPY (Sigma, UK) were administered using a 28 gauge infusion cannula attached to a 1µl Hamilton syringe at a concentration of 1ng/nl at a volume of 200nl. The infusion cannula extended 2.4 mm from the base of the guide cannula.

Neuropeptide Y and [Leu^{31}, Pro^{34}]NPY were dissolved in artificial cerebral spinal fluid (ACSF; in mM; NaCl 124, KCl 33, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 2.5, glucose 10). The vehicle consisted of ACSF only.

2.2.3 Experimental treatments

Prior to the start of the experiment all animals in group 1 were injected with NPY at CT 6 five days after being placed into constant conditions. Similarly 6 animals in group 2 were also injected with NPY at CT 6 and 4 animals were injected with GRP (150pmol, 200nl, dissolved in ACSF). These microinjections were used as functional assessments to verify cannula placements.

Group 1 (n=12) hamsters completed the following sequence of treatments: (1) a 15 minute light pulse (130 lux) at CT14, (2) NPY administration at CT14, (3) a 15 minute light pulse at CT14 in combination with NPY administration at CT14.25, (4) [Leu^{31}, Pro^{34}]NPY administration at CT14, (5) a 15 minute light pulse at CT14 followed by [Leu^{31}, Pro^{34}]NPY administration, (6) administration of the vehicle at CT14, (7) a 15 minute light pulse at CT14 directly followed by vehicle administration.

Group 2 (n=10) hamsters completed the following sequence of treatments: (1) a 15 minute light pulse (130 lux) at CT18, (2) NPY administration at CT18, (3) a 15 minute light pulse

at CT18 in combination with NPY administration at CT18.25, (4) [Leu^{31}, Pro^{34}]NPY administration at CT18, (5) a 15 minute light pulse at CT18 followed by [Leu^{31}, Pro^{34}]NPY administration, (6) administration of the vehicle at CT18, (7) a 15 minute light pulse at CT18 directly followed by vehicle administration.

2.2.4 Data analysis

For activity onset calculation see appendix III.

Data from each set of treatments was analysed by the one-way repeated measures ANOVA, followed by Bonferroni's method for multiple comparisons. All *t*-values reported were derived from multiple comparison tests.

2.2.5 Cannula Placement

See appendix IV for cannula placement assessment methods.

2.3 Results

2.3.1 Effects of [Leu^{31},Pro^{34}]NPY and NPY on light induced phase delays

Light stimulation at CT 14 resulted in phase shifts of –93 ± 7 minutes (mean ± SEM). Phase delays to light were attenuated by microinjection of NPY directly after photic stimulation with phase shifts of –37 ± 9 minutes ($F_{(5,55)}$= 25.2, p<0.01). Furthermore the administration of the NPY Y1/Y5 receptor agonist, [Leu^{31},Pro^{34}]NPY, following light exposure also resulted in the attenuation of light induced phase shifts (-49 ± 8 minutes; t=4.25, p<0.05). The combination of light and NPY administration was not significantly different from NPY microinjection alone (t= 1.60, p>0.05), however it was significantly different from light followed by vehicle treatment (-91 ± 6 minutes, t= 5.45, p>0.05). Neuropeptide Y or [Leu^{31},Pro^{34}]NPY produced phase shifts of –19 ± 8 minutes and -7 ± 6 minutes respectively. Phase shifts to light were not significantly altered by vehicle administration post light exposure (t=0.14, p>0.05).

2.3.2 Effects of [Leu^{31},Pro^{34}]NPY and NPY on light induced phase advances

Light induced phase advances (124 ± 8 minutes) were blocked by the administration of [Leu^{31},Pro^{34}]NPY and NPY with resulting phase shifts of –5 ± 12 minutes and 7 ± 15 minutes respectively ($F_{(6,48)}$= 34.7, p<0.01). Microinjections of NPY or [Leu^{31},Pro^{34}]NPY alone resulted in phase shifts of 6 ± 15 minute and 7 ± 6 minutes respectively. Vehicle administration following light stimulation did not result in phase shifts significantly different from those with light exposure alone (106 ± 4; t= 1.42, p>0.05).

2.3.3 Extended inhibition of activity by light pulses

Pulses of light at CT 14 resulted in the attenuation of activity compared to levels during prepulse days at the same CT time (t=5.53, p<0.05; Fig. 3). This extended inhibition following 15 minutes of light exposure lasted for, on average, 117 ± 19 minutes. The duration of quiescence following light stimulation was defined as the time until at least one wheel revolution was recorded in each of three successive 10 minute bins (Mistlberger and Antle, 1998). The combination of light with NPY or [Leu^{31},Pro^{34}]NPY resulted in a shortening of this extended inhibition due to light alone (52 ± 16 and 50 ± 16 minutes respectively, $F_{(5,50)}$= 8.59, p<0.01).

Pre-pulse days showed minimal activity levels during the late subjective night, therefore light pulses at CT 18 did not significantly attenuate home cage wheel running. During the hour following light exposure, NPY or [Leu^{31},Pro^{34}]NPY microinjections or a combination of light and either NPY or [Leu^{31},Pro^{34}]NPY wheel running activity was not significantly suppressed compared with control days i.e. pre-pulse days ($F_{(9,90)}$= 6.33, p<0.01).

2.3.4 Effects on Tau

The administration of light, NPY, [Leu^{31},Pro^{34}]NPY or a combination of light and NPY or [Leu^{31},Pro^{34}]NPY did not significantly alter tau (p>0.05), both during the early and late subjective night. These findings reflect those from previous studies (Biello et al., 1994).

2.3.5 Cannula placement verification

For functional assessment animals were injected with a known phase shifting stimulus. All phase shifts seen in this experimental group were comparable to those obtained in previous studies (Piggins et al., 1995; Biello and Mrosovsky, 1996; Mintz et al., 1999; McArthur et

al., 2000) . The following stimuli were used for functional assessment: NPY injected at CT 6 (200nl at a concentration of 1ng/nl, 133 min ± 9.6 [mean ± SEM], n=10); GRP injected at CT 14 (150pmol, 200nl, -191.1min ± 21.2, n=4). Further, histological placement verification confirmed that cannula were within 400μm of the SCN along the anterior-posterior, dorsal-ventral and lateral axes.

Figure 8

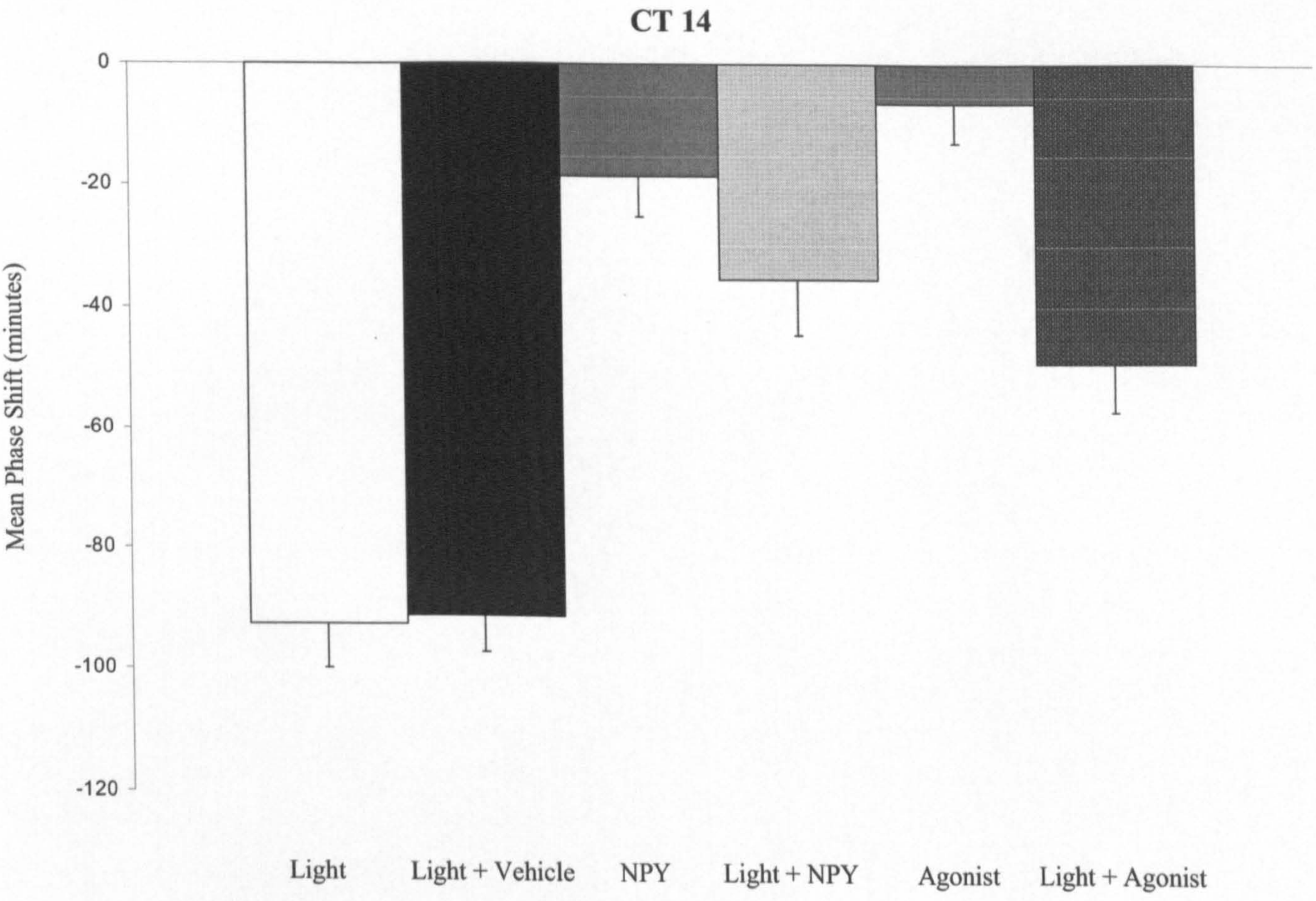

CT 14
0
-20
-40
-60
-80
-100
-120
Mean Phase Shift (minutes)
Light
Light + Vehicle
NPY
Light + NPY
Agonist
Light + Agonist

Figure 8. Phase delays to light were significantly attenuated by the administration of [Leu^{31},Pro^{34}]NPY, a NPY Y1/Y5 receptor agonist and NPY (Light + Agonist and Light + NPY respectively; $p<0.05$). Microinjections of NPY or [Leu^{31},Pro^{34}]NPY resulted in minimal phase delays (NPY and Agonist respectively). Vehicle administration post light exposure (Light + Vehicle) had no effect on the phase shifting properties of light at this time.

Figure 9

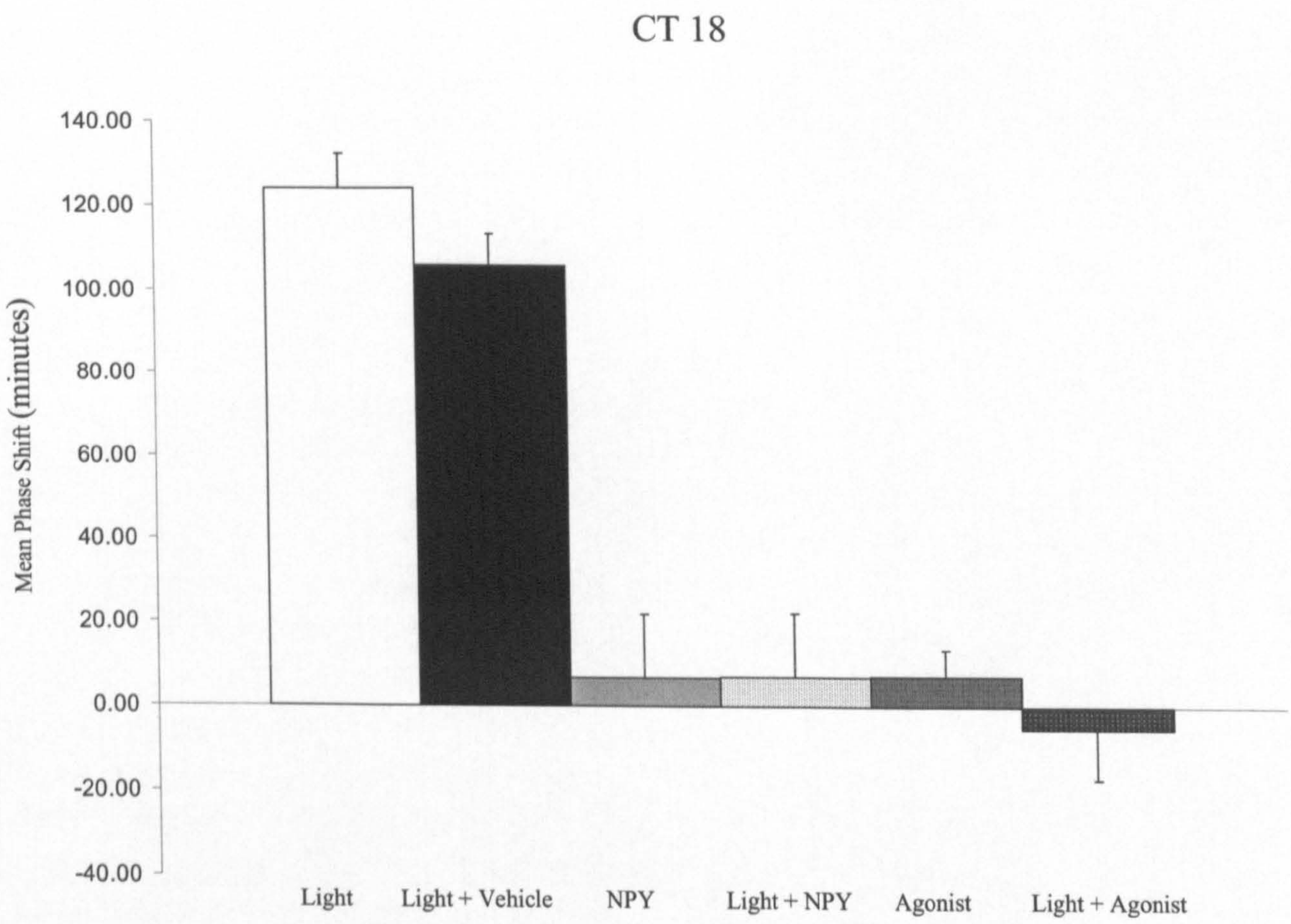
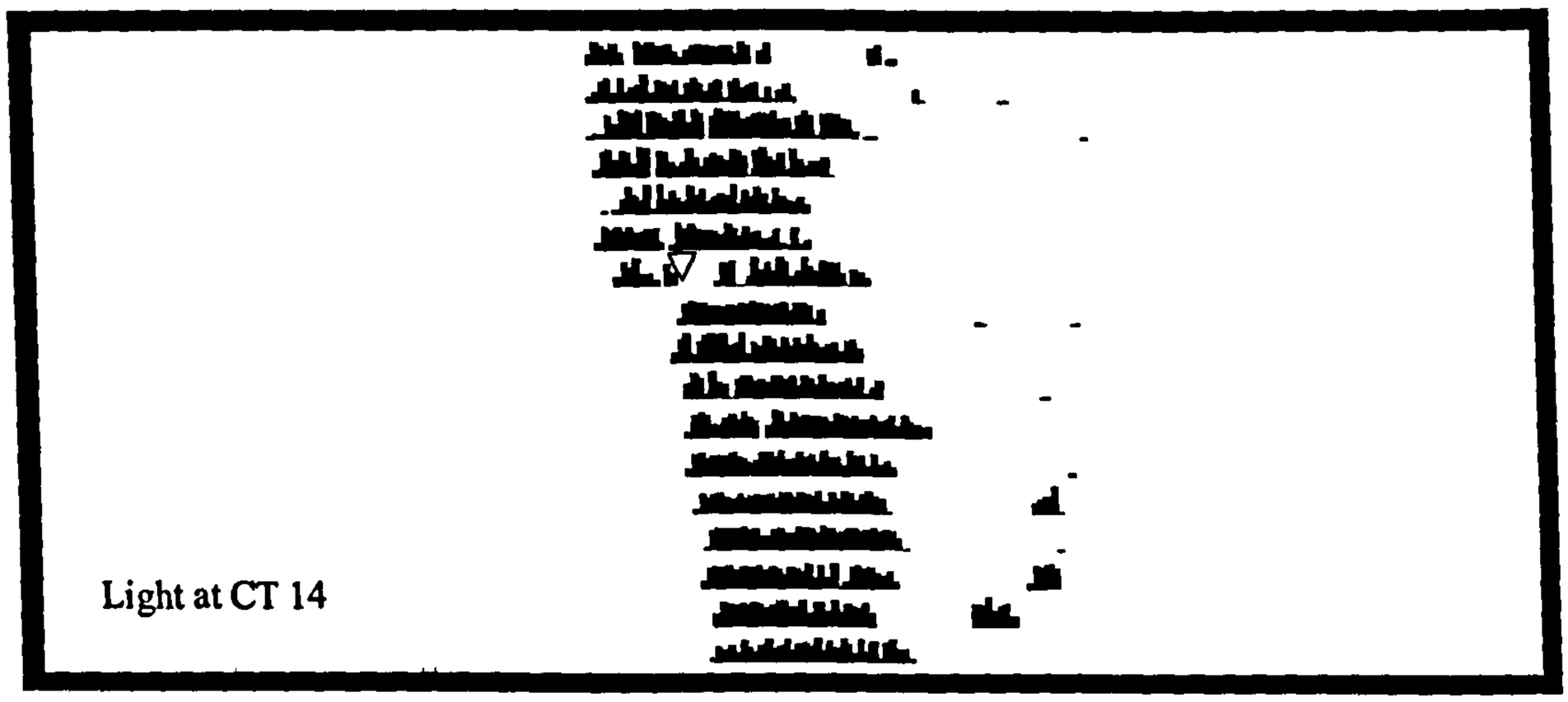
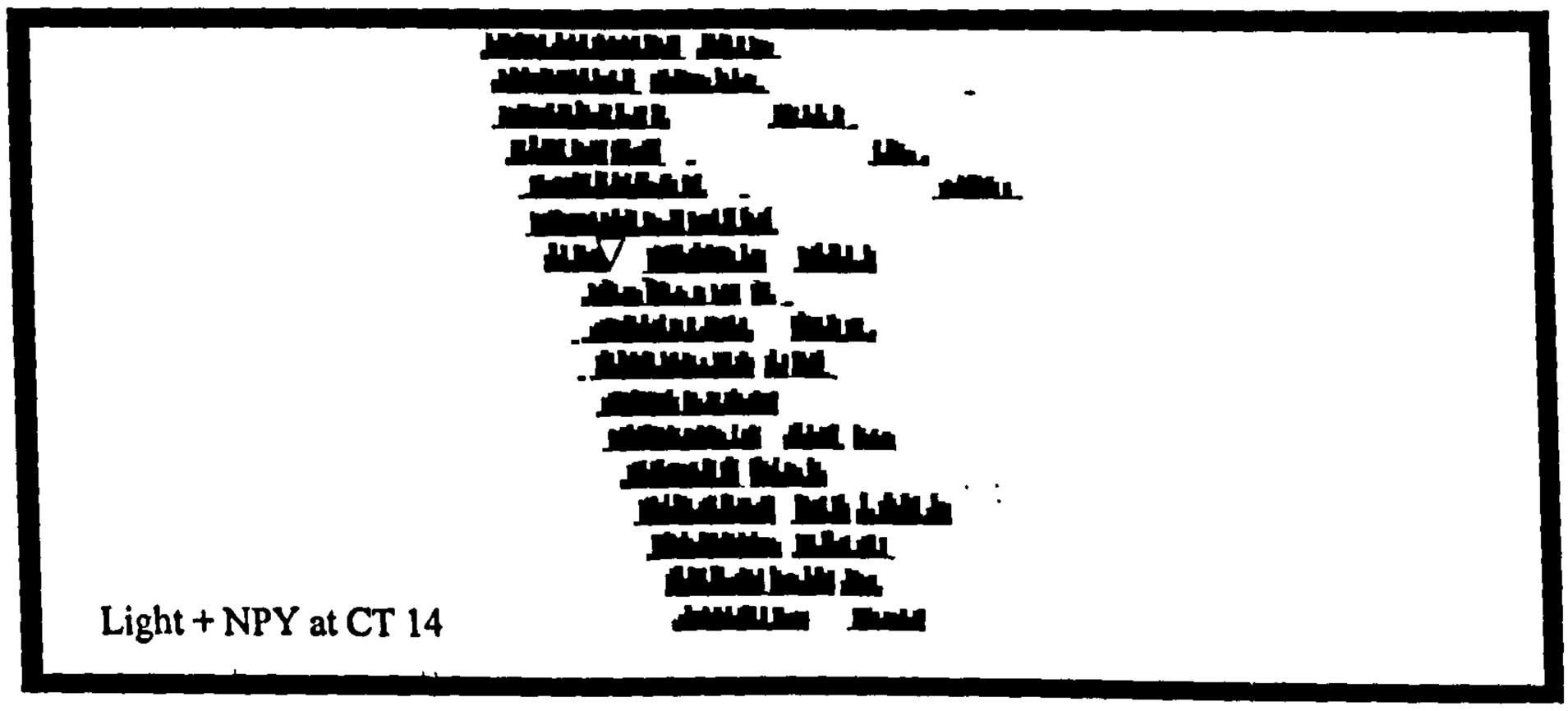

CT 18
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
Mean Phase Shift (minutes)
Light
Light + Vehicle
NPY
Light + NPY
Agonist
Light + Agonist

Figure 9. Light induced phase advances were blocked by microinjections of [Leu^{31},Pro^{34}]NPY and NPY (Light + Agonist and Light + NPY respectively; $p<0.05$). Little effect on phase was observed with administration of [Leu^{31},Pro^{34}]NPY or NPY alone (Agonist and NPY respectively). No significant effect on light induced phase shifts was observed by the administration of the vehicle post light exposure (Light + Vehicle, $p>0.05$).

Figure 10.


