MDMA ATTENUATES PHASE SHIFTS OF THE CIRCADIAN CLOCK TO THE $GABA_B$ AGONIST BACLOFEN

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Abstract

The mammalian suprachiasmatic nucleus (SCN) of the hypothalamus is the site of a circadian pacemaker that drives many rhythms of behaviour and physiology. The pacemaker displays a period of approximately 24 hours and can be entrained to external conditions by a variety of environmental cues. The principle entraining cue is the daily light-dark cycle but other non-photic stimuli can also reset the phase. In rodents, the pacemaker is sensitive to photic influences during the night while non-photic stimuli are only effective in resetting the pacemaker during the day. One afferent pathway to the SCN originating in the median raphe nucleus contains serotonin (5-HT) and has been implicated in the mediation of non-photic phase shifting of the pacemaker. Disruption of this pathway by neurotoxins can result in alteration of several parameters of circadian rhythmicity. Application of 5-HT or its receptor agonists during the day results in phase advances of pacemaker period. A role for the neurotransmitter γ-amino butyric acid (GABA) in circadian rhythmicity has also been shown. The $GABA_B$ receptor agonist baclofen has been shown to phase advance the pacemaker during the day in a manner similar to 5-HT. Baclofen has also been shown to result in an increase in the release of newly synthesised 5-HT within the SCN area during the day. The substituted amphetamine, 3,4-methylenedioxymethamphetamine (MDMA) is a common drug of misuse as well as being a selective 5-HT neurotoxin. At neurotoxic doses it is known to result in degeneration of 5-HT terminal areas. In this study, the magnitude of phase shifts due to daytime injection of baclofen to Syrian hamsters maintained in constant darkness was compared before and after administration of a neurotoxic dose of MDMA. Immunohistochemical techniques were employed to quantify neurotoxic damage to the 5-HTergic terminal field within the SCN and cell bodies within the raphe nuclei. It was found that the magnitude of baclofen induced phase

advances during the day were significantly greater than phase shifts induced by control injections of saline at the same time. There was a significant difference between the magnitude of phase advances to baclofen before and after treatment with MDMA. The difference in density of immunohistochemical staining for 5-HT was also different between MDMA treated animals and untreated controls. These results imply there may be interaction between the GABAergic and 5-HTergic systems within the circadian system for mediation of non-photic phase shifting of the pacemaker during the day in rodents.

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I confirm that this thesis is my own work carried out under normal conditions of supervision.

Abbreviations Used

τ	.....	tau
5,7-DHT	.....	5,7-dihydroxytryptamine
5-CT	.....	5-carboxamidotyptamine
5-HIAA	.....	5-hydroxyindoleacetic acid
5-HT	.....	5-hydroxytryptamine
5-HTP	.....	5-hydroxytryptophan
8-OH-DPAT	.....	8-hydroxy-dipropylaminotetralin
ABC	.....	avidin-biotin complex
AVP	.....	arginine vasopressin
Baclofen	.....	4-amino-3-[4-chlorophenyl]-butanoic acid
BMAL1	.....	brain and muscle Arnt-like protein
CKI	.....	casein kinase
CGP-35348	.....	3-amino-propyl (diethoxymethyl)phosphinic acid
CT	.....	circadian time
DAB	.....	diaminobenzidine
Dbp	.....	D-element binding protein
DD	.....	constant darkness conditions
ddH_2O	.....	double distilled water
DOI	.....	(±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride
DR	.....	dorsal raphe
GABA	.....	γ-aminobutyric acid
GAD	.....	glutamate decarboxylase
GHT	.....	geniculohypothalamic tract
GPCR	.....	G protein-coupled receptors
IHC	.....	immunohistochemistry
H_2O_2	.....	hydrogen peroxide
IGL	.....	intergeniculate leaflet
KCl	.....	potassium chloride
LD	.....	light-dark cycle
MDMA	.....	3,4-methylenedioxymethamphetamine
MR	.....	median raphe
Muscimol	.....	3-hydroxy-5-aminoethylisoxazole hydrobromide
NaCl	.....	sodium chloride
$NaHCO_3$	.....	sodium bicarbonate
OD	.....	optical density
p-CPA	.....	p-chloroamphetamine
PAM	.....	4% paraformaldehyde
PB	.....	phosphate buffer
PBS	.....	phosphate buffered saline
PBS+T	.....	phosphate buffered saline with Triton® X100
Phaclofen	.....	3-Amino-2-(4-chlorophenyl) propylphosphonic acid
PK2	.....	prokineticin 2
PRC	.....	phase-response curve
RHT	.....	retinohypothalamic tract
ROD	.....	relative optical density
SCN	.....	suprachiasmatic nucleus
T	.....	transmittance
TFMPP	.....	1-[3-(trifluoromethyl)phenyl]-piperazine
TGFα	.....	transforming growth factor α
TPH	.....	tryptophan hydroxylase
TTX	.....	tetrodotoxin
ZT	.....	zeitgeber time

1 Introduction

1.1 Circadian Rhythms

Circadian rhythms are biological cycles that follow a regular pattern of approximately 24 hours and are controlled by an endogenous pacemaker. They are manifest in numerous physiological and behavioural processes such as body temperature, hormone levels and wheel running behaviour. The natural period of any individual animal's clock, or tau (τ), is synchronised to environmental conditions by the process of entrainment. This involves the synchronisation of the clock by environmental stimuli. The most predominant stimuli, or zeitgeber, is light although other non-photic stimuli also reset the phase of the clock.