Light at CT 14


Light + NPY at CT 14

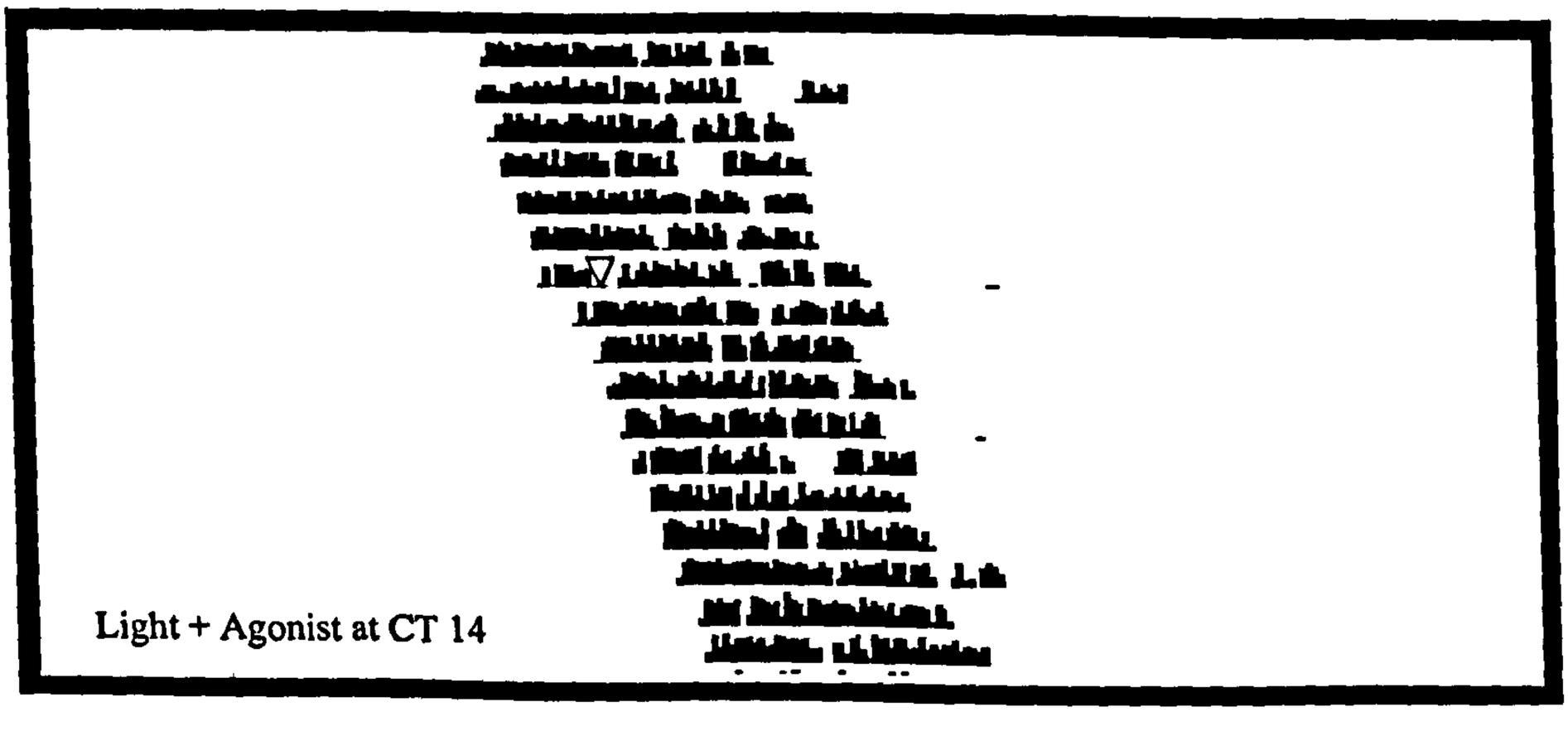
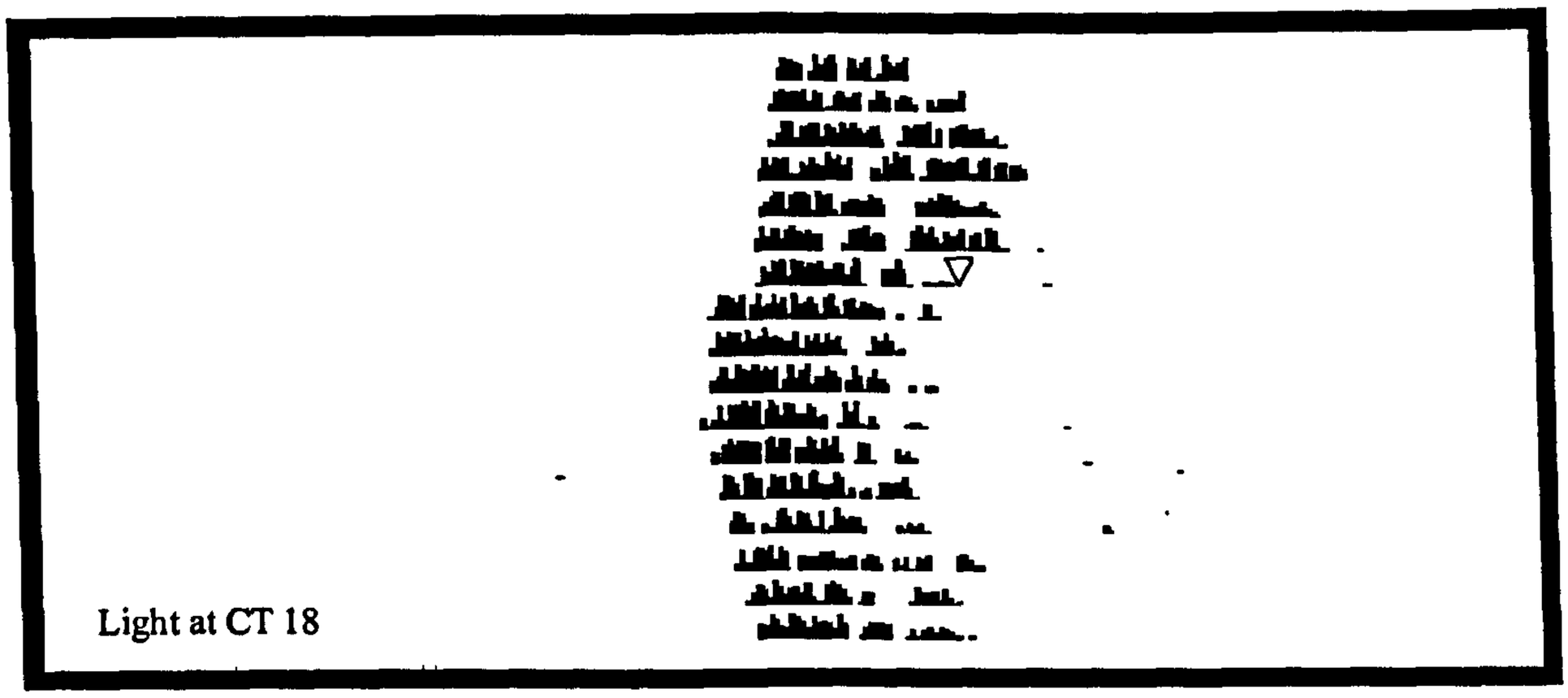
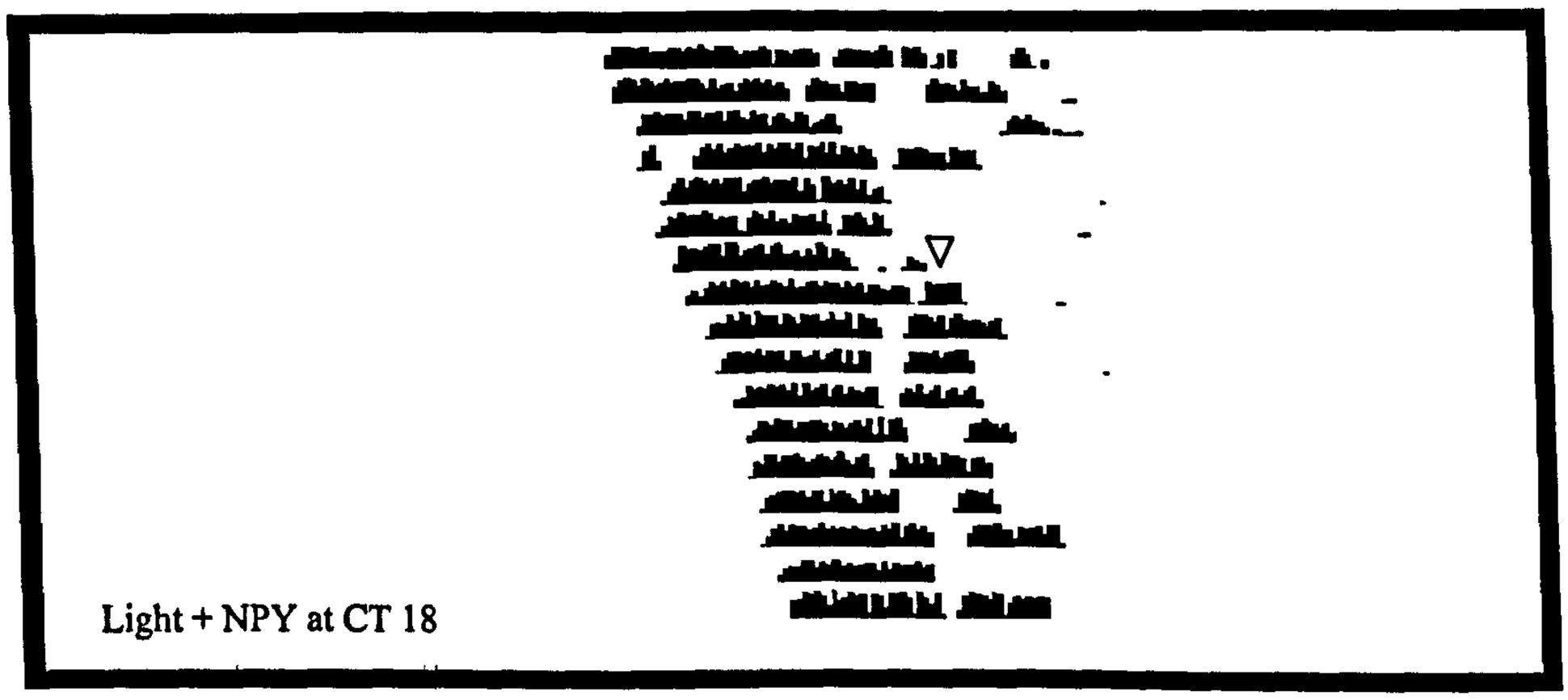
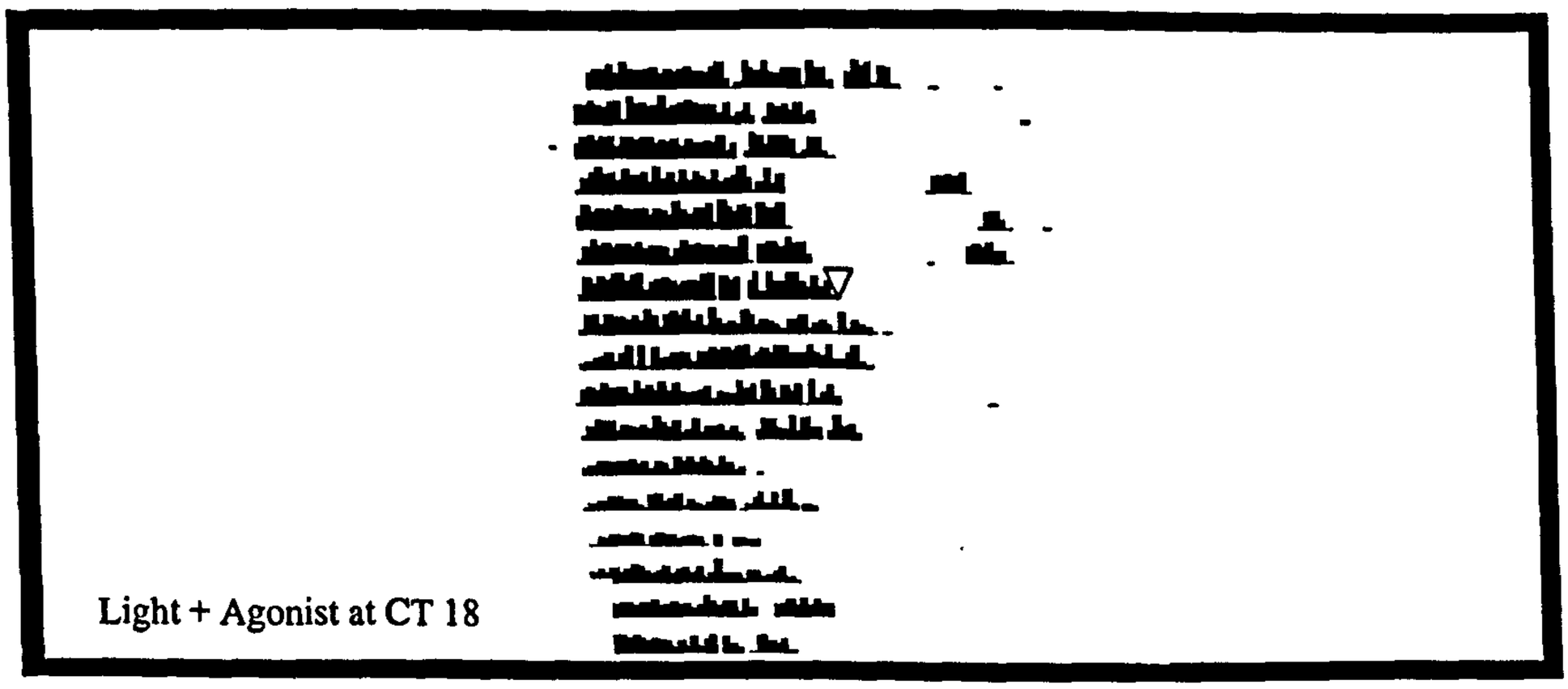

Light + Agonist at CT 14
0
Time (Hours)
24

Figure 10. Representative actograms demonstrating the attenuation of light induced phase delays at CT 14 by [Leu^{31},Pro^{34}]NPY and NPY. Light exposure for 15 minutes at CT 14 resulted in a phase delays of the free running rhythm in Syrian hamsters. However microinjections of either [Leu^{31},Pro^{34}]NPY or NPY, at the end of the light pulse, were able to attenuate these phase shifts. Times of light stimulation are indicated by inverted open triangles.

Figure 11.







0 Time (Hours) 24

Figure 11. Representative actograms demonstrating the inhibition of light induced phase advances at CT 18 by [Leu^{31},Pro^{34}]NPY and NPY. Phase advances to light were blocked by the administration of [Leu^{31},Pro^{34}]NPY or NPY. Times of light stimulation are indicated by inverted open triangles.

2.4 Discussion

The current study demonstrates the inhibitory effects of NPY on light induced phase shifts during the subjective night, *in vivo*. Microinjections of NPY were shown to attenuate light induced phase delays for the first time; furthermore NPY administration blocked phase advances to light during the late subjective night. Finally, and most significantly, the NPY Y1/Y5 receptor agonist, [Leu^{31}, Pro^{34}]NPY, was found to attenuate phase delays and block advances to light, in a manner similar to NPY.

Phase advances to light were attenuated by approximately 90% after administration of NPY during the late subjective night in our current study. However phase delays to light showed only a 40% attenuation after administration of NPY. This variation in results between the early and late night may be due to the difference in the way light resets the clock during these times. Glutamate is thought to be the principal neurotransmitter responsible for relaying photic information to the SCN. The presence of glutamate in presynaptic terminals of the SCN has been shown using ultrastructural methods (Ebling, 1996). Stimulation of the optic nerve *in vitro*, releases glutamate onto SCN cells (Liou et al., 1986), while optic nerve stimulation *in vivo* produces phase shifts that mimic those seen in response to light (de Vries et al., 1994). *In vitro* investigations have shown glutamate induced phase shifts to be blocked by NPY administration during the early and late night (Biello et al., 1997a). Recently, phase shifts to the glutamatergic agonist N-methyl-D-aspartate (NMDA) *in vitro*, were blocked by the administration of NPY during the early night (Yannielli and Harrington, 2001).

Light and glutamate phase shift the SCN mainly via NMDA receptors during the early subjective night (Ding et al., 1998) and via a mechanism involving protein kinase G (PKG) during the late subjective night (Weber et al., 1995). It is possible that the activation of the NMDA receptor results in a more rapid induction of the intracellular pathway mediating the resetting of the clock than that initiated by PKG activation. However, the inhibitory action of NPY may remain consistent between the early and late night, hence the ability of NPY to attenuate light induced phase shifts may be determined by the speed at which the glutamatergic intracellular pathway proceeds.

Hamsters maintained under constant conditions for long periods of time do show greater responses to photic stimuli (Shimomura and Menaker, 1994). To minimize this effect our animals were re-entrained to a light-dark cycle following a maximum of three treatments.

A previous study conducted by Weber and Rea (1997) found NPY microinjections blocked phase advances to light, but did not significantly alter phase delays. There were several methodological differences that may account for the attenuation of our photic phase delay shifts reaching significance. First, cannula guides were aimed at the SCN in the Weber & Rea study, while our cannula guides were aimed at the third ventricle, just above the SCN.

Thus, it is possible that NPY may be acting at a site other than that of the SCN in our study although unlikely due to *in vitro* evidence. Also, NPY administration times also differed, with Weber and Rea microinjecting 10 minutes prior to light stimulation. The administration of NPY prior to the light pulse may have induce induced physiological changes which altered the way photic information was conveyed to the SCN. There is support for this, as application of NPY, to rat SCN slices, prior to treatment with NMDA, resulted in the potentiation of NMDA induced phase shift (Shibata et al., 1994). However,

another *in vitro* study showed that NPY administration following NMDA treatment attenuated the phase shift (Yannielli and Harrington, 2001).

The IGL utilises NPY as its primary neurotransmitter (Harrington, 1997). Microinjections of anti-serum raised against NPY resulted in the potentiation of light induced phase shifts (Biello, 1995), indicating the involvement of NPY in the mediation of photic stimuli to the SCN. Lesions to the GHT have been shown to increase light induced phase delays (Pickard et al., 1987), implicating the role of the IGL and NPY in the regulation of photic phase shifts. Immunoassay studies have shown NPY levels to peak between CT 12 and CT 14 under constant conditions, in rats (Shinohara et al., 1993). Our results would suggest that the endogenous NPY release during the early subjective night sub-maximally saturates the NPY receptors at this time; hence NPY microinjections were only able to attenuate the phase shift to light. It is also possible that at CT 14 the cells of the SCN are less responsive to NPY due to the cycling rhythm of this neurotransmitter. Therefore only a minimal effect of NPY microinjection is seen on photic phase delay shifts in comparison to the late subjective night.

Changes in the free-running period of hamsters following GHT lesions have been variable. One group reported a lengthening of the free-running period following GHT lesions (Pickard et al., 1987), however another report indicated no change (Harrington and Rusak, 1986). Microinjections of NPY alone also do not show alterations in the free-running rhythm of hamsters (Biello et al., 1994). We show that microinjections of NPY or the NPY Y1/Y5 receptor agonist either in combination with light or alone did not alter the

free-running rhythm in our hamsters, suggesting that NPY is only acting as a direct inhibitor to light and not influencing the natural free running rhythm.

Light has been known to acutely suppress activity in nocturnal rodents, therefore 'masking' the phase of the circadian pacemaker (Aschoff et al., 1982). Pulses of light at CT 14 resulted in the suppression of activity for approximately 100 minutes, however the co-application of NPY or [Leu^{31}, Pro^{34}]NPY reduced this suppression by approximately 50%. It is possible that NPY influences photic phase shifts by reducing the duration of the suppression in activity induced by light. Hence supporting a role of NPY in the induction of activity.

At least three NPY receptor subtypes have been found in the SCN, NPY Y1, NPY Y2 and NPY Y5 receptors (Chen and van den Pol, 1996; Golombek et al., 1996; Larsen and Kristensen, 1998). Phase shifts induced by NPY are mediated by the NPY Y2 receptor (Golombek et al., 1996; Huhman et al., 1996). However, it is likely that the regulation of photic entrainment by NPY, may involve the NPY Y1 or NPY Y5 receptor subtypes. Due to the lack of commercially available specific NPY Y1 or NPY Y5 receptor agonists and antagonists we were unable to determine the relative contribution of either receptor in the inhibition of photic phase shifts. This is certainly an area on which to focus future *in vivo* work.

At the level of gene expression, light stimulation results in the increase of Per1 and Per2 mRNA levels (mPer1 and mPer2, respectively), in mice (Shearman et al., 1997; Zylka et al., 1998). Light induced phase delays have been strongly linked to increases in mPer2,

while phase advances to increases in *mPer1*, in the mouse (Albrecht et al., 2001). However, NPY administration has been shown to reduce *mPer1* and *mPer2* levels in hamsters and mice (Fukuhara et al., 2001; Maywood et al., 2002). In the early night NPY differentially suppresses *mPer1* and *mPer2* levels after light stimulation, such that there is a sustained suppression of *mPer2* (Brewer et al., 2002). It is possible that alternate differential suppression of *mPer1* and *mPer2* late in the night could explain the observed differences in photic suppression.

The modulation of light induced phase shifts by NPY is possibly a vital mechanism in the ability of mammals to synchronise to the environmental light dark cycle. We have shown NPY to attenuate both phase delays and inhibit phase advances to light during the subjective night, *in vivo*. Furthermore our results show, for the first time, that NPY suppresses both photic phase advances and delays via the NPY Y1 and/or the Y5 receptors, *in vivo*.

Chapter 3

Neuropeptide Y inhibits photic phase shifts via the Y5 receptor.

In the previous chapter it was shown that NPY utilized either or both the Y1 or Y5 receptor subtypes in its interaction with photic phase shifts. Due to the commercial unavailability of a specific Y5 receptor antagonist a Y1/Y5 agonist was used in the previous study. Recently, Johnson and Johnson pharmaceuticals have developed a Y5 receptor antagonist. We were fortunate to receive adequate samples of this novel drug to test, *in vivo*.

The administration of the Y5 receptor antagonist during light exposure followed by NPY administration prevented the attenuation of the photic phase shift by NPY during the early and late subjective night. Furthermore, microinjections of a specific Y1 receptor antagonist did not influence the inhibitory actions of NPY on light induced phase advances and delays.

This study clearly indicates that NPY utilizes the Y5 receptor subtype in its interaction with light during both the early and late subjective night.

3.1 Introduction

The precise mechanism by which NPY mediates its modulatory effects on photic stimuli is unclear. However a variety of NPY receptor subtypes have been found to mediate effects of NPY on pacemaker cells. Rat SCN has been shown to express NPY Y1 (Y1), NPY Y2 (Y2) and NPY Y5 (Y5) receptor subtypes (Weinberg et al., 1996; Larsen and Kristensen, 1998). The phase resetting effects of NPY during the day were found to be mediated via the Y2 receptor subtype (Golombek et al., 1996; Huhman et al., 1996). *In vivo* work indicates that either or both the Y1 and Y5 receptors are involved in the interaction between light and NPY during the night (Lall and Biello, 2003b). In this case a specific Y1/Y5 receptor agonist attenuated phase delays to light and inhibited phase advances in a similar manner to NPY microinjections. Furthermore, phase shifts to NMDA were blocked by NPY administration *in vitro* and this inhibition was prevented by the application of a specific Y5 receptor antagonist prior to NPY microinjection (Yannielli and Harrington, 2001).

Thus, it is possible that NPY mediates its inhibitory effect on photic stimuli via either or both the Y1 or Y5 receptor subtypes *in vivo* and *in vitro*. The current study was designed to determine the precise receptor or receptors utilized by NPY in its complex interaction with light, *in vivo*.

3.2 Materials and methods

3.2.1 Subjects

Experimental procedures were carried out under licence by the Home Office (UK) in accordance with the Animals (Scientific Procedures) Act (1986). Adult male Syrian hamsters (40 days old, Harlan Sprague-Dawley, Oxon, UK) were housed individually in plastic cages fitted with 16 cm running wheels connected to a computer that summed wheel revolutions in 10 minute activity bins (Dataquest Pro-Data software, Data Sciences Inc., Roseville, MN,USA). Food and water were available *ad libitum*. Animals (n=21) were maintained under a LD cycle of 14 hours light and 10 hours of darkness and then transferred to constant conditions (22 ± 2°C; 14-18 lux dim red light) 10 days before treatment. Animals were re-entrained by this method following a maximum of three treatments before continuing with further experimental conditions. All efforts were made to minimise the number of animals used and their suffering.

3.2.2 Surgery and injections

See appendix I for a detailed description of the surgical procedure.

Hamsters were anaesthetised with halothane gas throughout the length of the surgery. Animals received cannula guides, which were implanted to a depth of 5.6mm below the skull surface at a 10° angle and fixed to the skull with fine machine screws and dental cement. Cannula guides were stereotaxically aimed at the SCN (co-ordinates relative to bregma: AP+0.4mm, ML 1.0mm, upper incisor bar –2 mm). After recovery from surgery (1 week under LD 14:10h), animals were transferred into cages fitted with running wheels and maintained for 7-10 days under LD 14:10h before being placed into constant conditions. In all treatments the vehicle consisted of a 0.9% saline solution. Porcine NPY

(234μM in 200nl saline, Calbiochem, UK), Y1 antagonist BIBP 3226 BS (*R-N2*-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methyl-argininamide; 1μM in 200nl saline; Research Biochemicals) and Y5 antagonist RWJ57926 ([α-(3-pyridylmethyl)-β-aminotetralin-derived sulfonamide]; 2μM in 200nl saline; RW Johnson Pharmaceutical Research Institute), were administered using a 28 gauge infusion cannula attached to a 1μl Hamilton syringe. The infusion cannula extended 2.4 mm from the base of the guide cannula.

3.2.3 Experimental treatments

Prior to the start of the experiment all animals were injected with NPY at CT 6 five days after being placed into constant conditions. These microinjections were used as functional assessments to verify cannula placements.

Experiment 1 (n=10). Hamsters completed the following sequence of treatments: (1) a 15 minute light pulse (130 lux) at CT14 directly followed by vehicle administration, (2) saline administration at CT14 followed by NPY administration at CT14.08, (3) Y1 antagonist administration at CT14 followed by saline administration at CT14.08, (4) Y5 antagonist administration at CT14 followed by saline administration at CT14.08, (5) a 15 minute light pulse at CT14 with Y1 antagonist administration at CT14.08 and NPY administration at CT14.17, (6) a 15 minute light pulse at CT14 with Y5 antagonist administration at CT14.08 and NPY administration at CT14.17, (7) a 15 minute light pulse at CT14 with Y1 antagonist administration at CT14.08 and saline administration at CT14.17, (8) a 15 minute light pulse at CT14 with Y5 antagonist administration at CT14.08 and saline

administration at CT14.17, (9) a 15 minute light pulse at CT14 with saline administration at CT14.08 and NPY administration at CT14.17.

Experiment 2 (n=9). Hamsters completed the following sequence of treatments: (1) a 15 minute light pulse (130 lux) at CT18 directly followed by vehicle administration, (2) saline administration at CT18 followed by NPY administration at CT18.08, (3) Y1 antagonist administration at CT18 followed by saline administration at CT18.08, (4) Y5 antagonist administration at CT18 followed by saline administration at CT18.08, (5) a 15 minute light pulse at CT18 with Y1 antagonist administration at CT18.08 and NPY administration at CT18.17, (6) a 15 minute light pulse at CT18 with Y5 antagonist administration at CT18.08 and NPY administration at CT18.17, (7) a 15 minute light pulse at CT18 with Y1 antagonist administration at CT18.08 and saline administration at CT18.17, (8) a 15 minute light pulse at CT18 with Y5 antagonist administration at CT18.08 and saline administration at CT18.17, (9) a 15 minute light pulse at CT18 with saline administration at CT18.08 and NPY administration at CT18.17.

3.2.4 Data analysis

For activity onset calculation see appendix III.

Data from each set of treatments was analysed by the one-way repeated measures ANOVA, followed by Student-Newman-Keuls method for multiple comparisons. All *t*-values reported were derived from multiple comparison tests.

3.2.5 Cannula Placement

See appendix IV for cannula placement assessment methods.

3.2 Results

3.3.1 Effects of BIBP 3226 on the attenuation of photic phase delays by NPY

The administration of the specific Y1 receptor antagonist during light exposure and preceding NPY microinjection did not significantly alter the ability of NPY to attenuate the photic phase shift with delays of –29 ± 5 minutes (mean ± S.E.M) in the presence of the Y1 antagonist and –38 ± 5 minutes with a vehicle substitution of BIBP 3226 (p>0.05, fig. 12). Injections of the Y1 antagonist during light exposure followed by saline microinjection did not alter phase shifts to the photic stimulus at this time with resulting shifts of –58 ± 5 minutes and –64 ± 6 minutes with light and saline administration alone (p>0.05).

Neuropeptide Y administration following light exposure resulted in the attenuation of the photic phase shift observed with light and saline with average shifts of –38 ± 5 minutes and –64 ± 6 minutes respectively (p<0.01). Microinjections of NPY followed by saline or BIBP 3226 and saline resulted in shifts that were not significantly different from each other (-13 ± 6 minutes and 0 ± 4 minutes respectively; p>0.05), but both were significantly different from phase shifts resulting from light and saline treatment ($F_{(8, 81)}$= 29.1; fig. 14).

3.3.2 Effects of RWJ57926 on the attenuation of photic phase delays by NPY

Microinjection of the specific Y5 receptor antagonist prevented the attenuation of the photic phase delay by NPY (-74 ± 5 minutes) with shifts significantly different from those observed with saline substituted for the Y5 antagonist at this time (p<0.001, fig. 12). However administration of RWJ57926 during light exposure followed by saline injection

did not significantly alter the light induced phase shift with resulting shifts of -72 ± 8 minutes relative to –65 ± 6 minutes with light and saline alone (p>0.05).

The injection of RWJ57926 followed by saline resulted in phase shifts not significantly different from those with NPY and saline, with shifts of –3 ± 4 minutes and –12.9 ± 6 minutes respectively (p>0.05).

3.3.3 Effects of BIBP 3226 on the inhibition of photic phase advances by NPY

Light presented at CT 18 followed by saline administration resulted in phase shifts of 106 ± 4 minutes. These shifts were inhibited by NPY microinjection post light exposure with resulting shifts of 7 ± 15 minutes (p<0.001). The administration of the specific Y1 receptor antagonist during the photic pulse followed by NPY microinjection did not significantly alter the inhibitory effect of NPY with average shifts of 11 ± 9 minutes (p>0.05). Furthermore, the presence of the Y1 antagonist did not influence the phase shifting effects of light (119 ± 4 minutes), with shifts not significantly different from light and saline administration (p>0.05).

Microinjections of BIBP 3226 and saline resulted in phase shifts similar to those observed with NPY and saline administration, with average shifts of 8 ± 1 minutes and 7 ± 15 minutes respectively (p>0.05).

3.3.4 Effects of RWJ57926 on the inhibition of photic phase advances by NPY

Administration of the Y5 receptor antagonist inhibited the blocking effects of NPY on light induced phase advances. Phase shifts to light, followed by RWJ57926 and NPY

administration resulted in advances of 166 ± 10 minutes, which were significantly different from those observed with light, saline and NPY administration (8 ± 15 minutes; $p<0.001$). Furthermore, both treatment groups receiving the Y5 antagonist with light and either NPY or saline (170 ± 11 minutes) showed shifts greater than those observed with light and saline (106 ± 4 minutes) alone ($p<0.001$, in both instances).

Phase shifts to light, RWJ57926 and NPY were not significantly different from those obtained with light, RWJ57926 and saline administration ($p>0.05$). Microinjections of the Y5 antagonist and saline resulted in phase shifts that were not significantly different from those with NPY and saline treatments, with average shifts of 3 ± 2 minutes and 7 ± 15 minutes, respectively ($F_{(8, 72)}= 58.9$; $p>0.05$).

3.3.5 Cannula placement verification

For functional assessment animals were injected with a known phase shifting stimulus. All phase shifts seen in this experimental group were comparable to those obtained in previous studies (Huhman et al., 1995; Biello and Mrosovsky, 1996). Neuropeptide Y microinjections at CT 6 were used for functional assessment (0.023nmol in 400nl saline, 119 min ± 11 [mean ± SEM], n=19). Further, histological placement verification is set so that all placements lie within 400μm of the SCN along the anterior-posterior, dorsal-ventral and lateral axes.

Figure 12.

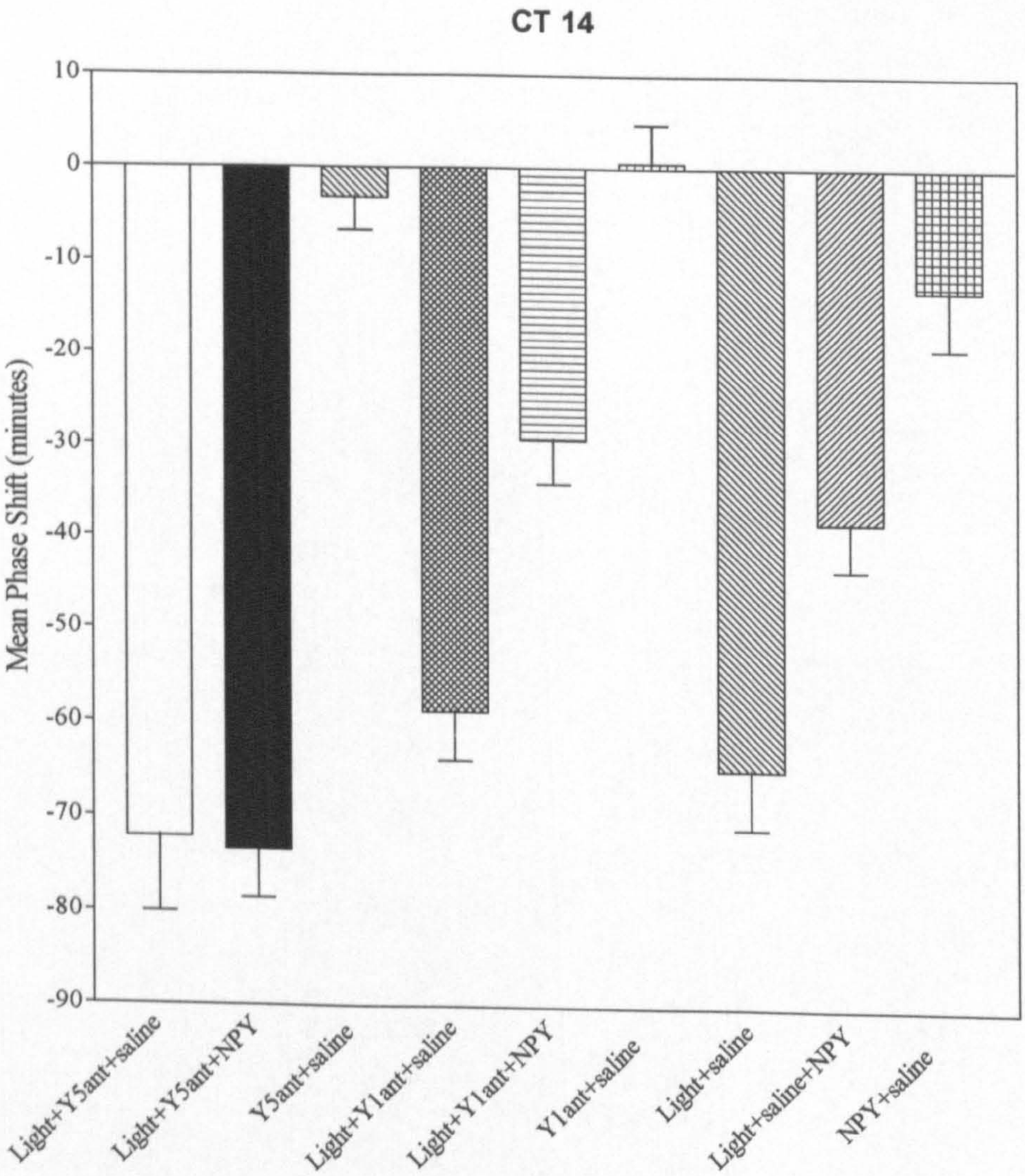

CT 14
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
Mean Phase Shift (minutes)
Light+Y5ant+saline
Light+Y5ant+NPY
Y5ant+saline
Light+Y1ant+saline
Light+Y1ant+NPY
Y1ant+saline
Light+saline
Light+saline+NPY
NPY+saline

Figure 12. Phase delays to light were significantly attenuated by NPY administration (Light+NPY; $p<0.05$). Microinjections of NPY were still able to attenuate phase shifts to light in the presence of a Y1 receptor antagonist (Light+Y1ant+NPY; $p<0.05$). However, the administration of the specific Y5 receptor antagonist was able to prevent the attenuation of the photic phase shift by NPY (Light+Y5ant+NPY).

Figure 13.

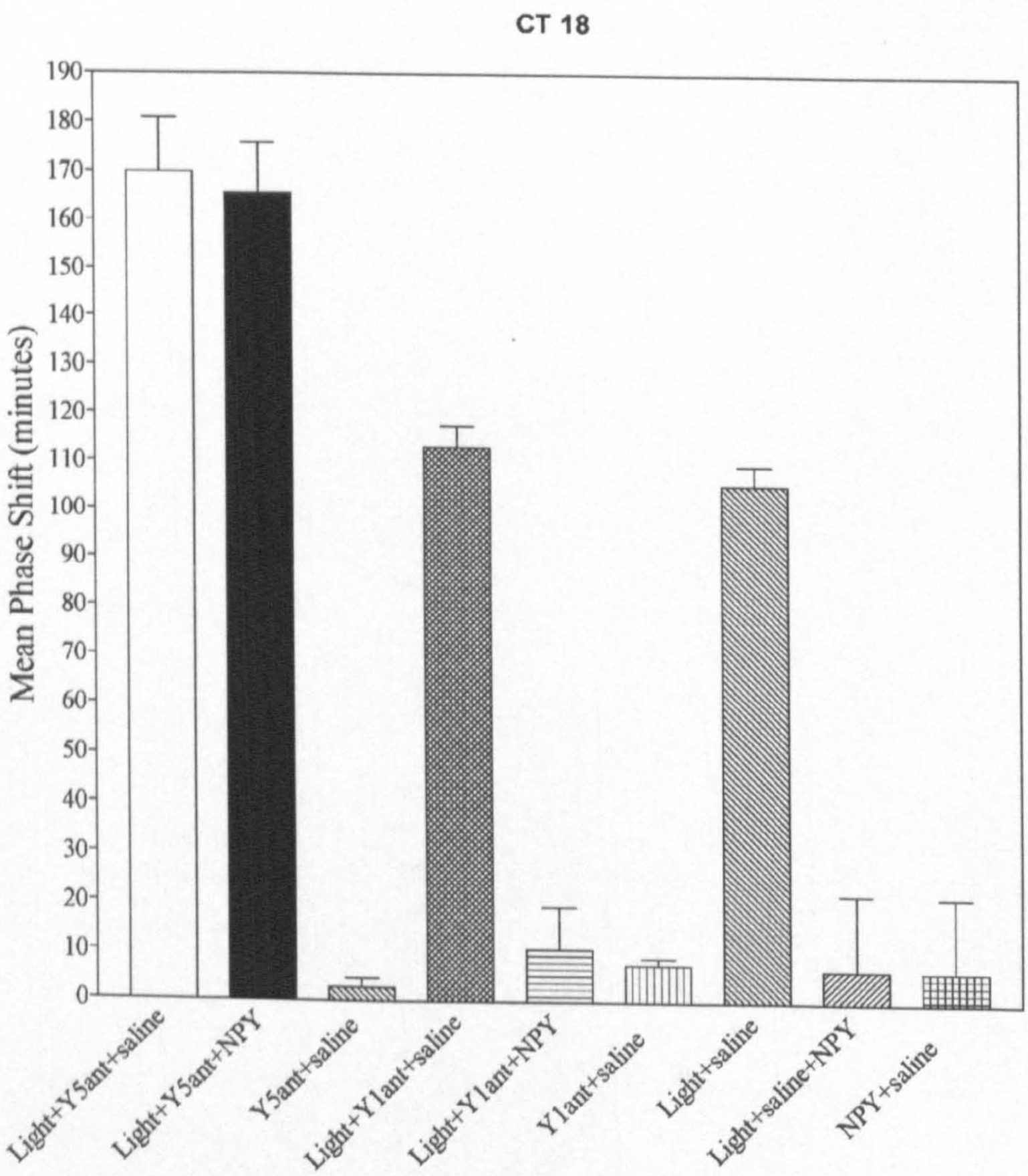
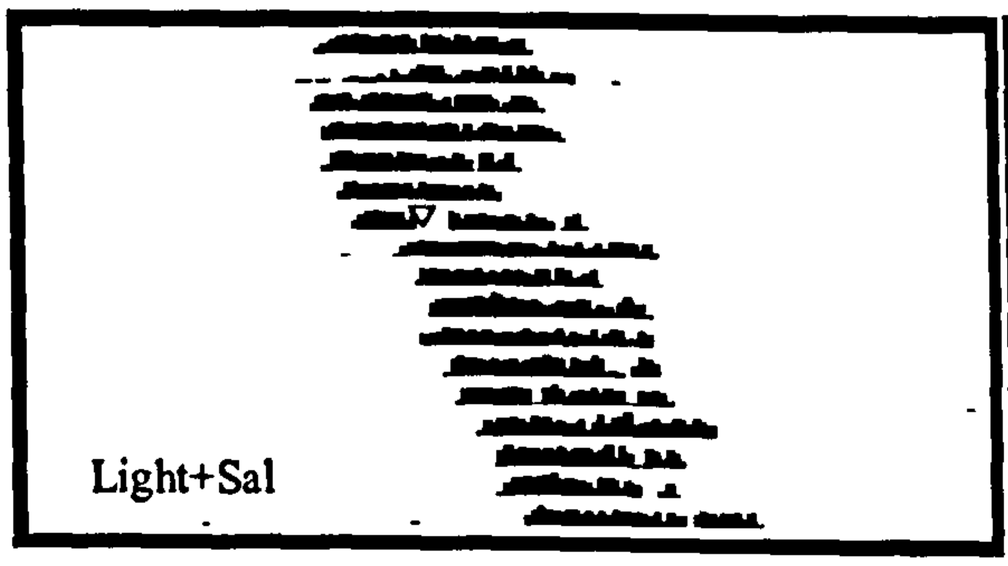
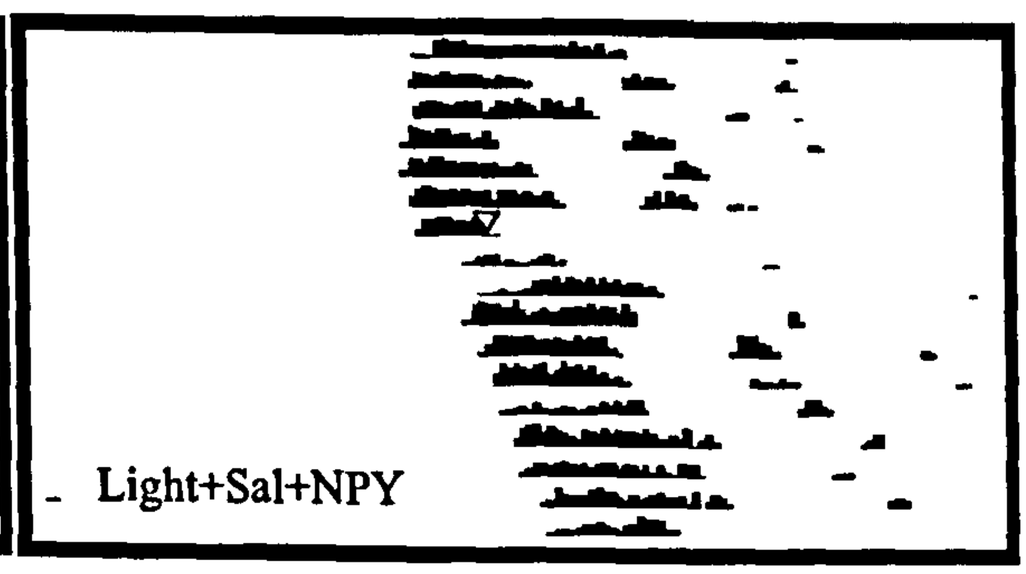

CT 18
Mean Phase Shift (minutes)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Light+Y5ant+saline
Light+Y5ant+NPY
Y5ant+saline
Light+Y1ant+saline
Light+Y1ant+NPY
Y1ant+saline
Light+saline
Light+saline+NPY
NPY+saline

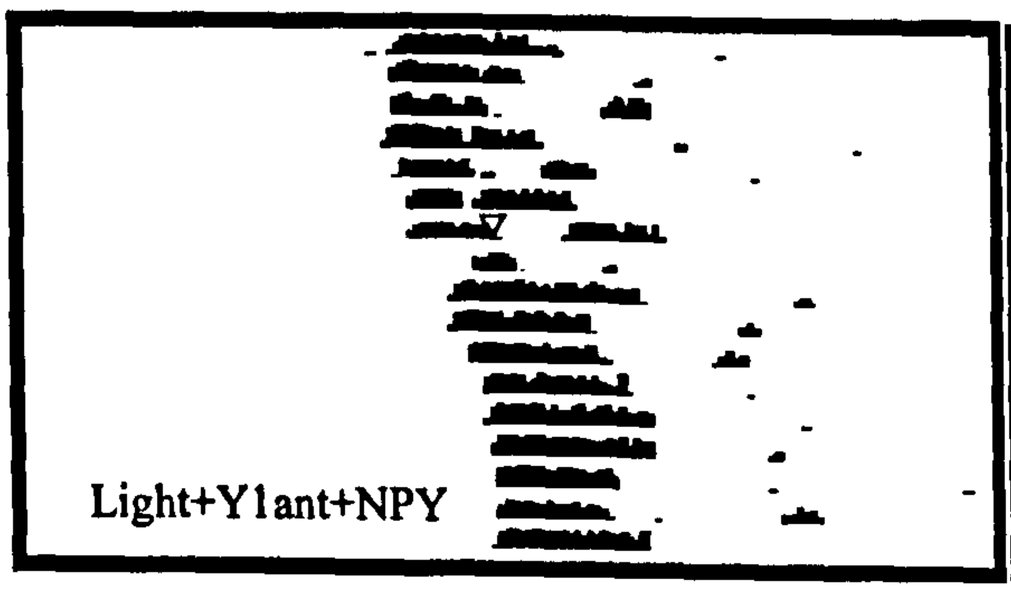
Figure 13. Phase advances to light were blocked by NPY administration (Light+NPY; $p<0.05$). Microinjections of NPY still blocked phase shifts to light in the presence of a Y1 receptor antagonist (Light+Y1ant+NPY; $p<0.05$). However, administration of the specific Y5 receptor antagonist was not only able to prevent the attenuation of the photic shift by NPY, but actually potentiated the phase shift to light an effect also observed with light and the antagonist alone (Light+Y5ant+NPY; Light+Y5ant+Saline, respectively).