Manipulation of the endogenous clock can be achieved under laboratory conditions by manipulation of environmental zeitgebers. The result of this can be observed as measurable changes in various physiological or behavioural processes, such as wheel-running activity in the case of laboratory animals. Through the removal of zeitgebers and establishment of constant environmental conditions, τ can be measured from changes in these activity patterns.

Circadian time (CT) measures the state of the endogenous clock under constant environmental conditions with CT12 being defined as the time of activity onset for nocturnal animals. Giving pulses of light at different circadian times results in phase shifts of the clock reflected as changes in daily activity patterns. The magnitude and direction of these phase shifts varies depending upon the time at which the light pulse is given. Graphically plotting time of day against phase shift produces in a phase-response curve (PRC) that varies slightly between species (Figure 1.1). For mammals, pulses given in the early subjective night result

in phase delays, in the late subjective night phase advances and during the subjective day no phase shift at all (Dann and Pittendrigh 1976).

Non-photic stimuli are also capable of resetting the circadian clock. These include food availability (Mistlberger 1992), social interaction (Mrosovsky 1988), behavioural activity (Mrosovsky 1995), exposure to a novel running wheel (Mrosovsky and Salmon 1987) and sleep deprivation (Antle and Mistlberger 2000). The PRC is different from that seen in response to photic stimuli, with phase advances during subjective day and very little effect during subjective night (Figure 1.2).

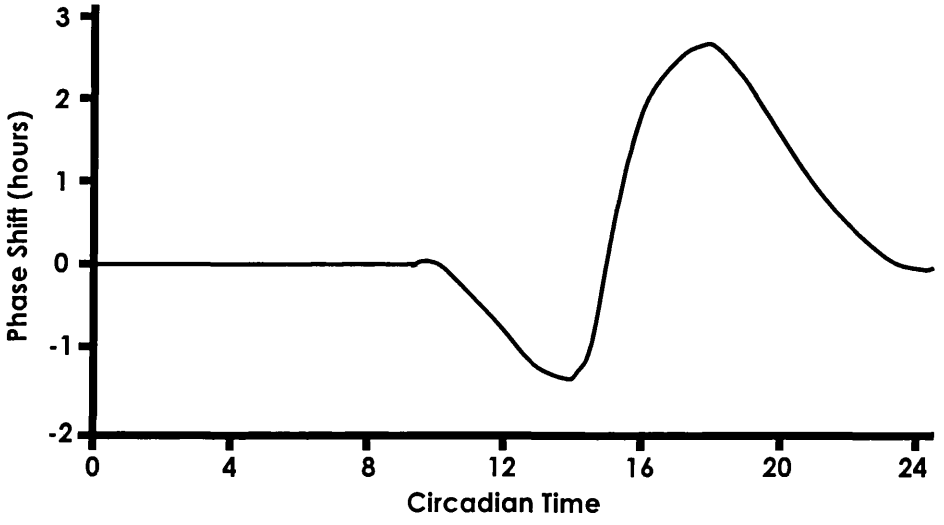


Figure 1.1 Photic phase response curve for the hamster. A pulse of light given early in the circadian night results in phase delays of circadian rhythms, while pulses given late in the night result in advances.

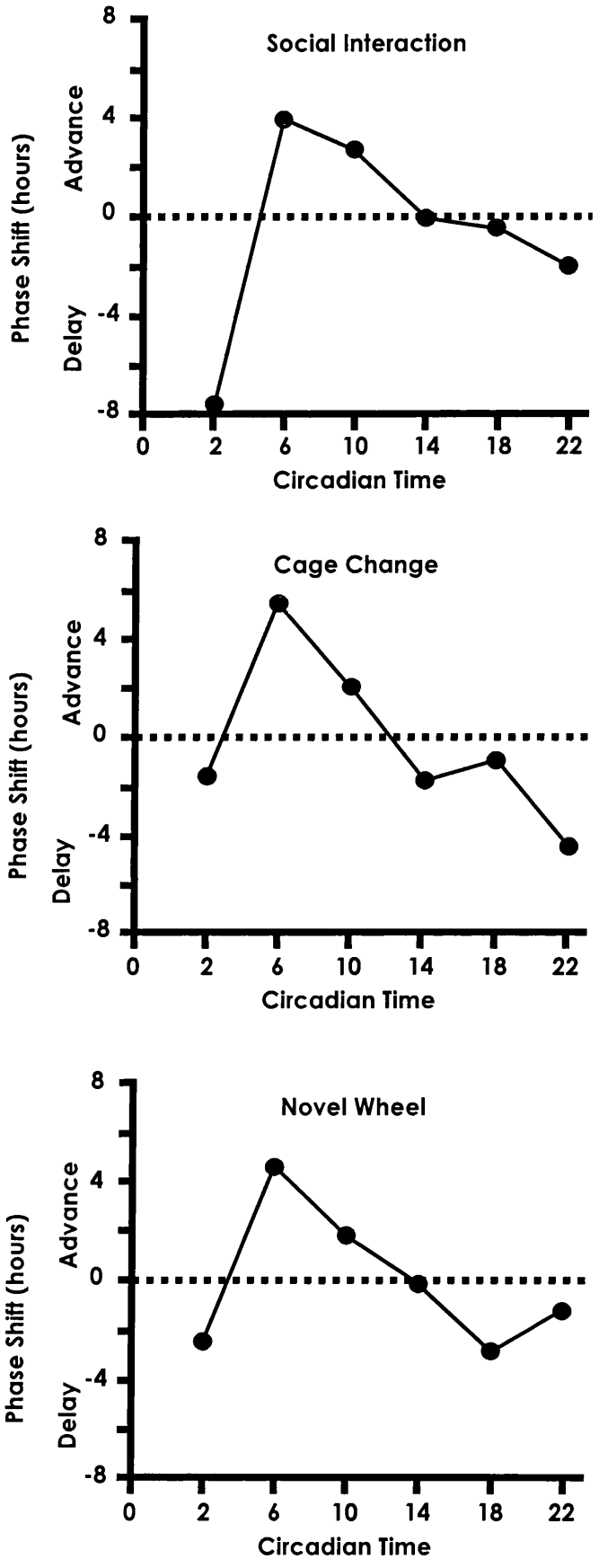


Figure 1.2 Examples of PRCs for three different non-photic stimuli. Each of these stimuli result in phase advances at CT6. (From (Mrosovsky, Reebs et al. 1989).

1.2 The Suprachiasmatic Nucleus

In mammals, the site of the pacemaker is the suprachiasmatic nucleus (SCN) of the hypothalamus (Meijer and Rietveld 1989; Moore 1995). For mammals, this is located above the optic chiasm and below the third ventricle. Lesions of the SCN result in the loss of rhythmicity in the animal (Rusak 1977). Cells of the SCN show circadian rhythms of electrical activity (Gillette 1986) and this rhythmicity continues after isolation of the nucleus from other brain areas, indicating its endogenous nature. Electrical stimulation of the nucleus results in phase shifts of circadian rhythms (Rusak and Groos 1982). Transplantation of SCN tissue from intact to lesioned animals results in re-establishment of rhythmicity, with τ being that of the donor animal (Ralph, Foster et al. 1990). These studies would indicate that the SCN is involved in the generation of circadian rhythms and in the absence of environmental cues or input from other brain areas, is capable of generating endogenous rhythms.

Despite the SCN being composed of a relatively small number of cells, the neuropil is complex with many synapse types. Axons enter the nucleus from all directions and dendrites extend outwith it. Some collaterals terminate on other neurones within the nucleus forming local circuits and some axons connect the left and right nuclei (Van den Pol 1991). Several lines of evidence point to the SCN being composed of subdivisions based upon cell size, afferent input or efferent output and transmitter content of neurone cell bodies (Card and Moore 1982; Hoorneman and Buijs 1982; Van den Pol 1991). Two major subdivisions are the ventrolateral and dorsomedial regions with other subdivisions based upon immunostaining for cellular components. Afferent pathways synapse on the ventrolateral SCN where they form an extensive and complex terminal field.

There are three main afferent inputs conveying information on environmental conditions to the SCN. The retinohypothalamic tract (RHT) is a monosynaptic tract originating in the retinal ganglion cells, which conveys photic information predominantly to the SCN (Johnson, Morin et al. 1988). The geniculohypothalamic tract (GHT) runs from the thalamic intergeniculate leaflet (IGL) to the SCN (Morin, Blanchard et al. 1992) and is thought to convey non-photic information that acts to regulate photic stimuli (Albers and Ferris 1984). The IGL itself receives direct input from the retina and this provides an indirect source of photic information for the SCN (Morin 1994). Non-photic information is also received from the midbrain raphe nuclei (Meyer-Bernstein and Morin 1996; Leander, Vrang et al. 1998). There is evidence for a direct neuronal projection from the retina to the raphe (Shen and Semba 1994) but not that this synapses onto cells that project to the SCN (Kawano, Decker et al. 1996).