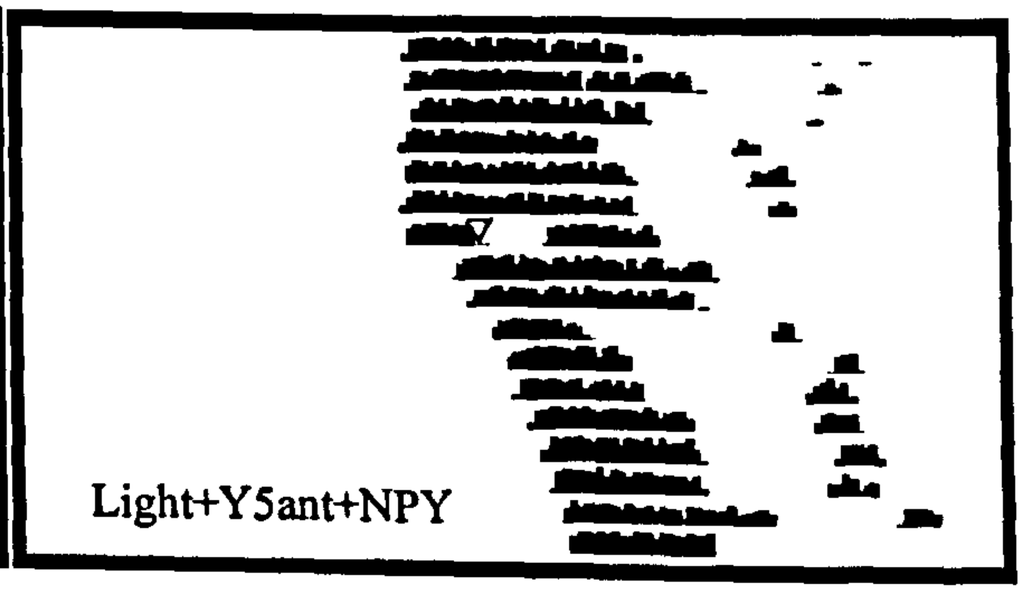
Figure 14

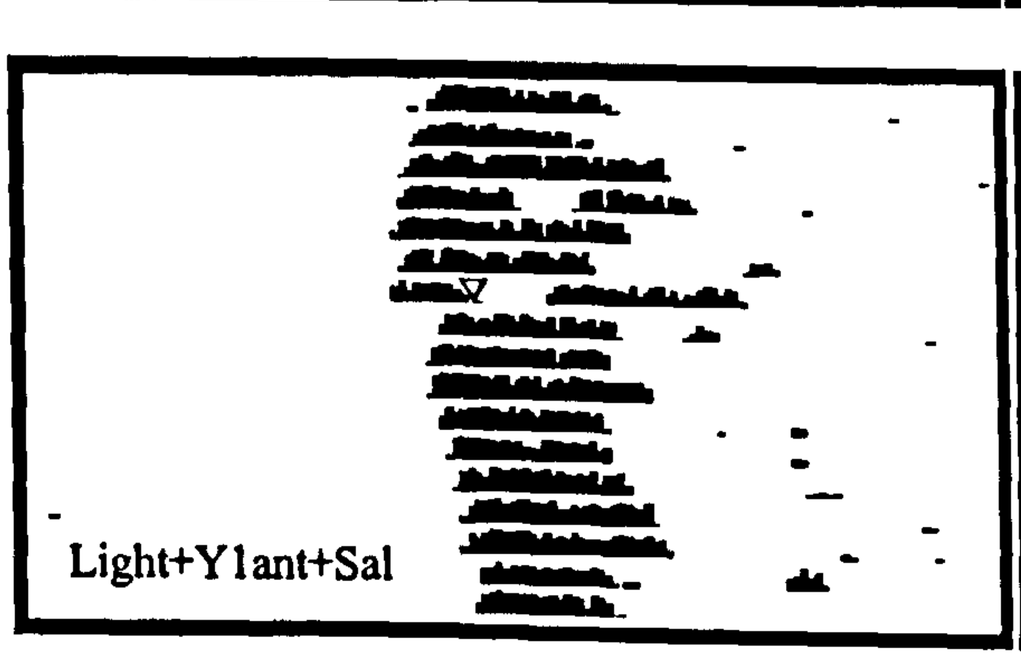
CT14











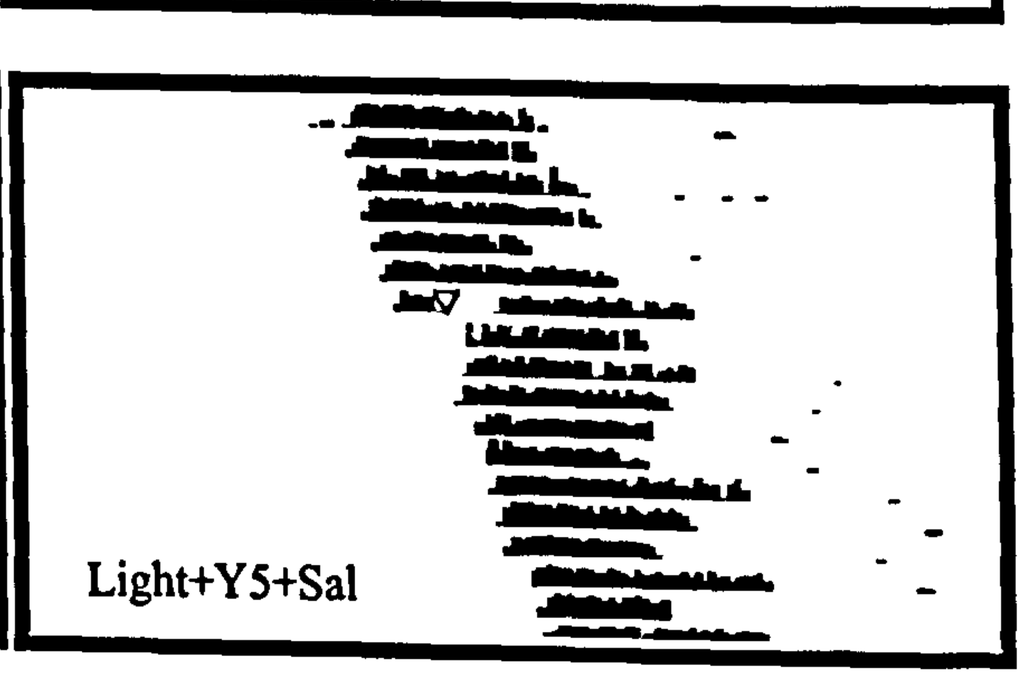


Figure 14. Representative actograms demonstrating the attenuation of light induced phase delays at CT 14 by NPY in the presence of either a Y1 or Y5 receptor antagonist. Light exposure for 15 minutes (Light+Sal) at CT 14 resulted in phase delays of the free running rhythm in Syrian hamsters. Microinjections of the Y1 receptor antagonist prior to NPY administration, during light exposure (Light+Y1ant+NPY), did not affect the attenuating effects of NPY at this time. However, the presence of the Y5 receptor antagonist prevented the attenuation of the photic phase shift by NPY when presented during the light pulse (Light+Y5ant+NPY). Times of light stimulation are indicated by inverted open triangles.

Figure 15

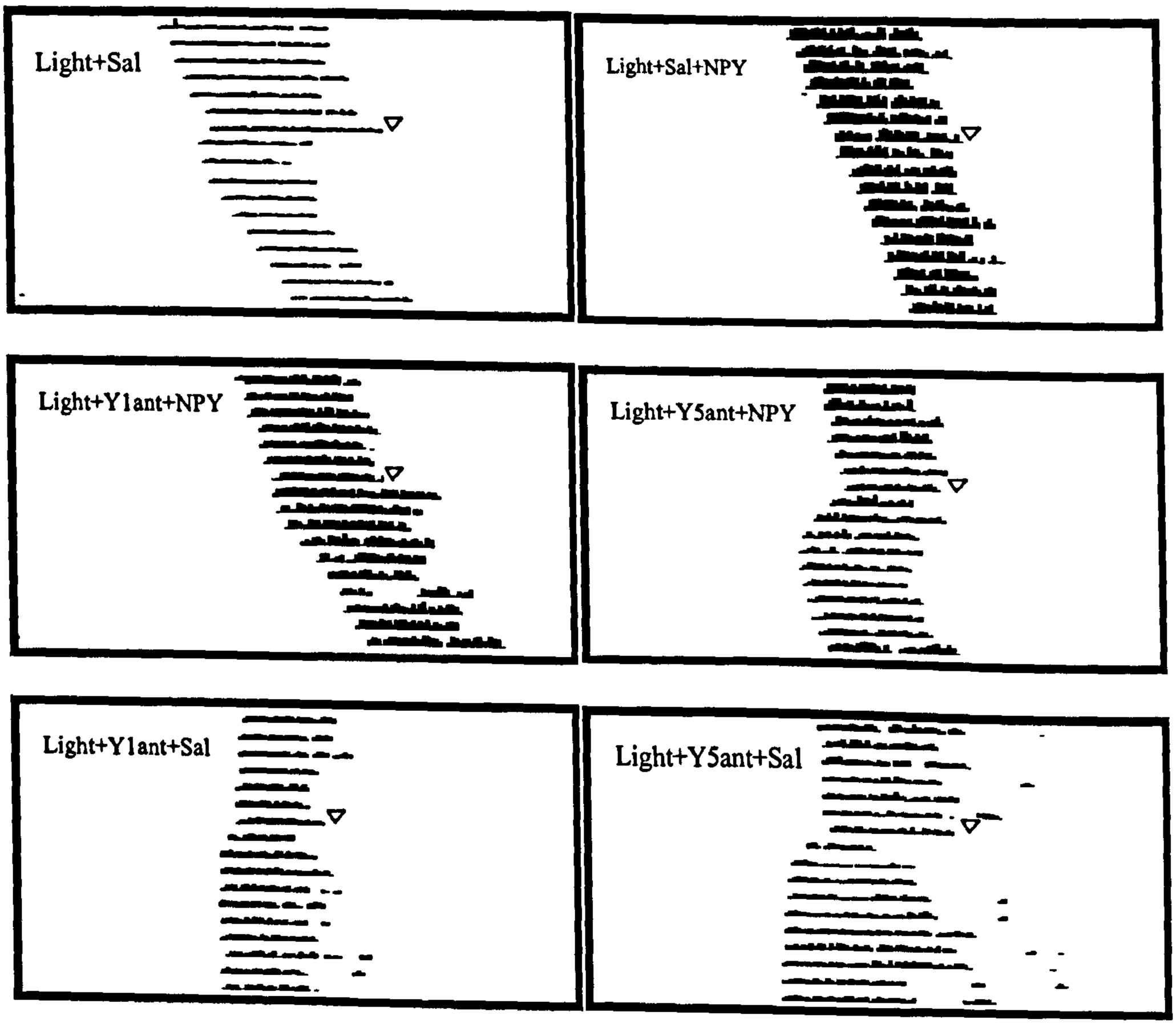

CT18
Light+Sal
Light+Sal+NPY
Light+Y1ant+NPY
Light+Y5ant+NPY
Light+Y1ant+Sal
Light+Y5ant+Sal

Figure 15. Representative actograms demonstrating the inhibition of light induced phase advances at CT 18 by NPY in the presence of either a Y1 or Y5 receptor antagonist. Phase advances to light were blocked by the administration of NPY with and without prior administration of the Y1 receptor antagonist (Light+Y1ant+NPY; Light+Sal+NPY, respectively). However, microinjection of the Y5 antagonist during light exposure followed by either saline or NPY resulted in the potentiation of the photic phase shift (Light+Y5ant+Sal; Light+Y5ant+NPY, respectively). Times of light stimulation are indicated by inverted open triangles.

3.4 Discussion

Microinjections of NPY, *in vivo*, have been shown to attenuate phase delays to light and inhibit phase advances (Weber and Rea, 1997; Lall and Biello, 2003b). Furthermore, a recent study reported that this inhibitory effect of NPY on photic phase shifts was mediated via either or both the Y1 and/or the Y5 receptors (Lall and Biello, 2003b). Our present study has shown that NPY attenuates photic phase delays and inhibits light induced phase advances via the Y5 receptor, *in vivo*. Microinjection of the Y5 receptor antagonist prevented NPY from influencing the phase shifting effects of light during the early and late night. Furthermore, during the late night the presence of the Y5 antagonist with light stimulation resulted in the potentiation of the phase advancing effects of light at this time. A similar result was also obtained combining NPY with light and the Y5 receptor antagonist, during the late night. Administration of the Y1 receptor antagonist did not influence the inhibitory effects of NPY on both advances and delays to light.

The administration of NMDA, *in vivo* and *in vitro*, onto the SCN has been shown to mimic the phase shifting effects of light (Colwell and Menaker, 1992; Ding et al., 1994; Shibata et al., 1994; Mintz et al., 1999). A recent study has shown that NPY applied to an SCN slice preparation *in vitro* can inhibit the phase shifting effects of NMDA during the night (Yannielli and Harrington, 2001). Further, the addition of a Y5 receptor antagonist blocked the effects of NPY on NMDA induced phase shifts, with the Y1 receptor antagonist having no effect on the interaction between NMDA and NPY (Yannielli and Harrington, 2001). Our study is the first to report that the interaction between light and NPY during the night is mediated via the Y5 receptor at the level of the SCN, *in vivo*. Hence further clarifying our previous findings indicating the involvement of either or both the Y1 and the Y5 receptors (Lall and Biello, 2003b).

At present there are at least six known subtypes of the neuropeptide Y receptor (Ingenhoven and Beck-Sickinger, 1999). These receptors have been pharmacologically defined by their ability to bind NPY, PYY, PP and synthetic ligands derived from these compounds. The Y2 receptor can be defined by its ability to bind NPY3-36 (Grandt et al., 1996), however [Leu^{31}, Pro^{34}]NPY has been used as a Y1 receptor agonist (Fuhlendorff et al., 1990), but also binds to the Y5 receptor (Ingenhoven and Beck-Sickinger, 1999). Recently a new Y5 receptor antagonist (RWJ5726, used in this study) has been developed and was shown to bind selectively and potently to the Y5 receptor subtype (Youngman et al., 2000). While the Y2 receptor mediates phase shifts to NPY during the mid subjective day (Golombek et al., 1996; Huhman et al., 1996), the inhibitory actions of NPY on photic phase shifts have been shown to involve different NPY receptor subtypes. [Leu^{31}, Pro^{34}]NPY microinjected into the SCN following light exposure during the early and late night attenuated photic shifts in the circadian pattern of wheel running behavior in hamsters (Lall and Biello, 2003b). This study indicated that either or both the Y1 and Y5 receptors were involved in the interaction between light and NPY during the subjective night. We have now shown that the Y5 receptor is responsible for the inhibitory effects of NPY on photic phase shifts and not the Y1 receptor. The presence of the Y5 receptor antagonist prevented the ability of NPY to attenuate both light induced phase advances and delays, however administration of the Y1 receptor antagonist had no effect on this interaction, with both delays and advances to light being reduced by NPY microinjection. Phase shifts to light during the late night were potentiated by the administration of the Y5 receptor antagonist. However, phase delays to light were unaffected by the Y5 receptor antagonist with no attenuation or potentiation being observed with treatments administered at this time. Previous work has shown that the administration of antiserum raised against

NPY also potentiated the photic phase shift during the late night (Biello, 1995). Neuropeptide Y levels in the SCN under a light-dark cycle have been shown to peak at the beginning and again at the end of the light portion of the photoperiod, in the rat (Shinohara et al., 1993). In the same study it was found that NPY levels peaked at approximately CT 12 under conditions of constant darkness. From this data it would appear that in the natural environment NPY levels peak at dawn and then again at dusk. It is likely that the peak in NPY observed at dusk is the result of an endogenous rhythm of NPY as opposed to an acute response to the light-dark cycle imposed on the animals, since Shinohara et al, also found that NPY levels tended to peak at times during the transition from lights off to lights on. Therefore it is possible that the peak observed at dawn is due to an acute action of the lights coming on.

This may suggest a role of NPY in the modulation of photic phase shifts, given the present data on the inhibitory influences of NPY on photic phase shifts. During the late night, phase shifts to light may be regulated by NPY release acutely induced by lights on. Therefore the presence of the antagonist would prevent the modulation of the photic stimulus by NPY resulting in a potentiation of the phase shifting effects of light during the late night. However, during the early night NPY levels are endogenously high, hence SCN cells are under the influence of NPY before the light pulse. Therefore the Y5 antagonist microinjected at CT 14 may not be able to prevent the actions of the previously released NPY at CT 12. Thereby limiting the light induced phase shift at this time. Furthermore, phase shifts to light in the hamster appear to be larger during the late subjective night than during the early subjective night. This may be a result of the endogenous rhythm of NPY within the SCN. Our study may also indicate a possible role for the NPY peak observed at

dawn under a light-dark cycle. This peak may act to modulate the photic pulse received during the advance portion when lights come on during the beginning of the light-dark cycle. Hence preventing the clock from falling out of synchronization with the photoperiod.

The neurotransmitter γ-amino butyric acid (GABA) is found in the majority of SCN neurons as well as in many SCN afferents (Decavel and Van den Pol, 1990; Moore and Speh, 1993). Furthermore, NPY and GABA are co-localized in synaptic terminals within the SCN (Francois-Bellan et al., 1990). The GABAergic system has been implicated in the phase advancing properties of NPY during the middle of the subjective day as phase shifts to NPY were blocked by the administration of the $GABA_A$ receptor antagonist bicuculline (Huhman et al., 1995). Also, the $GABA_A$ receptor agonist, muscimol, was shown to block both phase advances and delays to light (Gillespie et al., 1996; Gillespie et al., 1997). However, during the early and late night the presence of bicuculline did not influence the ability of NPY to inhibit photic phase shifts (Lall and Biello, 2003a). Bicuculline administration with light alone during the early night resulted in the potentiation of the photic phase shift, *in vivo* (Gillespie et al., 1996; Lall and Biello, 2003a), while light induced phase advances were unaffected by bicuculline microinjections (Lall and Biello, 2003a) [See Chapter 4]. It is possible that phase delays to light are under GABAergic regulation as well as NPY, but phase advances primarily rely on the acute effects of NPY on photic shifts at this time. Therefore, during the early subjective night phase delays to light are modulated by NPY and GABA, hence blocking NPY regulation via the Y5 receptor does not influence the photic phase shift, as the GABAergic tone is unaffected. Furthermore, bicuculline administration potentiates phase delays to light, adding further

support to the involvement of GABA in the modulation of photic shifts at this time. During the late night GABAergic tone may be much less than it is during the early night, hence the observed potentiation in photic phase shifts with the Y5 receptor antagonist and light stimulation. Taken together it is possible that NPY plays a greater role in the regulation of light induced phase advances.

The current study has shown the involvement of the Y5 receptor subtype in the interaction between NPY and light during the early and late subjective night. Furthermore, it gives and insight into the possible non-photic mechanisms that may act on the SCN to provide synchronization to the natural environment.

Chapter 4

Neuropeptide Y, GABA and circadian phase shifts to photic stimuli

Having established that NPY utilizes the NPY Y5 receptor subtype in its interaction with light, the next set of experiments were designed to investigate the possible neural mechanisms induced by NPY in its inhibitory actions on photic phase shifts

A possible pathway for the non-photic resetting of the clock is thought to originate from the IGL, which conveys information to the SCN through the geniculohypothalamic tract and utilizes among others neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) as neurotransmitters. Phase advances to NPY during the mid subjective day have been shown to utilize a GABAergic mechanism, as the administration of bicuculline, a $GABA_A$ receptor antagonist, blocked NPY phase shifts.

Photic and non-photic stimuli have been shown to interact during the early and late subjective night. Microinjections of NPY or muscimol, a $GABA_A$ receptor agonist, into the region of the SCN can attenuate light induced phase shifts during the early and late subjective night. The precise mechanism for these interactions is unknown.

In the current study we investigate if a GABAergic mechanism is involved in the interaction between NPY and light during the early and late subjective night. Microinjections of NPY significantly attenuated light induced phase delays and inhibited phase advances ($p<0.05$). The administration of bicuculline during light exposure, before

NPY microinjection did not alter the ability of NPY to attenuate light induced phase delays and block photic phase advances.

These results indicate that NPY attenuates photic phase shifts via a mechanism independent of $GABA_A$ receptor activation. Furthermore it is evident that NPY influences circadian clock function via differing cellular pathways over the course of a circadian cycle.

4.1 Introduction

The neurotransmitter γ-aminobutyric acid (GABA) is found in the majority of SCN neurones as well as in many SCN afferents (Decavel and Van den Pol, 1990; Moore and Speh, 1993). Furthermore, NPY and GABA have been co-localized in synaptic terminals within the SCN (Francois-Bellan et al., 1990). The GABAergic system has been implicated in the phase advancing property of NPY during the middle of the subjective day. Phase shifts to NPY were blocked by the administration of the $GABA_A$ receptor antagonist, bicuculline (Huhman et al., 1995). The blockade of sodium dependent action potentials by tetrotoxin blocked NPY, but not muscimol, a $GABA_A$ receptor agonist, induced phase advances (Huhman et al., 1997). These findings suggest that NPY utilises a GABAergic mechanism to phase advance the circadian rhythm in mammals during the subjective day.

$GABA_A$ active drugs microinjected into the SCN during the subjective night have also been shown to modulate photic phase shifts, in a manner similar to NPY. Muscimol administration attenuated both light induced phase advances and delays (Gillespie et al., 1996; Gillespie et al., 1997). Furthermore, bicuculline microinjections potentiated photic phase shifts during the early subjective night (Gillespie et al., 1996), a result similar to that observed with the administration of antiserum raised against NPY prior to a light pulse given during the late subjective night (Biello, 1995). However, *in vitro* data shows that NPY is able to influence SCN cells directly and independently of a GABAergic mechanism (Cutler et al., 1998).

Thus, it is possible that NPY utilises a GABAergic pathway in influencing photic phase shifts during the early and late subjective night. The purpose of the present study was to determine whether NPY mediates its modulatory effect on photic phase shifts via a GABAergic mechanism.

4.2 Experimental procedures

4.2.1 Subjects

Experimental procedures were carried out under licence by the Home Office (UK) in accordance with the Animals Scientific Procedures Act (1986). Adult male Syrian hamsters (40 days old, Harlan Sprague-Dawley, Oxon, UK) were housed individually in plastic cages fitted with 16 cm running wheels connected to a computer that summed wheel revolutions in 10 minute activity bins (Dataquest Pro-Data software, Data Sciences Inc., Roseville, MN,USA). Food and water were available *ad libitum*. Animals (n=21) were maintained under a LD cycle of 14 hours light and 10 hours of darkness and then transferred to constant conditions (22 ± 2°C; 14-18 lux dim red light) 10 days before treatment. Animals were re-entrained by this method following a maximum of three treatments before continuing with further experimental conditions. All efforts were made to minimise the number of animals used and their suffering.

4.2.2 Surgery and injections

See appendix I for a detailed description of the surgical procedure.

Hamsters were anaesthetised with halothane gas throughout the length of the surgery. Animals received cannula guides, which were implanted to a depth of 5.6mm below the skull surface at a 10° angle and fixed to the skull with fine machine screws and dental cement. Cannula guides were stereotaxically aimed at the SCN (co-ordinates relative to bregma: AP+0.4mm, ML 1.0mm, upper incisor bar –2 mm). After recovery from surgery (1 week under LD 14:10h), animals were transferred into cages fitted with running wheels and maintained for 7-10 days under LD 14:10h before being placed into constant conditions. In all treatments the vehicle consisted of a 0.9% saline solution. Porcine NPY

(0.023nmol in 200nl saline, Calbiochem, UK) and bicuculline methobromide, a competetive $GABA_A$ antagonist (0.18nmol in 200nl saline, Sigma, UK) were administered using a 28 gauge infusion cannula attached to a 1µl Hamilton syringe. The infusion cannula extended 2.4 mm from the base of the guide cannula.

4.2.3 Experimental treatments

Prior to the start of the experiment all animals were injected with NPY at CT 6 five days after being placed into constant conditions. These microinjections were used as functional assessments to verify cannula placements.