1.3 Pharmacology of the Circadian System

In vitro electrophysiological and pharmacological studies using isolated SCN slices demonstrate the effect on firing patterns of SCN neurones from application of agonists and antagonists of neurotransmitter receptors. *In vivo* pharmacological manipulations allow observation of changes in the timing of activity levels and thus interpretation of the state of the internal clock. Alteration of either phase or period of the clock may occur through the direct action of a drug on pacemaker cells. Alternatively, the site of action for the drug may be in another brain area such as the raphe nuclei where it acts to modulate afferent input to the SCN.

Several neurotransmitters and neuromodulators in the circadian timing system utilise several receptor subtypes for communicating environmental information.

The excitatory amino-acid glutamate is the principal neurotransmitter of the RHT (Ebling 1996), while non-photic stimuli act through the neuropeptide Y containing cells of the GHT (Card and Moore 1989) and serotonin (5-hydroxytryptamine or 5-HT), containing cells of the raphe (Meyer-Bernstein and Morin 1996). The principle neurotransmitter of the SCN is γ-aminobutyric acid (GABA) which is found along with its synthetic enzyme glutamate decarboxylase (GAD) in virtually all SCN neurones (Moore and Speh 1993).

1.4 Serotonin

Serotonin is an indolealkylamine, also known as 5-hydroxytryptamine (5-HT), synthesised from its dietary precursor the amino acid tryptophan. The synthetic reaction is catalysed by the enzyme tryptophan hydroxylase and results in the production of 5-hydroxytryptophan (5-HTP). Tryptophan hydroxylase is synthesised in serotonergic nerve cell bodies and undergoes axonal transport to terminal areas where the majority of 5-HT synthesis takes place (Meek and Neff 1972). Decarboxylation of 5-HTP by the enzyme aromatic L-amino acid decarboxylase results in the formation of 5-HT. Once synthesised, 5-HT is accumulated via a membrane transporter in storage vesicles within nerve terminals. The mechanism of release is by exocytosis which is sensitive to the sodium channel blocker tetrodotoxin (TTX) and to the presence of calcium ions, whether measured *in vitro* from brain slices (Carboni, Cadoni et al. 1989) or *in vivo* by microdialysis (Auerbach, Minzenberg et al. 1989; Carboni and Dichiara 1989).

Release of 5-HT is known to be regulated by the action of inhibitory autoreceptors, since release inhibition by 5-HT agonists was shown to be blocked by the 5-HT antagonist methiothepin (Starke, Gothert et al. 1989). Serotonergic

neurones have both somatodendritic and presynaptic autoreceptors, which differ in their pharmacological profiles. The somatodendritic autoreceptors suppress cell firing and are involved in collateral inhibition (Aghajanian 1981) as well as leading to reductions in synthesis and release of 5-HT in terminal areas. Presynaptic autoreceptors however are only involved in inhibition of 5-HT release.

The effects of synaptically released 5-HT are terminated by means of an reuptake process mediated by a membrane bound transporter protein (Graham and Langer 1992). In nervous tissue, 5-HT is metabolised by the process of oxidative deamination that is catalysed by the mitochondrial enzyme monoamine oxidase to produce 5-hydroxyindoleacetaldehyde. This is rapidly oxidised by aldehyde dehydrogenase to produce 5-hydroxyindoleacetic acid (5-HIAA), which diffuses into the cerebrospinal fluid and is eliminated from the brain.

1.5 Anatomy of the Serotonergic System

Techniques used to elucidate the anatomy of the 5-HT system have included neurotoxic lesioning; retrograde and anterograde tracing; autoradiography following *in vivo* and *in vitro* application of [^{3}H]5-HT; and immunohistochemistry for tryptophan hydroxylase or 5-HT.

The 5-HT system consists of a morphologically diverse group of neurones with cell bodies located in the midbrain raphe nuclei and some regions of the reticular formation (Steinbusch 1981). There are two distinct subdivisions, a rostral division with cell bodies located in the midbrain and rostral pons which projects to the forebrain and a caudal division located in the medulla oblongata with descending projections to the spinal cord (Lidov and Molliver 1982). The rostral

serotonergic division consists of the caudal linear nucleus, the dorsal raphe nucleus (DR), the median raphe nucleus (MR), the B9 group and in primates a large dispersed group of cells located in the nucleus pontis oralis (Azmitia and Gannon 1986). The DR and MR together account for approximately 80% of forebrain serotonergic terminals (Azmitia 1978).