Experiment 1 (n=12). Hamsters completed the following sequence of treatments: (1) a 15 minute light pulse (130 lux) at CT14 directly followed by vehicle administration, (2) a 15 minute light pulse at CT14 with bicuculline administration at CT14.08 and saline administration at CT14.17, (3) a 15 minute light pulse at CT14 with saline administration at CT14.08 and NPY administration at CT14.17, (4) a 15 minute light pulse at CT14 with bicuculline administration at CT14.08 and NPY administration at CT14.17, (5) bicuculline administration at CT14 followed by saline administration at CT14.08, (6) saline administration at CT14 followed by NPY administration at CT14.08.

Experiment 2 (n=9). Hamsters completed the following sequence of treatments: (1) a 15 minute light pulse (130 lux) at CT18 directly followed by vehicle administration, (2) a 15 minute light pulse at CT18 with bicuculline administration at CT18.08 and saline administration at CT18.17, (3) a 15 minute light pulse at CT18 with saline administration at CT18.08 and NPY administration at CT18.17, (4) a 15 minute light pulse at CT18 with

bicuculline administration at CT18.08 and NPY administration at CT18.17, (5) bicuculline administration at CT18 followed by saline administration at CT18.08, (6) saline administration at CT18 followed by NPY administration at CT18.08.

4.2.4 Data analysis

For activity onset calculation see appendix III.

Data from each set of treatments was analysed by the one-way repeated measures ANOVA, followed by Bonferroni's method for multiple comparisons. All *t*-values reported were derived from multiple comparison tests.

4.2.5 Cannula Placement

See appendix IV for cannula placement assessment methods.

4.3 Results

4.3.1 Effects of bicuculline on the attenuation of photic phase delays by NPY.

The administration of bicuculline during light exposure and preceding NPY microinjection did not significantly alter the ability of NPY to attenuate the photic phase shift with shifts of -14 ± 13 minutes (mean ± SEM) in the presence of bicuculline and -18 ± 8 minutes with a vehicle substitution of bicuculline ($t = 0.34$, $p > 0.05$) (Fig.16). However, microinjections of bicuculline following light and preceding saline administration resulted in phase shifts significantly greater than those of light with saline alone resulting in shifts of -96 ± 4 minutes and -61 ± 5 minutes respectively ($t = 3.38$, $p < 0.05$).

NPY administration following light exposure resulted in the attenuation of the photic phase shift observed with light and saline alone with average shifts of -18 ± 8 minutes and -61 ± 5 minutes respectively ($t = 4.19$, $p < 0.05$). Microinjections of NPY followed by saline, or bicuculline followed by saline, resulted in phase shifts not significantly different from each other (-19 ± 7 minutes and -5 ± 4 minutes respectively, $t = 1.31$, $p > 0.05$), but both were significantly different from shifts resulting from light followed by saline alone ($F_{(5,66)} = 23.6$, $p < 0.01$) (Fig.18).

4.3.2 Effects of bicuculline on the inhibition of photic phase advances by NPY.

The administration of bicuculline following light exposure and preceding NPY microinjection did not significantly alter the ability of NPY to block photic phase advances, with resulting phase shifts of –6 ± 5 minutes relative to those observed with light, saline and NPY treatments (7 ± 15 minutes, t=0.85, p>0.05) (Fig.17).

Microinjections of bicuculline and saline following light exposure were not significantly different from treatments with saline following photic stimulation during the subjective late night, with photic phase shifts of 98 ± 5 minutes and 106 ± 4 minutes respectively ($F_{(5,47)}$=23.1, p<0.01). The administration of bicuculline followed by saline, and NPY followed by saline, were not significantly different from each other with average shifts of 22 ± 10 minutes and 7 ± 15 minutes respectively (t=0.9, p>0.05) (Fig.19).

4.3.3 Cannula placement verification

Neuropeptide Y microinjections at CT 6 were used for functional assessment (0.047nmol in 200nl saline, 123 min ± 8 [mean ± SEM], n=21). All phase shifts seen in this experimental group were comparable to those obtained in previous studies (Huhman et al., 1995; Biello and Mrosovsky, 1996). Further, histological placement verification confirmed that cannula were within 400μm of the SCN along the anterior-posterior, dorsal-ventral and lateral axes.

Figure 16.

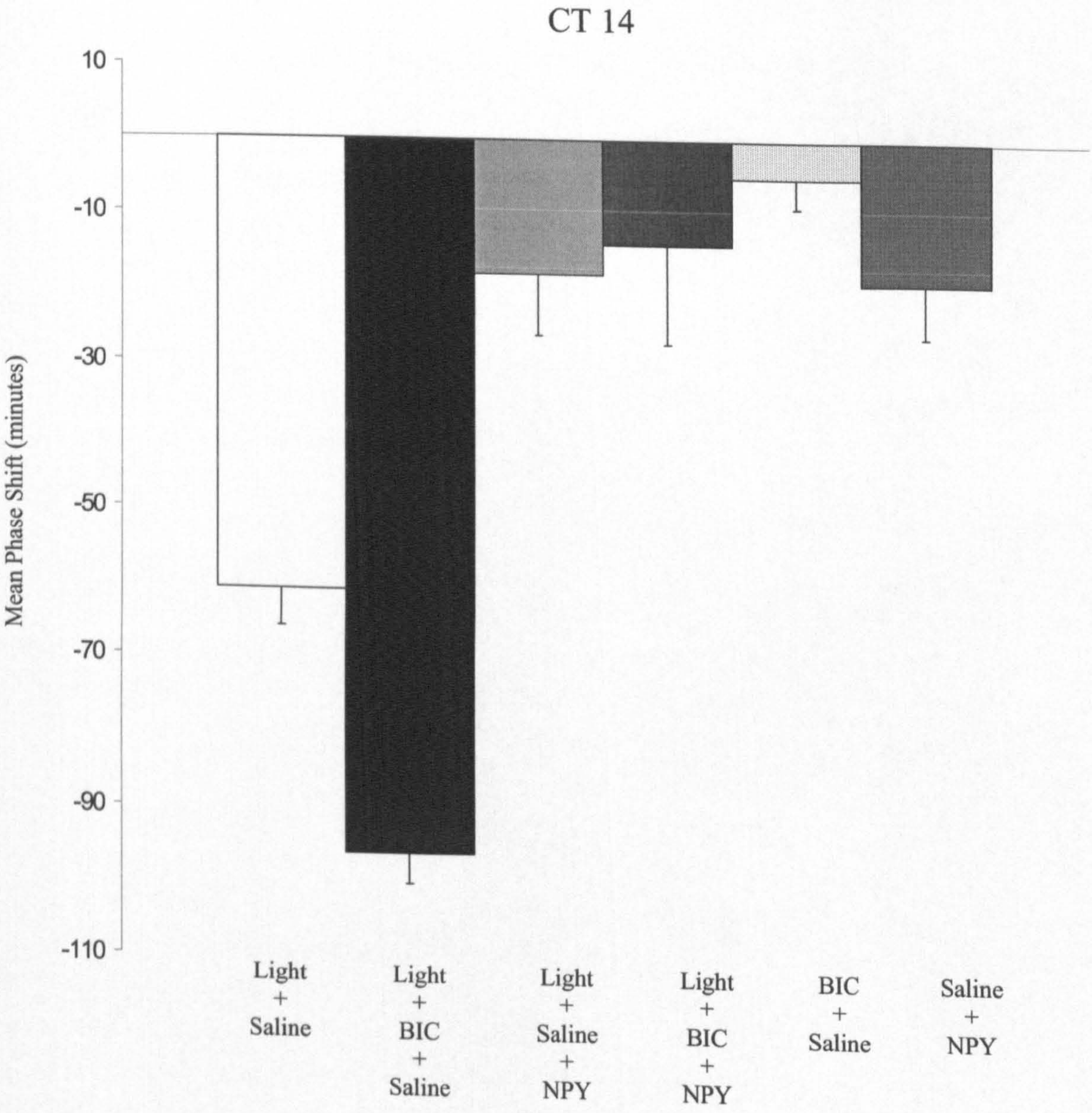

CT 14
10
-10
-30
-50
-70
-90
-110
Mean Phase Shift (minutes)
Light + Saline
Light + BIC + Saline
Light + Saline + NPY
Light + BIC + NPY
BIC + Saline
Saline + NPY

Figure 16. Phase delays to light were significantly attenuated following NPY administration ($p<0.05$). Microinjection of bicuculline during light exposure followed by NPY administration also resulted in a significant attenuation of the photic shift ($p<0.05$). However, bicuculline administration during the pulse of light followed by saline microinjection resulted in a significant potentiation of the light induced phase shift at this time ($p<0.05$; $n=12$).

Figure 17.

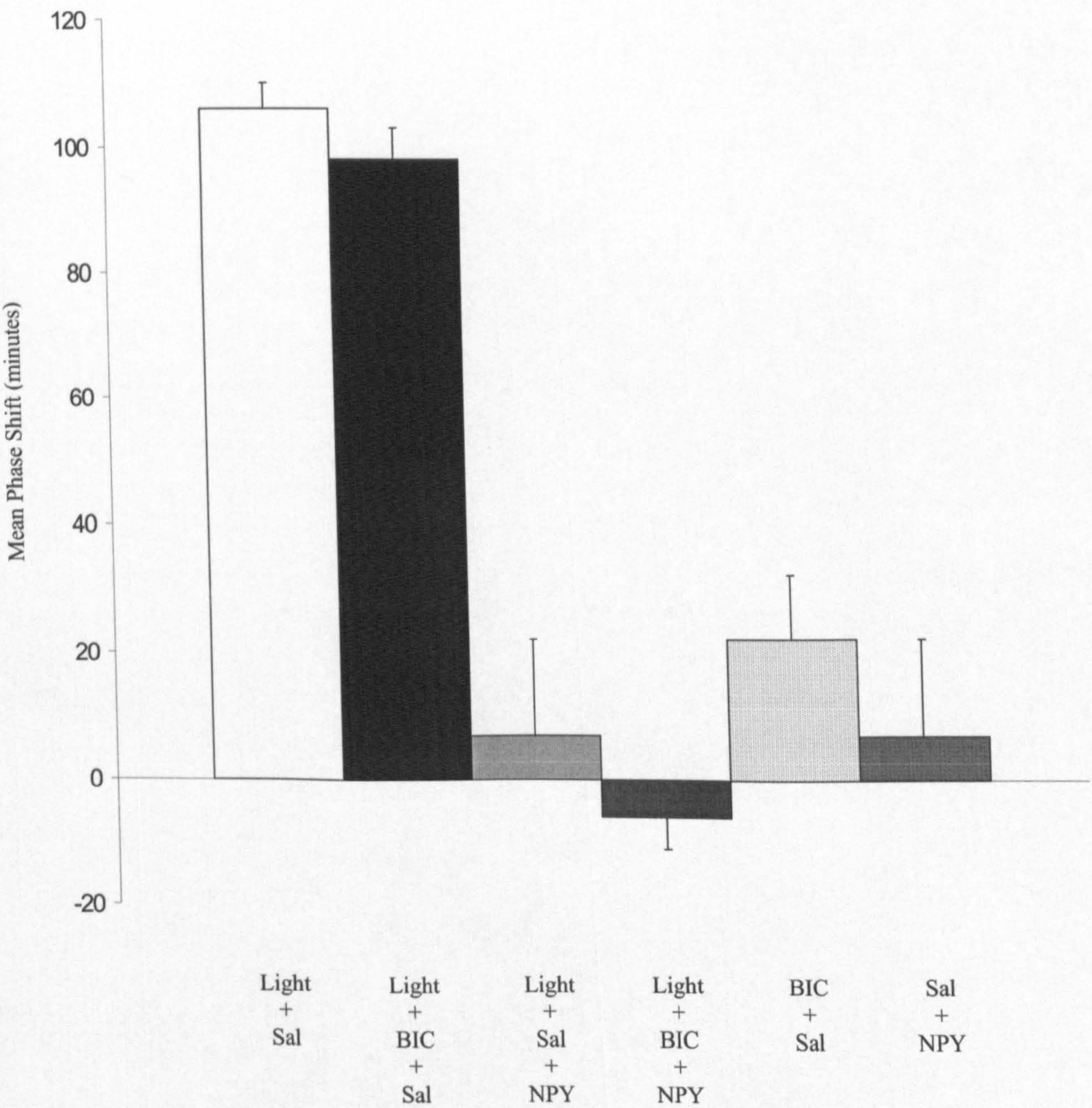

CT 18
120
100
80
60
40
20
0
-20
Mean Phase Shift (minutes)
Light + Sal
Light + BIC
Light + Sal
Light + BIC
BIC + Sal
Sal + NPY
+ Sal
+ NPY
+ NPY

Figure 17. Phase advances to light were significantly attenuated following NPY administration ($p<0.05$). Microinjection of bicuculline during light exposure followed by NPY administration also resulted in a significant attenuation of the photic shift ($p<0.05$). However, the administration of bicuculline during the pulse of light followed by saline microinjection did not alter the phase shifting properties of light at this time ($p>0.05$; $n=9$).

Figure 18.

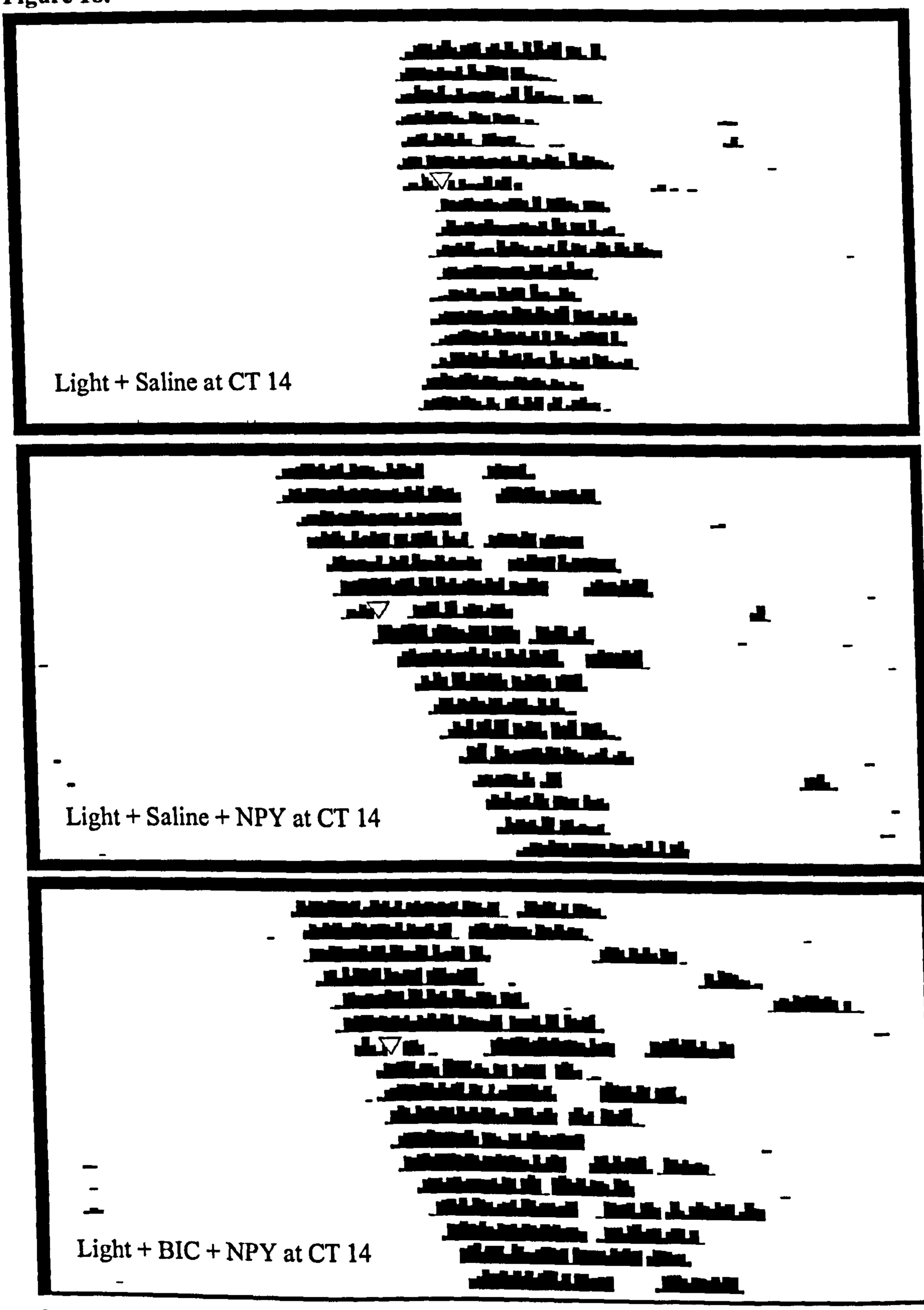

Light + Saline at CT 14
Light + Saline + NPY at CT 14
Light + BIC + NPY at CT 14
0
Time (Hours)
24

Figure 18. Representative actograms demonstrating the attenuation of light induced phase delays at CT 14 by NPY in the presence of bicuculline. Light exposure for 15 minutes at CT 14 resulted in phase delays of the free running rhythm in Syrian hamsters. However microinjections of bicuculline prior to NPY administration, during light exposure, did not affect the attenuating effects of NPY at this time. Times of light stimulation are indicated by inverted open triangles.

Figure 19.

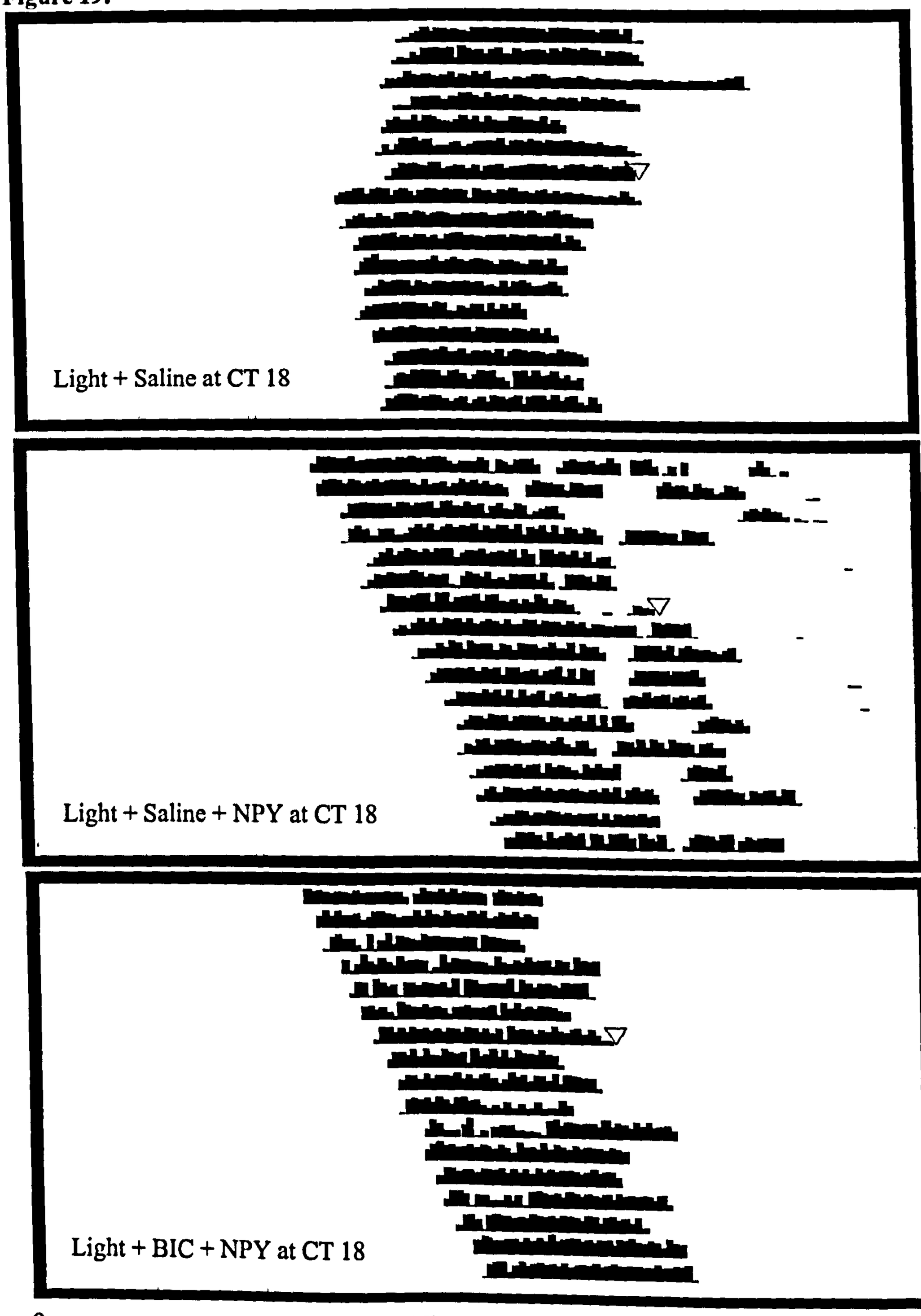

Light + Saline at CT 18
Light + Saline + NPY at CT 18
Light + BIC + NPY at CT 18
0
Time (Hours)
24

Figure 19. Representative actograms demonstrating the inhibition of light induced phase advances at CT 18 by NPY in the presence of bicuculline. Phase advances to light were blocked by the administration of NPY with and without prior administration of bicuculline. Times of light stimulation are indicated by inverted open triangles.

4.4 Discussion

Neuropeptide Y has been previously shown to attenuate phase delays to light and inhibit phase advances, *in vivo* (Weber and Rea, 1997; Lall and Biello, 2002). Microinjections of the $GABA_A$ antagonist, bicuculline, did not alter the inhibitory effects of NPY on photic phase shifts during the subjective night. Phase advances to NPY during the subjective day were shown to be blocked by the administration of bicuculline, (Huhman et al., 1995), suggesting that NPY phase shifts were mediated via a GABAergic mechanism. In the present study we have shown it is likely that NPY does not influence photic phase shifts via a mechanism involving the $GABA_A$ receptor subtype. This further strengthens previous evidence suggesting that the interaction site of NPY and light lies downstream from receptor binding sites within SCN cells. Phase shifts to light, presented *in vivo*, during the early subjective night were also blocked by the application of NPY *in vitro* up to 30 minutes post light exposure (Yannielli and Harrington, 2000). Together this evidence indicates the postsynaptic effects of NPY on photic phase shifting.

Microinjections of muscimol, a $GABA_A$ receptor agonist, have been shown to inhibit both phase delays and advances to light (Gillespie et al., 1996; Gillespie et al., 1997). Neuropeptide Y microinjections attenuated phase delays to light and inhibited phase advances (Lall and Biello, 2003b).Photic information is thought to be conveyed to the SCN via a monosynaptic pathway utilizing glutamate as its principal neurotransmitter (Ebling, 1996). Light and glutamate phase shift the SCN mostly via NMDA receptors during the subjective night (de Vries et al., 1994; Mintz et al., 1999). $GABA_A$ receptors conduct mostly chloride ions (Sivilotti and Nistri, 1991; Kaila, 1994). The conductance of these chloride ions through the $GABA_A$ receptor has been shown to rise quickly inducing a rapid

hyperpolarizing effect on cells (Mierlak and Farb, 1988; Tauck et al., 1988). Taken together, it is possible that the activation of $GABA_A$ receptors by muscimol inhibits both photic phase delays and advances via a mechanism involving changes in membrane conductance. However, NPY appears not to influence SCN cells at the cell surface, but at the level of protein synthesis (Hall et al., 1999; Maywood et al., 2002).

In agreement with a previous study (Gillespie et al., 1996) we found microinjections of bicuculline during the early subjective night potentiated the phase shifting effects of light at this time. Phase delays to light were increased by approximately 57% following bicuculline administration. However, this effect of bicuculline did not alter the ability of NPY to attenuate photic phase shifts when light, bicuculline and NPY were administered in sequence. During the early subjective night NPY levels have been shown to peak in nocturnal rodents, possibly because of increased activity levels at this time (Shinohara et al., 1993). Due to the co-localization of NPY and GABA within the same synaptic terminals (Francoisbellan et al., 1990) it is possible that GABA levels also rise. This increase in GABA will result in a baseline activation of $GABA_A$ receptors thereby generating a natural chloride conductance within SCN cells at this time. Hence the application of bicuculline could block this underlying chloride conductance and remove the hyperpolarizing effects of GABA resulting in potentiated phase shifts to light during the early night. During the late subjective night activity levels tend to be low, therefore it is possible that the underlying chloride conductance within SCN cells is also reduced. Hence phase advances to light maybe under little or no influence of $GABA_A$ receptor activation.

Bicuculline did not alter the attenuating effect of NPY on light induced phase delays. It may have been expected that if bicuculline increases phase delays to light, that NPY's ability to influence this shift would be substantially impaired. However this was not the case. Furthermore, bicuculline microinjections during the late subjective night following light exposure did not significantly alter the resulting phase shift in comparison to light administration alone. Taken together this result may indicate that the interaction between GABA and light lies at the level of receptor activation on SCN cells, during the early and late night. Further, the interaction between NPY and light probably lies at a level post receptor activation within SCN cells, possibly at the level of gene expression.

Light stimulation results in the increase of Per mRNA (*mPer*) levels, in mice (Shearman et al., 1997; Zylka et al., 1998). Neuropeptide Y administration has been shown to reduce mPer levels in hamsters and mice (Fukuhara et al., 2001; Maywood et al., 2002). The resulting potentiation of the light induced phase shift following bicuculline microinjection, during the early subjective night, may possibly act to increase the quantity of *mPer* expressed, thereby resulting in a shift of greater magnitude than that observed with light alone at this time. However, NPY administration following light and bicuculline microinjections was still able to attenuate the photic phase shift. This would indicate that NPY's ability to reduce the increase in *mPer* expression was not impaired by the presence of bicuculline. Taken together it is possible that bicuculline administration following light exposure during the early subjective night may increase the quantity of *mPer*, but does not increase the rate of synthesis, hence NPY is still able to inhibit *mPer* production long before the cytosolic concentrations of *mPer* start to rise above those expected with light

alone. The complex interaction between NPY and light may lie at the molecular level, and this should provide an area for future investigation.

It is clear that both GABA and NPY provide a modulatory role in the mediation of photic phase shifts observed in mammals. Both these non-photic stimuli interact at different sites within the circadian system to influence light induced phase shifts, but from this study it is evident that each stimuli interacts with light independently. Such a mechanism may be advantageous in the evolution and adaptation of mammals to our ever-changing environment.

Chapter 5

Attenuation of phase shifts to light by activity or neuropeptide Y: a time course study.

Previous work has shown that NPY can modulate both light induced phase advances and delays. Furthermore, NPY binds to the Y5 receptor in this interaction. Also, it appears that NPY does not utilize a $GABA_A$ dependent pathway to mediate its effects on photic phase shifts. Taken together evidence would suggest that the interaction between NPY and light occurs down stream from Y5 receptor binding sites, probably within SCN cells.

The next sets of experiments were designed to use a functional *in vivo* method to try and locate the possible site of interaction between NPY and light. In these studies I have also used the novel wheel stimulus and aim to see if this behavioural cue alters phase shifts to light in a similar manner to NPY microinjections. The novel wheel allows us to observe the effects of naturally stimulating NPY release rater than gross administration via cannula application. Furthermore, it also allows the correlation between the two types of experimental methods.

Interactions between photic and behavioural stimuli were investigated during the late subjective night, six hours after activity onset in Syrian hamsters (CT18). Light pulses of 130 lux for 15 minutes at this time resulted in phase advance shifts. Novel wheel exposure, for a period of 3 hours, following photic stimulation was able to attenuate the phase advancing effects of light. A time delay of up to 60 minutes between photic and behavioural stimuli also resulted in significant attenuation of light induced phase shifts

(p<0.05). A 90 minute interval between stimuli resulted in no significant attenuation. Novel wheel exposure mediates its effects via the intergeniculate leaflet, which conveys information to the SCN and utilises neuropeptide Y (NPY) as its primary neurotransmitter. Phase shifts to light pulses given at CT18 were attenuated by NPY administration. Neuropeptide Y injections up to 60 minutes post light exposure significantly attenuated phase shifts by 50% on average. However a 90 minute interval between light and NPY microinjection did not significantly affect light induced phase shifts.

These results confirm previous work indicating that novel wheel exposure and NPY administration can modulate light induced phase shifts during the late night. Further, they show for the first time that the time course for this interaction is similar between wheel running and NPY. Also, most significantly these results indicate that the interaction between NPY or novel wheel exposure and light probably occurs downstream from receptor binding sites within SCN cells.

5.1. Introduction

Non-photic stimuli affect the phase resetting property of light. Timed bouts of wheel running have been shown to attenuate the phase shifting effects of photic stimuli (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998). Light can also attenuate the phase resetting effects of activity during the mid-subjective day (the rest phase in nocturnal rodents) (Mrosovsky, 1991). These interactions provide evidence for a complex series of neural pathways that provide input onto the circadian pacemaker, the suprachiasmatic nucleus in mammals, and ultimately influencing biological and behavioural output.

Phase shifts to light during the subjective night (the active phase in nocturnal rodents) can be attenuated by NPY or serotonin agonists (Rea et al., 1994) or potentiated by antiserum to NPY (Biello, 1995). Phase shifts to glutamate, *in vitro*, can be blocked by the administration of NPY (Biello et al., 1997a). Current evidence has shown NPY to be involved in the ability of non-photic stimuli to attenuate light induced phase shifts, *in vivo*. NPY injections directly onto the SCN were shown to attenuate the phase shifting effects of light during the late subjective night (Weber and Rea, 1997).

The recent development of an *in vivo- in vitro* experimental design by Yannielli and Harrington has provided further evidence for the interaction of NPY and photic stimuli. Light pulses were presented to animals *in vivo* followed by NPY administration to the SCN in a slice preparation, *in vitro*. NPY was able to attenuate the phase shifting effects of light up to thirty minutes post light exposure during the early subjective night (Yannielli and Harrington, 2000). The fact that NPY administered *in vitro* can attenuate light induced

phase shifts up to 30 minutes post light pulse suggests that NPY influences mechanisms downstream from receptor binding sites on SCN cells.

It is possible that the modulation of light induced phase shifts is a mechanism for the control and maintenance of a circadian rhythm. Experiments were designed to test the ability of non-photic stimuli, novel wheel running and NPY, in the regulation of photic phase shifts.

5.2. Experimental procedures

5.2.1. Subjects

Adult male Syrian hamsters (40 days old, Harlan Sprague-Dawley, Oxon, UK) were housed individually in plastic cages fitted with 16 cm running wheels connected to a computer that summed wheel revolutions in 10 minute activity bins (Dataquest Pro-Data software, Data Sciences Inc., Roseville, MN,USA). Food and water were available *ad libitum*. Animals were maintained under a LD cycle of 14 hours light and 10 hours of darkness and then transferred to constant conditions (22 ± 2°C; 14-18 lux dim red light) 10 days before treatment. Group 1a (n=10) and group 1b (n=10) animals were treated with behavioural stimuli only. Group 2a (n=12) and group 2b (n=12) animals were treated with behavioural stimuli as well as intraventricular administration of NPY.

5.2.2. Surgery and injections

See appendix I for a detailed description of the surgical procedure.

Hamsters in group 2 were anaesthetised with halothane gas throughout the length of the surgery. Animals received cannula guides, which were implanted to a depth of 5.1mm below the skull surface and fixed to the skull with fine machine screws and dental cement. Cannula guides were stereotaxically aimed at the third ventricle, just above the SCN (co-ordinates relative to bregma: AP+0.6mm, ML 0.1mm, upper incisor bar –2 mm). After recovery from surgery (1 week under LD 14:10h), animals were transferred into cages fitted with running wheels and maintained for 7-10 days under LD 14:10h before being placed into constant conditions. Porcine NPY (Calbiochem, UK) was administered using a 28 gauge infusion cannula attached to a 1µl Hamilton syringe at a concentration of 1ng/nl

at a volume of 200nl. The infusion cannula extended 2.4 mm from the base of the guide cannula.

NPY was dissolved in artificial cerebral spinal fluid (ACSF; in mM; NaCl 124, KCl 33, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 2.5, glucose 10). The vehicle consisted of ACSF only.

5.2.3. Experimental treatments

Hamsters in group 1a completed the following sequence of treatments: (1) a 3 hour bout of wheel running induced by confinement to a novel running wheel at circadian time 18 (CT18; six hours after activity onset), (2) a 15 minute light pulse (130 lux) at CT18, (3) a 15 minute light pulse at CT18 with exposure to a novel running wheel at one of the following CT times: 18.25, 18.5, 18.75, 19.25, 19.75. Additional control animals (group 1b) received a 3 hour period of novel wheel exposure at the following circadian times: 18.5, 19. 19.5, 20.

Hamsters in group 2a completed the following treatments (1) a light pulse at CT18, (2) NPY administration at CT18, (3) a light pulse at CT18 in combination with NPY administration at one of the following CT times: 18.25, 18.5, 18.75, 19.25, 19.75, (4) administration of the vehicle at CT18, (5) a 15 minute light pulse at CT18 directly followed by vehicle administration. Additional control animals (group 2b) received NPY microinjections at the following circadian times: 18.5, 19. 19.5, 20.

5.2.4. Data analysis

For activity onset calculation see appendix III.

Data from each set of treatments was analysed by the one-way ANOVA, followed by Bonferroni's method for multiple comparisons.

5.2.5. Cannula placement verification

See appendix IV for cannula placement assessment methods.

5.3. Results

5.3.1. Phase shifts to light and activity at CT 18

Photic stimulation alone resulted in phase advances of 124 ± 8 minutes (mean ± SEM). Wheel running induced by confinement to a novel wheel at CT18 resulted in a phase change of –17 ± 10 minutes (Fig.20A). However presentation of both stimuli (light followed by novel wheel confinement) resulted in an approximate 77% attenuation in the phase shifting effect of light (mean shift 28 ± 13 minutes; t=6.26, p<0.01). Novel wheel exposure 15, 30 and 60 minutes post photic stimulation also resulted in the attenuation of light induced phase shifts with mean shifts of 35 ± 14, 27 ± 13 and 59 ± 15 minutes respectively ($F_{(7,60)}$= 12.1, p<0.05) (Fig.20A). Phase shifts observed from novel wheel confinement directly after light stimulation and 15 to 30 minutes post light pulse showed no significant differences ($F_{(2,24)}$= 0.1, p>0.05). A 90 minute period of DD between the presentation of the photic and non-photic stimuli did not result in a significant attenuation of the phase shifting effects of light alone, with a mean shift of 83 ± 13 minutes (t=2.47, p>0.05) (Fig.21). Additional control group 1b receiving novel wheel exposure at CT18.5, CT19, CT19.5 and CT20 resulted in phase shifts of –1 ± 5 minutes, 1 ± 5 minutes, –4 ± 6 minutes and –5 ± 5 minutes respectively and were not significantly different from novelty induced wheel running at CT18 ($F_{(4,52)}$= 1.1, p>0.05).

5.3.2. Effects of NPY on light induced phase shifts at CT 18

Vehicle (ACSF) administration directly after photic stimulation resulted in a phase advance of 105 ± 7 minutes, which was not significantly different from light stimulation alone (t=1.07, p>0.05). Phase shifts observed with microinjection of NPY, at CT 18, were

not significantly different from shifts obtained with vehicle administration alone. However the administration of NPY directly after 15 minutes of light exposure resulted in an approximate 94% attenuation of the light induced phase shift (mean shift 6 ± 15 minutes; t=6.78, p<0.05) (fig.20B). NPY injected 15 and 30 minutes post light stimulation resulted in the attenuation of the light phase shift with mean shifts of 3 ± 15 and 10 ± 7 minutes respectively ($F_{(7,64)}$= 17.6, p<0.05). A 60 minute delay between light and NPY administration resulted in an approximate 50% decrease in the phase shift to light with a mean shift of 61 ± 16 minutes being observed (t=3.65, p<0.05). Phase shifts resulting from microinjections of NPY directly after light stimulation and 15 to 30 minutes post light pulse showed no significant differences ($F_{(2,24)}$= 0.08, p>0.05) (Fig.20B). However a delay of 90 minutes between photic stimulation and NPY administration was unable to significantly affect the phase shift to light, resulting in a mean shift of 97 ± 9 minutes (t=1.57, p>0.05) (Fig.21). Additional control group 2b receiving icroinjections of NPY at CT18.5, CT19, CT19.5 and CT20 resulted in phase shifts of –6 ± 4 minutes, -9 ± 4 minutes, –2 ± 4 minutes and –5 ± 4 minutes respectively and were not significantly different from shifts observed at CT18 following NPY administration ($F_{(4,52)}$= 0.7, p>0.05).

5.3.3. Functional cannula placement verification

For functional assessment animals are injected with a known phase shifting stimulus. All phase shifts seen in this experimental group were comparable to those obtained in previous studies (Piggins et al., 1995; Biello and Mrosovsky, 1996; Mintz et al., 1999; McArthur et al., 2000). The following stimuli were used for functional assessment: NPY injected at CT 6 (200nl at a concentration of 1ng/nl, 133 min ± 9.6 [mean ± SEM], n=10); GRP injected

at CT 14 (150pmol, 200nl, -191.1min ± 21.2, n=4); NMDA injected at CT 18 (10mM, 200nl, 46 min ± 10.5, n=2).

Figure 20.

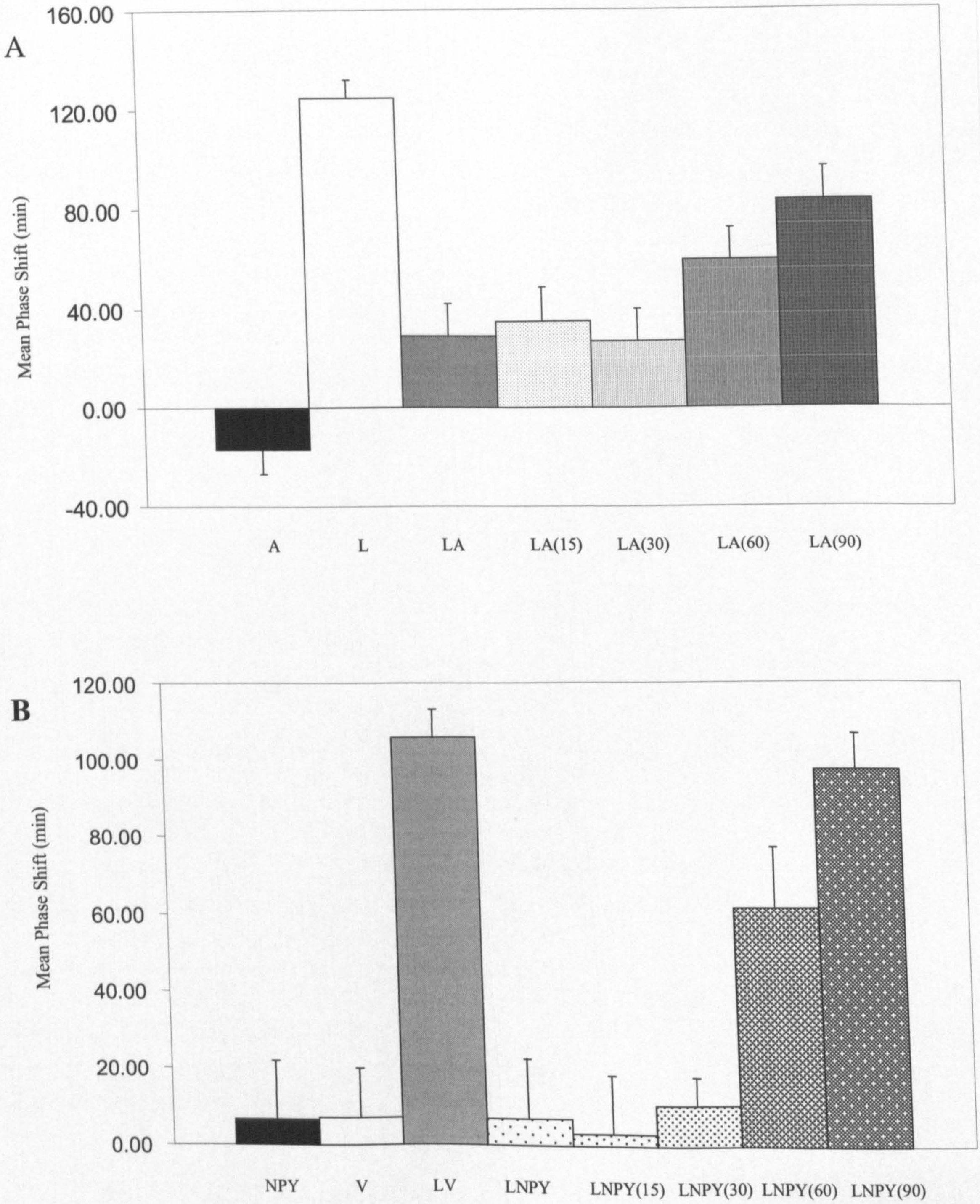

A
160.00
120.00
80.00
40.00
0.00
-40.00
Mean Phase Shift (min)
A
L
LA
LA(15)
LA(30)
LA(60)
LA(90)
B
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Mean Phase Shift (min)
NPY
V
LV
LNPY
LNPY(15)
LNPY(30)
LNPY(60)
LNPY(90)

Figure 20A. Light induced phase advances (L) are attenuated by novel wheel exposure directly following light stimulation (LA). A delay of 15, 30 and 60 minutes also resulted in a significant attenuation of the light induced phase advance at CT 18, (LA(15), LA(30) and LA(60) respectively; $p<0.05$). Novel wheel exposure 90 minutes post light stimulation (LA(90)) was unable to significantly attenuate the phase shift to light. An activity pulse alone at CT 18 (A) resulted in a minimal phase delay, however the ability of novel wheel exposure to attenuate light stimulation at CT 18 was not merely an additive effect due to novel wheel exposure.

Figure 20B. NPY administration following light stimulation (LNPY) resulted in an attenuation of the phase advancing effects of light at CT 18 ($p<0.05$). This effect of NPY was observed with administration 15, 30 and 60 minutes post light pulse (LNPY(15), LNPY(30) and LNPY(60) respectively). A 90 minute delay between light stimulation and NPY injection (LNPY(90)) was unable to attenuate the phase advancing effects of light at this time. Administration of the vehicle (V) at CT 18 had little effect on the free running rhythm. Vehicle administration directly following the photic pulse (LV) had no significant effect on light induced phase shifts ($p>0.05$).

Figure 21.

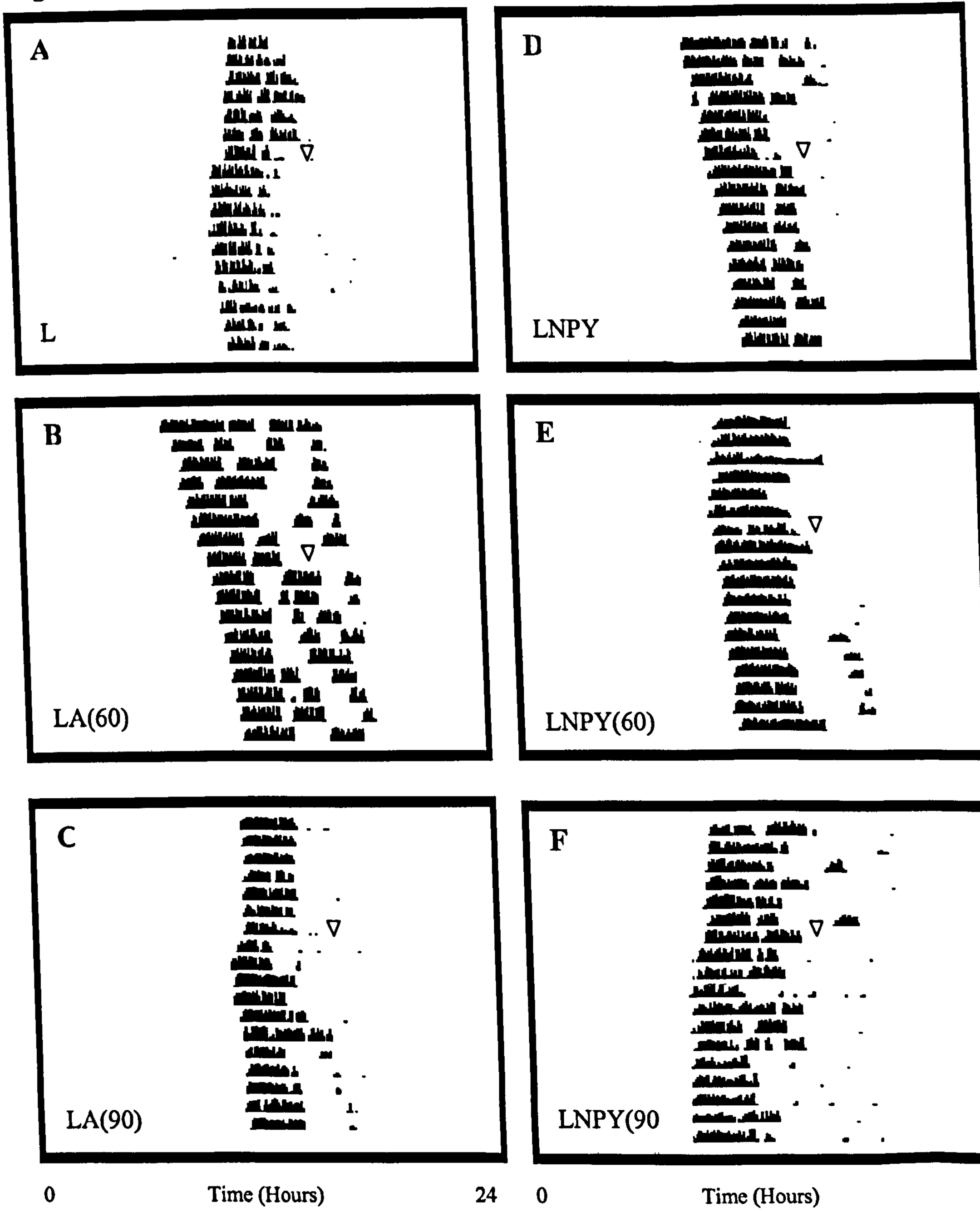

A
L
D
LNPY
B
LA(60)
E
LNPY(60)
C
LA(90)
F
LNPY(90
0
Time (Hours)
24
0
Time (Hours)
24

Figure 21. Representative actograms demonstrating the attenuation of light induced phase advances at CT 18 by novel wheel exposure and NPY. Light stimulation alone resulted in a phase advance at CT 18 (A). A delay of up to 60 minutes before exposure to a novel wheel was able to attenuate the phase advancing effects of light (B), however this was not possible 90 minutes post light exposure (C). NPY administration directly after a photic pulse resulted in a ~94% attenuation of the phase advance due to light (D). NPY was able to attenuate the photic phase shift when administered up to 60 minutes post light stimulation (E); this was not possible 90 minutes after the light pulse (F). Times of light stimulation are indicated by inverted open triangles.