The DR is located in the ventral part of the periaqueductal grey matter of the midbrain and extends into the periventricular grey matter of the rostral pons. It displays bilateral symmetry and has a diverse neuronal population, with the smallest cells on or near the midline and the largest cells in the most lateral and dorsal regions of the nucleus (Törk 1985). Several sub regions are distinguishable by differences in cell density, cell morphology and area of projection (O'hearn and Molliver 1984; Waterhouse, Mihailoff et al. 1986). The MR is found largely in the rostral pons with constituent cells arranged in two adjacent regions. Small cells with short dendrites are arranged around the midline and aligned parallel to the mid-sagittal plane. Outside the midline area the cells are loosely arranged without any particular orientation and the boundary of this nucleus is not sharply defined (O'hearn and Molliver 1984; Törk 1985; Waterhouse, Mihailoff et al. 1986).

The ascending serotonergic projections are extensive and contain many collaterals innervating diverse regions of the forebrain. These projections initially travel in the medial forebrain bundle with axons extending from this to other fibre pathways and on to their target areas. There are two types of serotonergic axons in the forebrain (Kosofsky and Molliver 1987). The most common are thin, with fusiform shaped varicosities of less than 1 μm, which extend over large areas of the forebrain. These fibres tend to branch frequently with the branches being of the same structure as the parent fibres. The second fibre type has large round or oval varicosities on thin fibres and displays a beaded appearance under the

microscope. The varicosities are swellings in terminal areas that contain the vesicles responsible for 5-HT storage and release. Anterograde tracing studies have shown that the different axon types have different origins with the fine axons originating in the DR while the beaded axons arise in the MR (Kosofsky and Molliver 1987). Serial section analysis in the cat has also demonstrated that the two axon types form two independent fibre systems which may also have distinct functions (Mulligan and Tork 1988).

Within the circadian system, a combination of anterograde tracing, retrograde tracing and neurotoxic lesioning has shown that serotonergic innervation of the hamster SCN originates in the MR while that of the IGL originates in the DR (Meyer-Bernstein and Morin 1996). Additionally, some evidence has been found in the rat, for a direct serotonergic pathway to the SCN originating in the DR (Kawano, Decker et al. 1996).

1.6 Serotonin in the Circadian System

Serotonin is thought to be involved in the transmission of non-photic information to the SCN. It is known that numerous types of non-photic stimuli are capable of adjusting the phase or period of circadian rhythms in mammalian species. Included among these are several which directly effect serotonergic activity such as exercise or sleep deprivation. Wheel running activity, itself regulated by the circadian system, can modulate oscillation of the clock in rodents indicating that certain behaviours could have a feedback effect on the clock. Sleep deprivation is also known to result in increased activity of 5-HT neurones within the raphe (Grossman, Mistlberger et al. 2000). Within the SCN, release of 5-HT has been shown through microdialysis to be increased in the mid-subjective day by three hours of either wheel running or sleep deprivation, while wheel running at

night results in reduction of 5-HT release (Dudley, DiNardo et al. 1998; Mistlberger, Antle et al. 2000). This pattern of release follows a similar pattern to the non-photic PRC of maximal phase advances during mid-subjective day and smaller delays during the night.

The effect on circadian rhythms of lesions of the raphe nuclei or chemical disruption of 5-HT cells has been investigated *in vivo* in a number of studies. Intraventricular administration of the 5HT-specific neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), demonstrates that loss of serotonergic innervation to the SCN results in changes in the entrained rhythm of wheel running in the hamster (Smale, Michels et al. 1990; Morin and Blanchard 1991). Lesions of the MR (but not the DR) produce patterns of locomotor activity similar to those resulting from the general intraventricular lesions (Meyer-Bernstein and Morin 1996). In general, it was found that the rhythms became irregular, had lower overall amplitude, an advanced phase angle of entrainment and the active period within a circadian cycle was generally lengthened. The conclusion was drawn that 5-HT plays a modulatory role that enhanced the stability of circadian rhythmicity.

Several experimental techniques that result in reduced levels of 5-HT within the SCN have been used to investigate the involvement of 5-HT in non-photic phase shifting. In hamsters, the drug triazolam is thought to phase shift the clock by inducing locomotor activity. This can be blocked by 5-HT depletion using the neurotoxins 5,7-DHT or p-chloroamphetamine (p-CPA) (Cutrera, Kalsbeek et al. 1994; Penev, Turek et al. 1995; Meyer-Bernstein, Blanchard et al. 1997). However, other studies have shown that phase shifts induced by running in a novel wheel are unaffected by 5,7-DHT lesions (Bobrzynska, Godfrey et al. 1996; Meyer-Bernstein, Blanchard et al. 1997). Administering a tryptophan-free diet to animals

results in a reduction of 5-HT release and when administered with three hours of sleep deprivation there was no effect on phase shifts in the mid-subjective day (Mistlberger, Antle et al. 2000). However, inhibition of 5-HT synthesis using p-CPA combined with ritanserin, a serotonin antagonist, does result in significant attenuation of phase shifts to sleep deprivation (Mistlberger, Antle et al. 2000). In mice, 5,7-DHT lesions eliminate entrainment to either restricted daily access to a home cage wheel or forced running in a treadmill (Edgar, Reid et al. 1997; Marchant, Watson et al. 1997). These results would indicate that 5-HT plays an important role in the regulation of circadian rhythms although its importance may vary between species.