5.4. Discussion

Our results demonstrate the ability of non-photic stimuli to attenuate the resetting effects of light during the late subjective night and thus confirm results from previous studies (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998). Furthermore the present study was designed to test the limits of behavioural and neurochemical stimuli in the attenuation of light induced phase shifts. Exposure to a novel running wheel and microinjections of NPY did not have significant phase shifting effects late in the subjective night, therefore the attenuation of light induced phase advancing were not due to an additive effect. Novel wheel confinement and NPY administration directly following light stimulation and up to 30 minutes post light exposure showed no significant differences in the extent to which light induced phase shifts were attenuated. Furthermore it would appear that following light exposure, a 30 minute window of opportunity exists in which time novel wheel running or NPY administration can maximally affect the resulting phase shift to light. Hence indicating that NPY release may occur early in the period of novel wheel exposure.

Behavioural activity is thought to modulate the transmission of photic information within the circadian system. Our results confirm previous work showing the attenuation of light induced phase shifts by activity during the late night, furthermore they shown that novel wheel exposure up to 60 minutes after a light pulse can also modulate the light induced phase advance (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998). Such a result indicates a complex interaction between photic and non-photic stimuli. We were unable to significantly attenuate the light phase shift with novel wheel exposure 90 minutes post light pulse.

Photic phase shifts have been shown to be completed within 1-2 hours of light stimulation (Best et al., 1999). Therefore our results indicate that novel wheel running administration must be within 60 minutes of the light pulse if the attenuation is to be observed. It would appear that at 90 minutes post light stimulation a majority of SCN cells have already been reset by light and so novel wheel exposure has very little effect. It may be that the signal transduction cascade utilised by light has either completed or is beyond the interaction site of the non-photic stimuli.

Photic input to the SCN is conveyed via a direct monosynaptic projection from the retina to the SCN, known at the retinohypothalamic tract (RHT). This pathway is thought to utilise glutamate as its primary transmitter to relay photic information to the SCN. The presence of glutamate in presynaptic terminals of the SCN was shown using ultrastructural methods (Ebling, 1996). Stimulation of the optic nerve *in vitro* releases glutamate onto SCN cells (Liou et al., 1986), while optic nerve stimulation *in vivo*, produces phase shifts that mimic those seen in response to light (de Vries et al., 1994).

A second indirect pathway to the SCN arises from the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN) and utilises neuropeptide Y as a primary neurotransmitter (Harrington, 1997). The geniculo-hypothalamic tract (GHT) links the IGL to the SCN and is thought to relay both photic and non-photic information. Stimulation of the GHT produces non-photic like phase shifts (Rusak et al., 1989). Neuropeptide Y administered *in vivo* and *in vitro* also produces non-photic like phase shifts (Albers and Ferris, 1984; Medanic and Gillette, 1993; Biello et al., 1997b). Antiserum to NPY can block the phase shifts to novelty induced activity, indicating NPY may be important in phase shifts to

wheel running (Biello et al., 1994). Lesions of the GHT or IGL have been shown to interfere with phase shifts to non-photic stimuli (Biello et al., 1991; Maywood et al., 1997). Non-photic stimuli have also shown to induce Fos-like immunoreactivity in the IGL, which is co-localised with NPY immunoreactivity (Janik et al., 1995).

It is likely that the IGL and its primary neurotransmitter, NPY, are involved in altering phase shifts to light by non-photic behavioural stimuli, such as novelty induced arousal. Neuropeptide Y containing fibres, projecting from the IGL, have been located in the ventrolateral portion of the SCN which is also innervated by the RHT (Card and Moore, 1989). NPY inputs are also likely to synapse on SCN cells receiving direct retinal information (Bosler and Beaudet, 1985; van den Pol and Gorcs, 1986). In addition, neurones activated by RHT stimulation are more likely to respond to application of NPY than those not activated (Shibata and Moore, 1988). Furthermore this interaction shown by *in vitro* experiments is supported by data from *in vivo* work. Lesions of the GHT alter the pattern of entrainment (Pickard et al., 1987). Behavioural experiments have also shown that administration of NPY can modulate the effects of light pulses (Weber and Rea, 1997). *In vitro* experiments indicate that application of NPY can alter phase shifts to glutamate in hypothalamic slice preparations of the SCN (Biello et al., 1997a). Finally *in vivo- in vitro* model showed that light pulses given *in vivo* were blocked by the administration of NPY *in vitro* during the early night (Yannielli and Harrington, 2000).

The inhibition of photic phase shifts up to 60 minutes post light stimulation by NPY or novel wheel exposure would suggest an intracellular inhibitory mechanism downstream from glutamatergic receptors. The precise mechanism by which NPY or behavioural non-photic stimuli inhibit photic phase shifts is unclear. However, NPY has been shown to

produce long-term depression in intracellular calcium levels in SCN cells stimulated with glutamate (van den Pol et al., 1996). It is possible that this neural depression may form part of the inhibitory pathway induced by NPY. Cells of the SCN are depolarised by glutamate (Meijer et al., 1993) and hyperpolarised by NPY (Hall et al., 1999). Neuropeptide Y activates a K^+- selective conductance in the SCN, but K^+ channel blockers apparently attenuated NPY phase shifts during the subjective day by inducing glutamate release (Hall et al., 1999). It is possible that NPY may influence light induced phase shifts via changes in K^+ conductance. During the subjective day, NPY phase shifts are mediated via the NPY Y2 receptor (Golombek et al., 1996). However NPY blocks phase shifts to light via the NPY Y5 receptor and NMDA induced phase shifts were inhibited by a NPY Y5 agonist, *in vitro* (Yannielli and Harrington, 2001). NPY Y5 receptor activation has been shown to inhibit the accumulation of the second messenger cyclic AMP (Gerald et al., 1996). Glutamate induced phase shifts are blocked by the inhibition of protein kinase A, *in vitro* (Tischkau et al., 2000). These findings suggest further possible sites for the inhibition of photic phase shifts by NPY.

Light or glutamate reset the circadian clock by utilising two different intracellular pathways. Phase delays to light in the early night are thought to be mediated via neuronal ryanodine receptors (Ding et al., 1998). However, phase advances late in the night have been linked to a cyclic GMP-dependent pathway (Weber et al., 1995). It would appear that phase delays to light and glutamate involve ionotropic receptors, whilst phase advances utilise mainly metabotrophic glutamatergic receptors. Our own *in vivo* work and previous *in vitro* studies have shown NPY to attenuate both phase delays and advances to light with a similar time course (Yannielli and Harrington, 2000). Both phase delays and advances

show attenuation by NPY administration up to 30 minutes post light exposure, hence indicating that NPY is acting downstream from glutamatergic receptor binding sites. Furthermore, the interaction between NPY and light during the early and late night must lie at the level of gene expression, where both the phase resetting pathways of light converge.

Period 1 (Per1) and Period 2 (Per2) are two key clock components localised to the SCN (Shearman et al., 1997; Tei et al., 1997). Per1 and Per2 mRNA levels (*mPer1* and *mPer2*, respectively) are increased upon light stimulation during the subjective night (Shearman et al., 1997; Zylka et al., 1998). Antisense oligodeoxynucleotides to the Per1 sequence have been shown to block light or glutamate induced phase shifts (Shigeyoshi et al., 1997; Akiyama et al., 1999; Wakamatsu et al., 2001). Photic stimulation results in increased levels of *mPer1* and *mPer2* expression which peak after 1hr and 2hr respectively (Albrecht et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998). NPY reduces *mPer1* and *mPer2* levels after 0.5hr and 2hr of NPY administration respectively, furthermore NPY Y1/Y5 receptor agonists have also been found to decrease Per1 and Per2 mRNA levels with a time course similar to that of NPY (Fukuhara et al., 2001).

Both NPY and light stimulation influence *mPer2* levels with maximum effects observed 2hr post stimulation. However NPY reduces *mPer1* within 0.5hr, while light increases *mPer1* within 1hr, indicating that NPY is faster than light in inducing its effects on *mPer1*. In our current study we found that NPY was able to attenuate the phase shift to light up to 60 minutes post light exposure, but was unable to effect the photic phase shift when administered 90 minutes post light stimulation. It would appear that the rapid effect of NPY on *mPer1* levels allows NPY to reduce the increase in *mPer1* initiated by light

stimulation up to 60 minutes post light. By 90 minutes post light exposure mPer1 levels would have peaked resetting the clock, therefore NPY administration at this time would cause a maximal reduction in *mPer1* 2hr post light stimulation and thus would have no effect on the resetting of the clock.

However recent evidence has shown NPY to differentially suppress *mPer1* and *mPer2* levels induced by light (Brewer et al., 2002). Neuropeptide Y administration following light, during the early subjective night, resulted in *mPer1* levels rapidly decreasing, but levels increase to match controls (light stimulation only) within 60 minutes. Conversely a sustained suppression of *mPer2* was observed throughout the expected peak for *mPer2* levels normally induced by light exposure alone. Light induced phase delays have been found to be strongly linked to the increase in *mPer2* levels and phase advances to an increase in *mPer1*, in mice (Albrecht et al., 2001). Given these current results it is possible that during the late subjective night NPY rapidly decreases *mPer2* levels and has a long-term suppressive effect on *mPer1* levels when administered post light exposure.

In view of the present investigation novel wheel running and NPY appear to be powerful stimuli in the regulation and inhibition of photic phase shifts during the late subjective night. It is clear that the attenuation of light induced phase shifts must occur via a more rapid signal transduction cascade than that utilised by light and glutamate at this time. It is evident that many possible sites for the interaction of photic and non-photic stimuli are present within SCN cells. Therefore it will be necessary to gain a further understanding of the intracellular signal transduction pathways utilised within this interaction, in order to elucidate the underlying mechanisms of such a complex series of events.

Chapter 6

General Conclusions

6.1 NPY and photic phase shifts

In the environment mammals display the ability to adjust to naturally changing photoperiods throughout the year. The mechanism used by the circadian clock to synchronize to such changes is not completely unclear. However, previous work, along with my own data strongly implicates the involvement of NPY in the modulation of photic stimuli, hence ultimately in the entraining mechanism of the SCN in mammals.

6.2 Importance of the photic phase shift

Under constant conditions of darkness mammals generally show circadian patterns of either a little over or a little under 24 hours, i.e. the tau's (τ) of specific individuals can be greater or less than 24. Thus in order for such individuals to synchronize to a light-dark cycle of 24 hours the circadian clock must be reset each day. It must either be pushed forward or backwards depending on the individuals τ. For mammals with τ's greater than 24 hours the clock must advance each day to remain synchronized with a 24 hour light-dark cycle. Consequently, mammals with τ's less than 24 hours must delay their clocks.

The mechanism that allows the entrainment of the circadian clock has been previously studied and my data now adds to the evidence indicating that non-photic stimuli play a significant role in this complex regulation.

6.3 NPY as a regulator

Neuropeptide Y has been previously shown to convey non-photic information to the SCN. Microinjections of antiserum, raised against NPY, directly onto the SCN blocked phase shifts to novel wheel exposure, a known non-photic stimulus (Biello, 1995). Furthermore, lesions of the IGL prevented hamsters from phase shifting to the novel wheel (Janik and Mrosovsky, 1994). Also, PRC's from GHT stimulation resembles those for NPY microinjections *in vivo* (Turek and Losee-Olson, 1986; Reebs and Mrosovsky, 1989; Meyer et al., 1993). Taken together, these studies show the importance of NPY in the circadian system and its ability to convey non-photic information to the SCN.

Previous work and now my own, show the ability of NPY to interact with photic phase shifts during the night. The significance of such an interaction is that it is likely to be vital for the entrainment of mammals to naturally changing environmental photoperiods. Thus NPY provides one possible mechanism for the modulation of photic phase shifts, enabling mammals to obtain precise synchronization with the environment.

6.4 Interaction between NPY and light

Interactions between NPY and light have been previously shown. Phase advances to light were blocked by NPY microinjection, *in vivo* (Weber and Rea, 1997). Furthermore, both phase delays and advances to light were blocked by NPY administration *in vitro* (Harrington, 1997). My work using a slightly different *in vivo* paradigm, mainly involving the microinjection of NPY following light exposure, has shown that NPY inhibits phase advances to light and also attenuates phase delays. Taken together these studies indicate a role for NPY in the inhibition of photic phase shifts. The fact that NPY inhibits phase

advances and attenuates phase delays may be of importance and may suggest differences in the regulatory mechanisms in place for the maintenance of light induced phase shifts.

6.5 Mechanisms for the interaction between NPY and light

Phase shifts to NPY were found to utilize the Y2 receptor subtype (Huhman et al., 1996; Biello et al., 1997b). However, *in vitro* data had indicated that the Y2 receptor was not involved in the inhibitory actions of NPY on photic phase shifts. In my first series of experiments, I found that a specific Y1/Y5 receptor agonist was able to influence photic phase shifts in a similar manner to NPY. With the next set of experiments, I found that by preventing NPY from binding to the Y5 receptor using a novel Y5 antagonist I was able to block the inhibitory effects of NPY on light induced resetting of the clock. The Y1 receptor antagonist had no effect on the interaction between NPY and light during the night. Thus it would appear that NPY initiates its inhibitory effects on photic phase shifts via the activation of the Y5 receptor subtype.

Having established the receptor subtype utilized by NPY in its complex interaction with light the next step was to identify a possible neural or intracellular pathway by which NPY mediates its influence on photic phase shifts. It has been previously shown that during the day NPY resets the circadian clock via a pathway involving GABAergic interneurons. In this study bicuculline, a $GABA_A$ antagonist blocked the phase resetting effects of NPY, *in vivo*. My next stage of investigation was to see if NPY utilized this same GABA dependent mechanism in the inhibition of light induced phase shifts. I found that bicuculline did not prevent NPY from blocking photic phase advances and attenuating

phase delays. Further, I also found that bicuculline potentiated light induced phase delays, but had no effect on phase advances.

The increases in the phase delays to light observed with bicuculline are possibly due to the disruption of a natural GABAergic tone present on SCN cells at this time. Muscimol, a $GABA_A$ agonist, is able to block the phase delays to light. Hence if a continuous tone were present, SCN cells would be under the influence of GABA during the early night, therefore photic phase shifts would be naturally reduced i.e. the magnitude we see in a normal photic PRC. Thus, the addition of a GABA antagonist would ultimately reduce the GABAergic tone on the SCN and prevent the inhibitory actions of GABA on light induced phase shifts. During the late night it is possible that GABAergic tone is reduced therefore administration of bicuculline has no effect on photic phase shifts.

Interestingly, microinjections of the Y5 antagonist resulted in the potentiation of the phase advances to light, but had no effect on phase delays. Taken together, it is possible that during the early night a GABA dependent mechanism is active in the regulation of the photic phase shift. However, during the late night NPY may modulate phase advances to light.

This interpretation of the experimental results displays the plasticity of the circadian system and the diversity of the modulatory mechanisms present for the regulation and preservation of the photic phase shift and ultimately entrainment.

6.6 Other possible mechanism of NPY and photic interactions at the physiological level

General conclusions from my data would suggest that the interaction between NPY and light occurs at the level of the SCN. It is likely that NPY opposes the cellular effects of light in order to prevent photic phase shifts. One way of doing this would be to interfere with the intracellular pathway induced by light via glutamate release from the RHT (Ebling, 1996). Throughout the night phase shifts to light require the activation of multiple glutamatergic receptor subtypes, of which NMDA receptors are critical (Colwell and Menaker, 1992; Ding et al., 1994; Shibata et al., 1994; Mintz et al., 1999). Activation of such receptors results in the influx of Ca^{2+} ions, which in turn activate nitric oxide synthase (NOS) to produce nitric oxide (NO) (Ding et al., 1994; Amir et al., 1995). At this point the photic pathway diverges. In the early night, light/glutamate requires the release of intracellular Ca^{2+} ions via the activation of neuronal ryanodine receptors (RYR) in order to phase shift. However, during the late night photic stimuli utilize a cGMP/protein kinase G (PKG) dependent signal transduction cascade (Weber et al., 1995; Mathur et al., 1996; Ding et al., 1998). Finally, light /glutamate signals during the early and late night induce the phosphorylation of Ca^{2+}/cAMP response element binding protein (CREB) which leads to CRE-mediated transcription activation (Ginty et al., 1993; Ding et al., 1997; Obrietan et al., 1999).

The intracellular pathway utilized by NPY is unclear. Currently, studies have been aimed at investigating the signaling molecules activated by NPY during the day, i.e. at times when NPY induces phase shifts. It is possible that NPY utilizes the same or a similar mechanism in its ability to interact with photic stimuli. In the first instance, NPY produces

long-term depression in intracellular calcium levels within SCN cells stimulated with glutamate (van den Pol et al., 1996). This would suggest that NPY might interact with photic phase shifts upstream from NOS activation. However, this is unlikely due to the differences observed with NPY and during the early and late night. Therefore the site of interaction is most probably post NO synthesis. The inhibition of protein kinase C (PKC) blocks NPY phase advances (Biello et al., 1997b). Also NPY increases selective K^+ conductance across SCN cell membranes (Hall et al., 1999), however this is not required for NPY phase shifts, and may be possibly linked to NPY's interaction with light. Furthermore, protein synthesis inhibitors did not prevent NPY induced phase shifts (Hall et al., 1999), suggesting that NPY may be acting to reduce protein synthesis within the SCN.

The divergence in the photic pathway may account for the difference observed with NPY's ability to attenuate phase delays and inhibit advances to light. The signal transduction cascade for delays to light are likely to be completed faster than those for advances due to the activation of RyR's and the rapid influx of Ca^{2+} ions from intracellular stores (Ding et al., 1998). Therefore, the key site for the interaction between NPY and light may lie at the rate of CRE-mediated transcriptional activation. With activation being faster with phase delays. This would suggest that the site of interaction lies at the level of gene transcription.

From this evidence it is possible to speculate a physiological pathway utilized by NPY in its interaction with photic phase shifts. Initially, during the night NPY binds to the Y5 receptor (Lall and Biello, Submitted), this then may activate a PKC dependent pathway and/or a cAMP/PKA mechanism. Both PKC and cAMP agonists show phase shifts similar to those observed with NPY (Biello et al., 1997b). Furthermore, PKC inhibitors block

NPY phase shifts. Ultimately this pathway will prevent the production of clock related genes normally increased by light stimulation.

Other neurotransmitters involved in non-photic phase shifts may also play a role in the mechanism of interaction between NPY and light. GABA agonists inhibit both phase delays and advances to light (Gillespie et al., 1996; Gillespie et al., 1997). Further, both NPY and GABA are co-localized within the same synaptic terminals (Francoisbellan et al., 1990). My data shows that the $GABA_A$ dependent pathway is not a mechanism used by NPY in its inhibitory effects of light. However, it is possible that NPY utilizes a $GABA_B$ and/or $GABA_C$ dependent pathway in this interaction. Serotonergic fibres, from the raphe nuclei, synapse onto SCN cells that also receive NPY and glutamatergic innervation (Morin et al., 1992). Activity induced phase shifts, mediated via NPY release, are unaffected by depletion of serotonin input to the SCN (Bobrzynska et al., 1996). However, the behavioural inhibition of light induced phase advances was prevented by the administration of a serotonergic antagonist (Mistlberger and Antle, 1998). This would suggest that serotonin and GABA also play a role in the inhibition of photic phase shifts.

Overall, it is likely that the majority of neurotransmitters mediating non-photic stimuli have the ability to interact with photic stimuli. The integration of all these various subsystems probably allows the plasticity and precision required for the functioning and ultimately the entrainment of the circadian clock.

6.7 NPY and light at the molecular level

Previous studies have shown NPY to oppose the actions of light at the molecular level. Together with my own data (chapter 5) it is likely that the interaction between NPY and light occurs at the level of gene transcription and/or protein synthesis.

Light stimulation results in the increase of the circadian gene period (Per) mRNA (*mPer*) levels (Shearman et al., 1997; Zylka et al., 1998). Neuropeptide Y administration has been shown to reduce *mPer* levels in hamsters and mice (Fukuhara et al., 2001; Maywood et al., 2002).

Cytosolic PER protein concentrations are regulated via three major cellular mechanisms. A dimer formed from the Clock and Bmal1 proteins (CLK and BMAL1, respectively) initiate *mPer* synthesis. The CLK/BMAL1 dimer binds to the promotor site of the numerous homologs of the period gene and starts the expression of *mPer*. The second mechanism involves an enzyme called casein kinase 1ε (ck1ε), which acts to phosphorylate PER. The phosphorylated PER (pPER) forms homodimers with other pPER proteins, which prevents *mPer* expression. Finally the synthesis of cryptochrome proteins (CRY) also aid the regulation of *mPer* synthesis by forming heterodimers with pPER and down regulating *mPer* transcription (see (van Esseveldt et al., 2000) for review of molecular genetics of the SCN).

This molecular mechanism presents various sites for NPY to inhibit the synthesis of *mPer* initiated by photic stimulation. It is possible that NPY is able to prevent the binding of the CLK/BMAL1 dimer to the promoter sites of the period genes. Neuropeptide Y may also

act to degrade the CLK/BMAL1 dimer during the night. An increase in the cytosolic concentration of ck1ε by NPY stimulation would result in the rise of pPER, ultimately leading to the increase of PER/PER homodimers, again, preventing *mPer* synthesis. Also the increase in the rate of synthesis of CRY would promote the formation of PER/CRY heterodimers, thereby inhibiting *mPer* synthesis. These speculations also fit into various molecular models proposed for the regulation of entrainment in mammals (Shearman et al., 2000).

Photic phase shifts have been linked to the expression of differing homologs of PER. Light induced phase delays have been strongly linked to increases in *mPer2*, while phase advances to increases in *mPer1* (Albrecht et al., 2001). Recently, during the early night NPY was shown to differentially suppress *mPer1* and *mPer2* levels following light stimulation, such that there was a sustained suppression of *mPer2* (Brewer et al., 2002). However, during the late night novelty induced wheel running, mediated via NPY release, did not inhibit photic phase shifts via a mechanism involving the down regulation of *mPer1* (Edelstein et al., 2003). Taken together this may indicate that either NPY interacts with photic phase shifts via the long-term suppression of *mPer2* or that, during the late night NPY inhibits photic resetting via a mechanism independent of PER regulation.

It is clear that from these speculations more work is required in the understanding of the molecular actions of NPY on the circadian clock.

6.8 The Future

It is evident that from the experiments included in this thesis that NPY input from the IGL is important in the regulation of photic phase shifts and ultimately in the entrainment of the mammalian circadian clock.

Further studies are required in order to fully understand the mechanism underlying the interaction between NPY and light during the night. My studies have shown that NPY utilizes the Y5 receptor subtype, at the level of the SCN, in its inhibition of the resetting effects of light.

Future work should be focused on determining the intracellular signaling cascade associated with the Y5 receptor and ultimately the molecular influences bought about by its activation. Further investigations into the differences between NPY's ability to interact with photic phase delays and advances also need to be addressed. It is unclear if the reason for such a difference is due to the photic intracellular pathway or the mechanism used by NPY to influence light induced phase shifts at varying times throughout the night. Also the role of other non-photic mediators need to be investigated, and their part in this complex interaction between NPY and light. The long-term aim of such research is to establish the precise mechanism by which the circadian system fine-tunes entrainment to the natural environment.