Techniques which can enhance 5-HT levels or activity within the SCN have also been used to investigate the involvement of 5-HT in non-photic phase shifting. The 5-HT antagonist WAY100635 increases 5-HT levels by blocking the actions of presynaptic inhibitory autoreceptors. When given systemically, this drug was found to increase levels of 5-HT within the SCN by approximately 50% as measured by *in vivo* microdialysis, which is comparable to the levels produced by wheel running in the mid-subjective day (Antle, Marchant et al. 1998; Dudley, Dinardo et al. 1999). However, when administered at this time, WAY100635 neither induced phase shifts nor potentiated shifts induced by wheel running (Antle, Marchant et al. 1998). Administration of the 5-HT precursor tryptophan can significantly increase 5-HT levels within the SCN during mid-subjective day by as much as 200% (Glass, Selim et al. 1995). This did not induce significant phase shifts even when combined with the 5-HT reuptake inhibitor fluoxetine, nor did it potentiate phase shifts induced by running in novel wheels.

Electrical stimulation of either the MR or the DR results in release of 5-HT within the SCN that is comparable to the levels released subsequent to injection of either

WAY100635 or tryptophan (Dudley, Dinardo et al. 1999). Electrical stimulation of either the MR or the DR during mid-subjective day also results in small phase advance shifts (Meyer-Bernstein and Morin 1999). Since the pharmacological manipulations do not result in phase shifts even though they release the same amount of 5-HT, it has been suggested that the shifts due to electrical stimulation are not mediated by 5-HT release within the SCN. It was also found that the general 5-HT antagonist metergoline was able to block the release of 5-HT in the SCN after electrical stimulation of the DR but not the MR. Since the DR does not synapse on the SCN, it may be that these effects on 5-HT transmission are due to stimulation of 5-HT receptors on a direct pathway to the MR (Dudley, Dinardo et al. 1999; Meyer-Bernstein and Morin 1999).

If 5-HT is involved in the mediation of non-photic phase shifts, then the use of agonists for the various 5-HT receptors should mimic these shifts. Direct manipulation of circadian rhythms can be achieved in vitro using isolated brain slice preparations containing the SCN. Under these conditions, cells of the pacemaker generate circadian rhythms of spontaneous electrical activity that can be phase shifted by exogenous stimulation. Application in a perfusate, of both 5-HT and the non-specific 5-HT agonist quipazine, results in phase advances during the middle of the subjective day and in delays during the middle of subjective night (Prosser, Miller et al. 1990; Prosser, Dean et al. 1993). These phase shifts were blocked by the non-specific 5-HT antagonist metergoline, indicating the shifts were due to stimulation of 5-HT receptors. The phase shifts to quipazine were not blocked by the presence of TTX or high concentration of magnesium ions, indicating a direct postsynaptic action on cells of the SCN (Prosser, Heller et al. 1992). Another study that applied the 5-HT agonists 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) and 5-carboxamidotyptamine (5-CT) by microdrop directly onto the ventrolateral SCN obtained different results

(Medanic and Gillette 1992). Daytime advances were found to be much larger by this method while there was no effect of these drugs at night. These differences in results suggest that serotonergic modulation may be regionally specific within the SCN.

In vivo studies have produced conflicting results with regard to the phase shifting effects of 5-HT agonists. Systemic administration of 5-HT agonists during mid-subjective day to both rats (Edgar, Miller et al. 1993) and hamsters (Tominaga, Shibata et al. 1992; Cutrera, Ouarour et al. 1994; Rea, Glass et al. 1994; Mintz, Gillespie et al. 1997) has been found to induce phase advances. These shifts were found to be much smaller than those produced by three hour bouts of locomotor activity or sleep deprivation, but similar to those from one hour bouts of wheel running in hamsters with blocked access to running wheels (Wickland & Turek, 1991). Administration during the night has been found to produce either no phase shift (Tominaga, Shibata et al. 1992; Edgar, Miller et al. 1993; Rea, Glass et al. 1994) or small delays (Tominaga, Shibata et al. 1992). Other studies in the rat have shown that systemic administration of 5-HT agonists results in a PRC similar to that of photic stimulation. This consists of phase delays in early subjective night, advances in late subjective night but with no phase shifts during the subjective day (Kennaway, Rowe et al. 1996; Kennaway and Moyer 1998; Kohler, Kalkowski et al. 1999).

Localisation of the site of drug action is possible using *in vivo* intra-cerebroventricular injection. In rats, injections to the SCN of either quipazine or 8-OH-DPAT induces phase advances during the day, but had no effect at night (Edgar, Miller et al. 1993). One study found injection of 8-OH-DPAT during the day, into the SCN of hamsters, induced slight advances (Challet, Scarbrough et al. 1998). Another found no effect of injection to either the SCN or IGL of

hamsters, but did find advances when injected into the raphe (Mintz, Gillespie et al. 1997). Yet another study found that SCN injection during the night in hamsters, of either 5-CT or 8-OH-DPAT resulted in no phase shift (Weber, Gannon et al. 1998).

These inconsistencies between experimental results may be due to species differences (Kohler, Kalkowski et al. 1999) or even differences between strains of the same species (Kohler and Wollnik 1998). Alternatively, it is known that different 5-HT receptor subtypes are expressed throughout the circadian system and their response to exogenous stimulation varies, depending upon the drug used and route of administration.

1.7 Serotonin Receptor Subtypes

Several 5-HT receptor subtypes have been differentiated by their pharmacological profiles and from the signal transduction mechanisms to which they are linked. All belong to the G protein-coupled superfamily with the exception of the 5-HT_3 subtype, which is a ligand-gated ion channel. G protein-coupled receptors (GPCR) are membrane bound proteins consisting of a single subunit with seven transmembrane regions. They function by coupling to members of a family of membrane bound proteins, the G proteins. Binding of agonist to GPCR results in activation of a G protein, regulated by the binding of a guanyl nucleotide, and leads to stimulation of one or more membrane effectors that mediate the cellular response. The effectors can be ion channels or alternatively enzymes that participate in synthesis or degradation of second messengers that can influence a variety of cellular processes. These cellular effects have latencies measured in hundreds of milliseconds making them suitable for slow but sustained signalling or for modulation of fast

neurotransmission. Within the circadian system, anatomical evidence exists for the presence of several of the 5-HT receptor subtypes.

Receptor binding studies using radio-ligands selective for different 5-HT receptors, indicate that the 5-HT_{1B} receptor is present in the SCN of the rat (Prosser, Dean et al. 1993) and its gene expression has been confirmed by *in situ* hybridisation (Roca, Weaver et al. 1993). Combined electron microscope-immunohistochemistry examination of the SCN of the mouse indicates the presence of 5-HT_{1B} receptors associated with the plasma membrane of retinal terminals (Pickard, Smith et al. 1999) although not at the synaptic terminal zone (Belenky and Pickard 2001). High levels of mRNA for 5-HT_{2C} receptors was found in the SCN by *in situ* hybridisation (Roca, Weaver et al. 1993) and subsequent immunohistochemical analysis found immunoreactivity for both 5-HT_{2C} receptors and to a lesser extent for 5-HT_{2A} (Moyer and Kennaway 1999). In the hamster, immunohistochemical techniques have shown that the $5HT_{5A}$ receptor is present in all four main components of the circadian system, the SCN, IGL, MR and DR (Duncan, Jennes et al. 2000) with very high levels in the SCN (Oliver, Kinsey et al. 2000). In neurones of the raphe nuclei, $5HT_{5A}$ receptor-immunoreactivity is found co-localised with 5HT-immunoreactivity, suggesting a functional role as presynaptic autoreceptor for regulation of 5HT neuronal activity (Duncan, Jennes et al. 2000). Further analysis of this receptor is hindered by the lack of ligands selective for this particular subtype.

Two other 5-HT receptors that are thought to be present within the circadian system are the 5-HT_{1A} and 5-HT_7 receptors. The recently cloned 5-HT_7 receptor shares some pharmacological overlap with the 5-HT_{1A} receptor, including high affinity for 8-OH-DPAT and 5-CT and there is some dispute as to which of these receptors mediate the effects of serotonin within the SCN. An early

autoradiographic study found high levels of [^{3}H]8-OH-DPAT binding in the SCN of the rat and this was attributed to 5-HT_{1A} receptors (Prosser, Dean et al. 1993). Other binding studies in the hamster have used [^{3}H]8-OH-DPAT in conjunction with pindolol and ritanserin, antagonists for the 5-HT_{1A} and 5-HT_7 receptors respectively. These studies concluded that 20 to 50% of receptor binding in the SCN is due to the 5-HT_7 receptor, with the remainder being attributed to the 5-HT_{1A} receptor (Duncan, Short et al. 1999; Rea and Pickard 2000). In the rat, use of [^{3}H]5-CT as the radioligand failed to find binding for 5-HT_7 receptors in the SCN (Gustafson, Durkin et al. 1996) although another study used this method successfully to identify binding in other areas of the rat brain (Mengod, Vilaro et al. 1996). *In situ* hybridisation in the rat found very little sign of gene expression for the 5-HT_{1A} receptor in the SCN (Roca, Weaver et al. 1993) while some evidence for the expression of the 5-HT_7 receptor has been found. One study using oligonucleotide probes to rat 5-HT_7 receptor mRNA failed to detect a hybridisation signal (Gustafson, Durkin et al. 1996), another using ribonucleotide probes to different 5-HT_7 receptor isoforms did find low to moderate levels of 5-HT_7 mRNA (Heidmann, Szot et al. 1998) while the most recent detected high levels (Neumaier, Sexton et al. 2001). One study has detected 5-HT_7 mRNA in the hamster (Rea and Pickard 2000). Immunohistochemical staining in the rat failed to detect immunoreactivity for the 5-HT_7 receptor in the SCN (Moyer and Kennaway 1999), while another laboratory detected prominent staining co-localised with c-Fos immunoreactivity (Neumaier, Sexton et al. 2001). In the mouse, 5-HT_7 immunoreactivity is found throughout the rostrocaudal extent of the SCN (Belenky and Pickard 2001).