Both photic and non-photic stimuli are essential for the regulation and entrainment of the circadian clock to our natural environment. Understanding the underlying mechanisms by which such stimuli interact will provide invaluable information regarding circadian clock

function. Ultimately such information would be beneficial in the treatment of many circadian related conditions and disorders, such as jet lag and depression.

Appendix I

Cannulation surgical procedure

Apparatus Preparation: all surgical instruments were autoclaved before use. The stero-taxic frame and accessories were all swabbed down with 70% alcohol as well as surrounding surgical surfaces.

Anesthetic setup: halothane was used throughout this procedure. Animals were anaesthetized in an enclosed chamber with inlet valves for oxygen and anesthetic. Oxygen flow into the chamber was set to 1 liter per minute. Halothane levels were set to 6% whilst the animal was confined to the chamber. Once the animal had been placed into the stero-taxic and the anesthetic mask fitted, halothane levels were reduced to 3%, but oxygen levels remain unaltered.

Animal preparation: male Syrian hamsters weighing approximately 90 –100 grams were used for this procedure. Initially animals were weighed and placed into the anesthetic chamber. Once the animal became ataxic it was taken out of the chamber and the head shaved using an electric shaver and then placed back into the chamber. At this stage the animal was left in the chamber until it was completely under the influence of the anesthetic. A typical test for this was the absence of a toe pinch reflex.

Stero-taxic preparation: one of the ear bars was set to 0.5 and locked into position. The platform on which the animals was to lie was set so that once the animal was in the stero-taxic its head was level with the plane of the ear bars. The guide cannula was placed into cannula holder and secured.

Placing the animal into the stero-taxic: once the animal was under the anesthetic in the chamber it was placed onto the stero-taxic platform with its nose firmly in the anesthetic mask and ears level with the ear bars. One ear was placed over the secure ear bar. This was used to anchor the animal's head. Once the head felt as though it was anchored via a bone ridge on the skull the head was held flat and the second ear bar placed into the other ear. Finally the incisors were secured in the incisor bar and pulled tort.

Cannulation: using a number 10A scalpel blade an incision of approximately 1.5 cm was made along the skull to reveal the skull surface. Then surrounding muscle was scraped back to uncover the skull markings. Using a cotton swab a little ethanol was applied to the skull surface whilst drying the skull using a blow dryer. Once bregma was visually predominant the guide cannula was lined up at the point at which the midline and bregma met. The co-ordinates were noted for the vertical, horizontal and longitudinal axis. These were then adjusted according to the sterio-taxic co-ordinates for aiming the guide cannula to the third ventricle or suprachiasmatic nucleus (see individual chapter sections on experimental procedures). The new position of the guide cannula was marked and a hole of approximately 0.1cm was drilled using an electronic hand drill. Three screws (approximately 0.2 cm in length) were fixed around the drilled hole in a triangular formation. The guide cannula was then lowered to the desired depth. Small pieces of gel foam were placed between the skull surface and the base of the guide cannula pedestal. Dental cement was applied around the guide cannula and the screws to form a smooth dome. Once dry the skin was sutured together using surgical glue. The animal was taken

out of the ear bars, anesthetic mask and incisor bar and placed into a warm box until the animal recovered from the anesthetic.

After care: post surgery animals were injected with 0.5 mls of saline and buprenorphine (0.5mg/Kg). Animals were also given mashed diet, which consisted of food being crushed and turned into a pulp by adding water. The animal's body weight was also monitored over a seven-day period following surgery.

Appendix II

Dose response analysis of RWJ57926 in the inhibition of photic phase shifts by NPY

Introduction

Previous work has shown NPY to inhibit photic and phase advances and attenuate phase delays. Furthermore, this interaction was shown to involve the activation or either the Y1 or Y5 receptor (Lall and Biello, 2003b). A recent *in vitro* study found that a Y5 receptor antagonist inhibited the attenuating effects of NPY on NMDA induced phase shifts (Yannielli and Harrington, 2001).

This study was designed to establish a dose response relationship between a novel NPY Y5 antagonist and its effects on the interaction between NPY and light.

Materials and methods

Subjects

Experimental procedures were carried out under licence by the Home Office (UK) in accordance with the Animals (Scientific Procedures) Act (1986). Adult male Syrian hamsters (40 days old, Harlan Sprague-Dawley, Oxon, UK) were housed individually in plastic cages fitted with 16 cm running wheels connected to a computer that summed wheel revolutions in 10 minute activity bins (Dataquest Pro-Data software, Data Sciences Inc., Roseville, MN,USA). Food and water were available *ad libitum*. Animals (n=12) were maintained under a LD cycle of 14 hours light and 10 hours of darkness and then transferred to constant conditions (22 ± 2°C; 14-18 lux dim red light) 10 days before treatment. Animals were re-entrained by this method following a maximum of three treatments before continuing with further experimental conditions. All efforts were made to minimise the number of animals used and their suffering.

Surgery and injections

See appendix I for a detailed description of the surgical procedure.

Hamsters were anaesthetised with halothane gas throughout the length of the surgery. Animals received cannula guides, which were implanted to a depth of 5.6mm below the skull surface at a 10° angle and fixed to the skull with fine machine screws and dental cement. Cannula guides were stereotaxically aimed at the SCN (co-ordinates relative to bregma: AP+0.4mm, ML 1.0mm, upper incisor bar –2 mm). After recovery from surgery (1 week under LD 14:10h), animals were transferred into cages fitted with running wheels and maintained for 7-10 days under LD 14:10h before being placed into constant conditions. In all treatments the vehicle consisted of a 0.9% saline solution. Porcine NPY

(234μM in 200nl saline, Calbiochem, UK), Y5 antagonist RWJ57926 ([α-(3-pyridylmethyl)-β-aminotetralin-derived sulfonamide]; 4μM, 2μM, 1μM, 0.5μM in 200nl saline; RW Johnson Pharmaceutical Research Institute), were administered using a 28 gauge infusion cannula attached to a 1μl Hamilton syringe. The infusion cannula extended 2.4 mm from the base of the guide cannula.

Experimental treatments

Prior to the start of the experiment all animals were injected with NPY at CT 6 five days after being placed into constant conditions. These microinjections were used as functional assessments to verify cannula placements.

Animals were randomly divided into four groups each consisting of three hamsters. Group 1 received a 15-minute light pulse at CT18 with Y5 antagonist (4μM) administration at CT18.08 and NPY administration at CT18.17. Group 2 received a 15-minute light pulse at CT18 with Y5 antagonist (2μM) administration at CT18.08 and NPY administration at CT18.17. Group 3 received a 15-minute light pulse at CT18 with Y5 antagonist (1μM) administration at CT18.08 and NPY administration at CT18.17. Group 4 received a 15-minute light pulse at CT18 with Y5 antagonist (0.5μM) administration at CT18.08 and NPY administration at CT18.17.

Data analysis

For activity onset calculation see appendix III.

Data from each set of treatments was analysed by the one-way repeated measures ANOVA, followed by Bonferroni method for multiple comparisons.

Cannula Placement

See appendix IV for cannula placement assessment methods.

Results

Table 1 shows the phase shifts observed during the late night with varying concentrations of RWJ57926 during light stimulation followed by NPY microinjection ($F_{(8, 11)}$= 198). Figure 22 illustrates the dose relationship of the Y5 antagonist with light and NPY treatments.

Table 1

[RWJ57926] μM	Phase shift (mean minutes ± SEM; n=3 in all groups)
4*	486 ± 20
2	164 ± 9
1	162 ± 6
0.5	171 ± 4

Table 1. Phase shifts obtained from microinjects of the Y5 antagonist during light exposure followed by NPY administration (* indicates p<0.001).

Figure 22

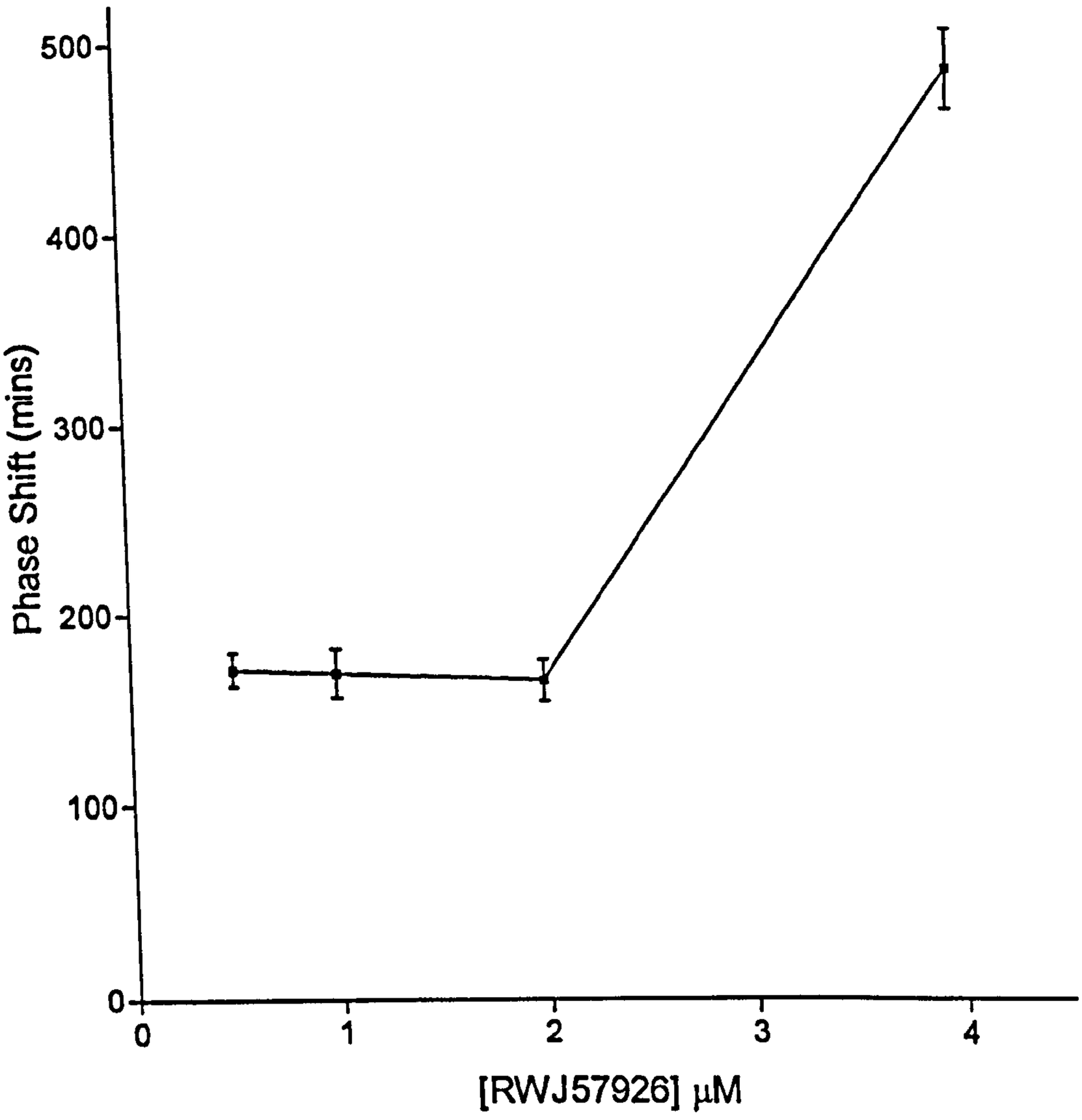
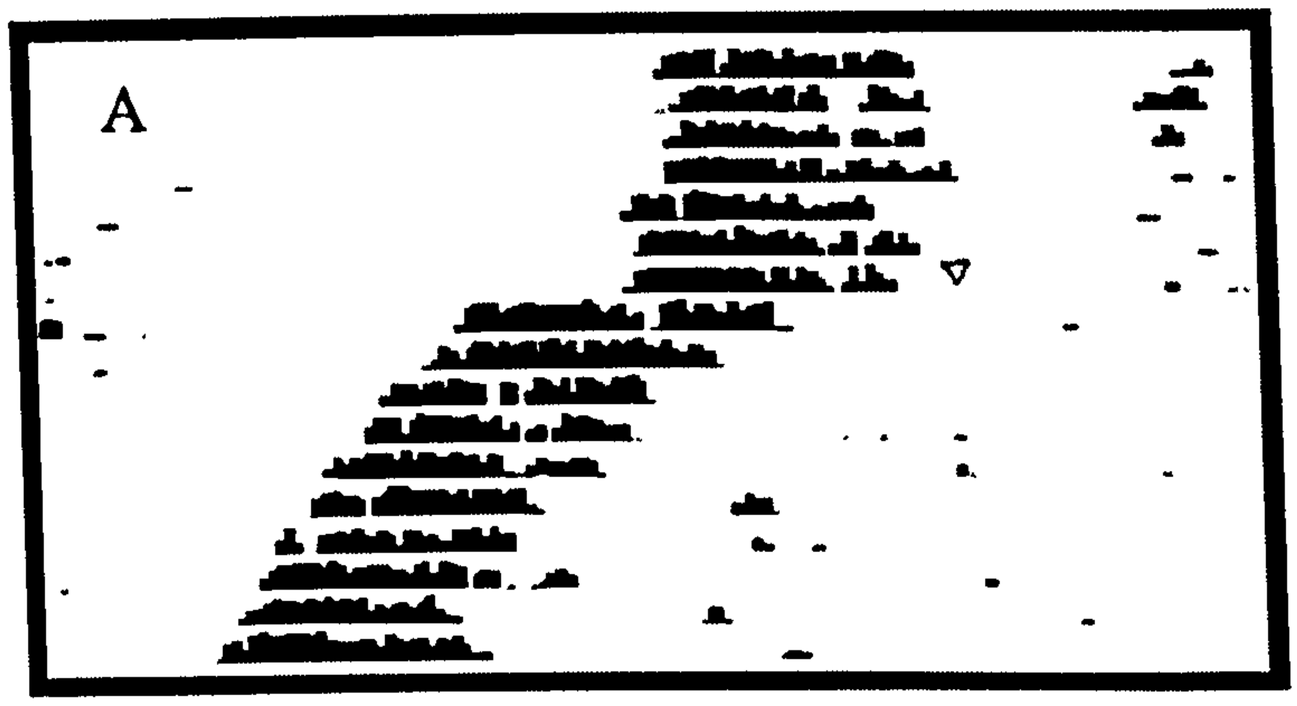
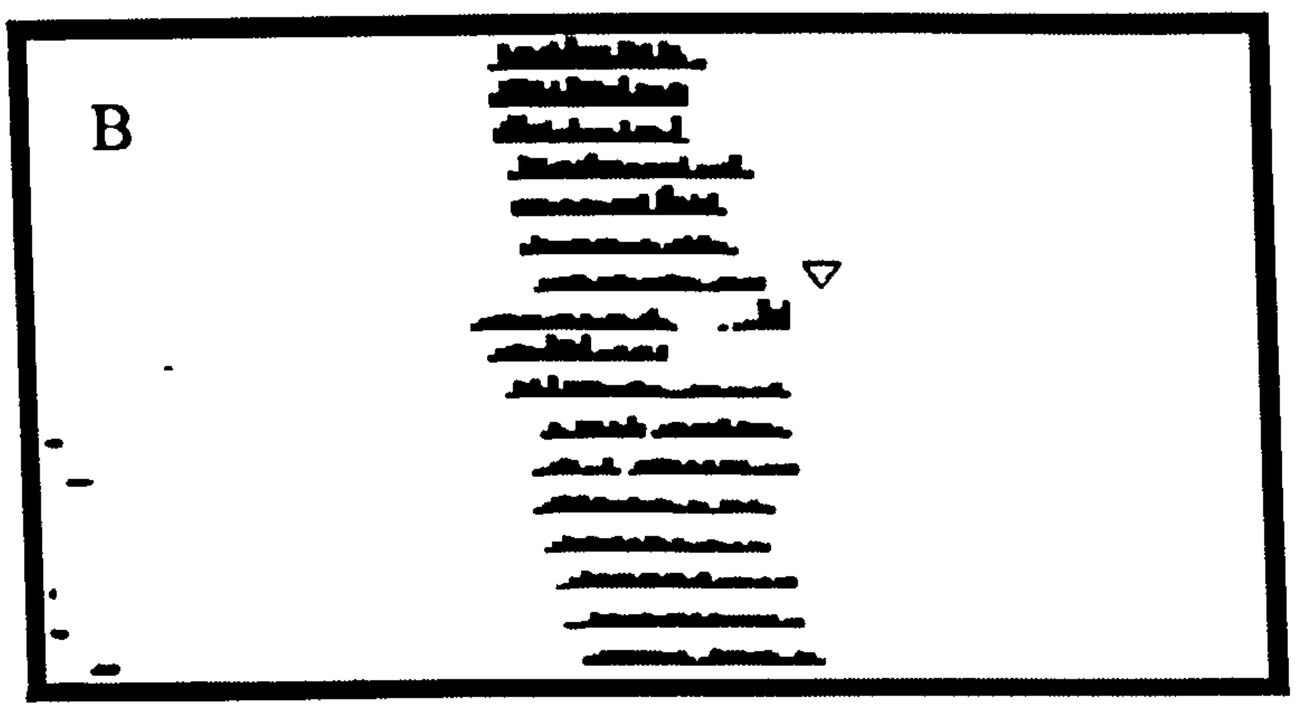


Figure 22 illustrates the dose response relationship observed with the Y5 antagonist, light and NPY.

Figure 23



A. Light + Y5 antagonist [4μM] + NPY



B. Light + Y5 antagonist [2μM] + NPY

Figure 23. Representative actograms illustrating the phase shifts obtained with the microinjection of varying concentrations of RWJ57926 with light and NPY.

Conclusion

The ability of NPY to block photic phase shifts was prevented by the presence of the NPY Y5 receptor antagonist. The higher dose of the antagonist resulted in the significant potentiation of the photic phase shift. Lower doses of the drug showed no variation in the phase shift to light. A recent *in vitro* study shows that a 2μM dose of RWJ57926 was effective in inhibiting the ability of NPY to block NMDA induced phase shifts (Yannielli and Harrington, 2001). From this set of experiments and those preformed *in vitro*, it was concluded that a 2μM concentration of RWJ57926 be used for the series of experiments preformed in chapter 3.

Appendix III

Onset calculation

Activity onset times (CT12) for the day of treatment were calculated by forward extrapolation of a regression line fitted to seven onsets pre-treatment. Activity onsets were defined as the first 10 minute bin with at least 50 wheel revolutions, following a period of no activity for at least 6 hours, followed by a second 10 minute bin with 50 or more wheel revolutions within 30 minutes. Post-treatment activity onsets for the day of treatment were calculated by back extrapolation of a regression line fitted to seven activity onsets post-treatment. The initial 3 days post-treatment were excluded from the regression calculations to allow for any transient effects. Phase shifts were calculated as the difference between pre-treatment and post-treatment activity onsets.

Appendix IV

Cannula placement analysis methods

Cannula placements were identified by histological and/or functional assessment.

Histology: animals were killed using an overdose of sodium pentobarbital (about 160 mg/kg, i.p.) and given an intracerebral injection of Indian ink (400nl) with the same length of injector used in the experiment. They were then perfused transcardially with 0.9% saline and 10% formaldehyde. The brains were postfixed in 10% formalin with 30% sucrose. Frontal sections (40 μm) were cut using a cryostat. Slices containing the SCN were mounted on Polysine microslides and stained for Nissl substance with Cresyl Violet. The distance from the injection site to the margin of the SCN was measured along the anterior-posterior, dorsal-ventral and lateral axes. The greatest of the three measurements was taken as the distance from the SCN. No data from animals with placements more than 400μm from the margin of the SCN was included in subsequent analyses.

Appendix V

Cannula placement site

Figure 24.

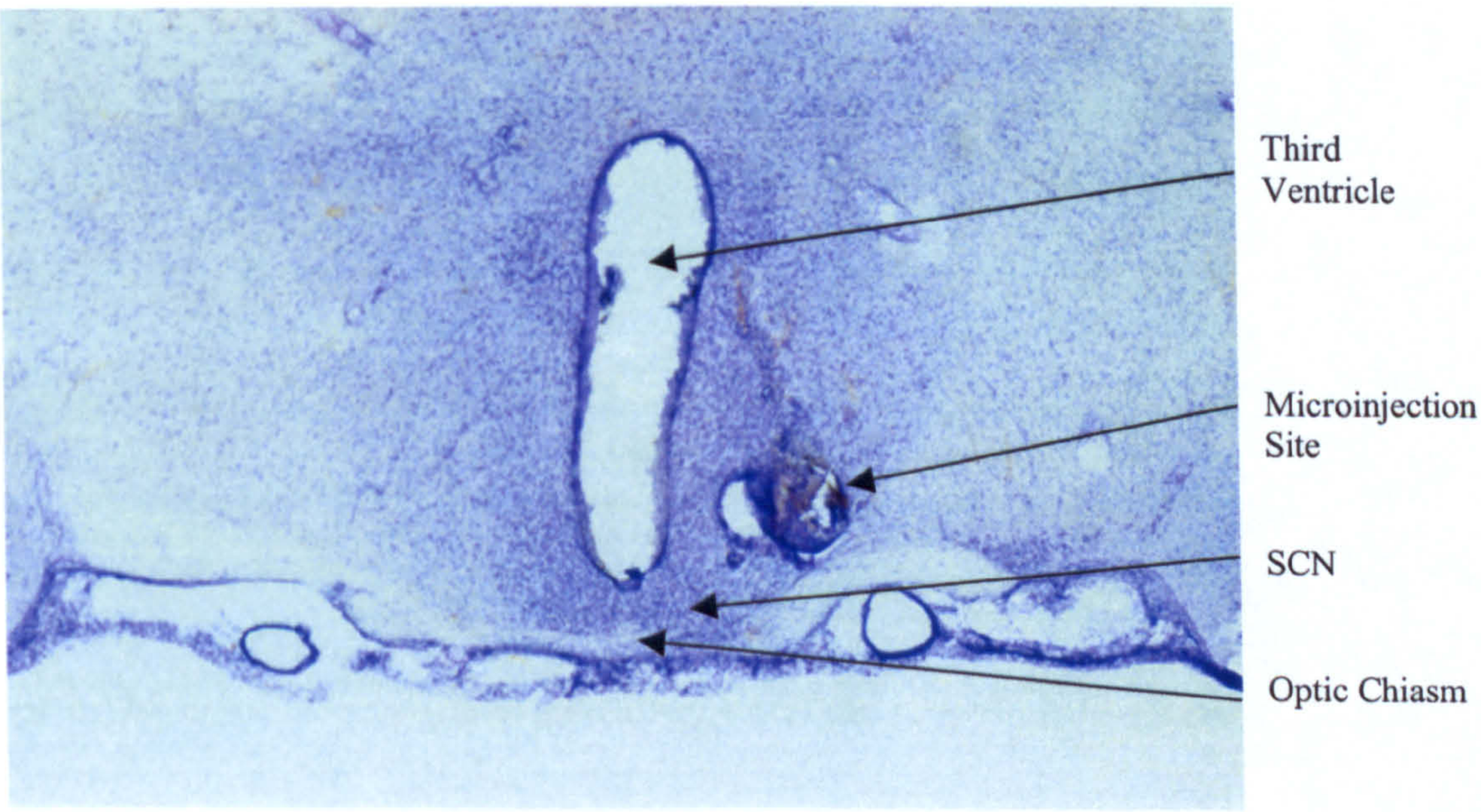


Figure 24. A representative coronal photomicrograph of a hamster brain detailing the verification of the cannula microinjection site.

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