Several *in vitro* studies have implicated 5-HT_7 receptors, in addition to or instead of 5-HT_{1A} receptors, as mediators of 5-HT effects on SCN neurones. Advances in spontaneous electrical activity in the rat brain slice induced by 8-OH-DPAT can

be blocked by ritanserin but not by pindolol (Lovenberg, Baron et al. 1993). Inhibition of $GABA_A$ responses by 5-HT and 8-OH-DPAT within the rat SCN was similarly antagonised by ritanserin, but not by pindolol (Kawahara, Saito et al. 1994). Spontaneous neuronal firing rates in the hamster have been studied *in vivo* and it was found that inhibition of firing by 5-HT, 5-CT and 8-OH-DPAT could be blocked by ritanserin but not by cyanopindolol (Ying and Rusak 1997).

1.8 Serotonergic Interactions with light

5-HT is thought also to act as a modulator of photic phase shifting in the SCN. *In vitro* application of the $5\text{-}HT_{1B}$ receptor agonist 1-[3-(trifluoromethyl)phenyl]-piperazine (TFMPP), to the hamster hypothalamic slice results in a reduction in the amplitude of optic nerve evoked excitatory post synaptic potentials (Pickard, Smith et al. 1999). When systemically administered *in vivo*, TFMPP attenuates the phase shifting effects of light as well as blocking the induction of Fos protein (an alternative marker for light stimulation), both in the hamster (Pickard, Weber et al. 1996) and in the mouse (Pickard and Rea 1997). These results along with those from the anatomical studies would indicate that these receptors function as modulators of glutamate release from RHT terminals.

The $5\text{-}HT_{1A}$ receptor is present in the circadian system both as presynaptic autoreceptor and postsynaptic heteroreceptor (Moriya, Yoshinobu et al. 1998). Systemic injection of the $5\text{-}HT_{1A}$ receptor antagonist WAY100635 results in augmentation of phase delays to light in early subjective night while having no effects on phase advances late at night (Smart and Biello 2001).

There is some evidence suggesting that, in the rat, 5-HT may modulate light induced phase shifts through action at $5\text{-}HT_{2C}$ and/or $5\text{-}HT_{2A}$ receptors. Systemic

injection of the 5-$HT_{2A/2C}$ receptor agonist (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride resulted in delays in the rhythms of activity, melatonin secretion, and temperature (Kennaway and Moyer 1998). This agonist also induced expression of the immediate early gene c-fos in a manner similar to light (Moyer and Kennaway 1999).

In vitro, the $5HT_{1A/7}$ agonist 8-OH-DPAT reduces the amplitude of optic nerve-evoked excitatory post-synaptic potentials in SCN neurones from the mouse via a $5HT_7$ receptor mediated mechanism (Smith, Sollars et al. 2001). The effects of 5-HT_7 antagonists on the expression of c-Fos expression has been examined in the rat. One study using metergoline and ritanserin failed to prevent the photic induction of c-Fos in the SCN (Kennaway and Moyer 1998). Another showed that c-Fos expression induced by 5-CT and 8-OH-DPAT at CT22 without light, could be blocked by ritanserin in the presence of pindolol (Mullins, Gianutsos et al. 1999).

1.9 The Role of GABA in the Clock

γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter found extensively throughout the central nervous system. It is synthesised in a single step from L-glutamate, catalysed by the enzyme glutamate decarboxylase (GAD). A large number of SCN neurones contain GABA as well as GAD (Card and Moore 1984; Van den Pol 1986; Moore and Speh 1993) and 48% of synaptic boutons in the SCN are GABAergic (Decavel and Van den Pol 1990). GABA has also been found co-localised in the SCN with other neurochemicals, such as vasoactive intestinal peptide in the ventrolateral SCN (Francois-Bellan, Kachidian et al. 1990) and arginine vasopressin in the dorsomedial SCN (Moore and Speh 1993). GABAergic interneurones comprise a dense network within the SCN (Moore and Speh 1993; Van den Pol 1993), including reciprocal innervation between each of

the two nuclei (Buijs, Hou et al. 1994). GABAergic neurones comprise part of one afferent, the GHT, where they are co-localised with NPY (Van den Pol 1986; Card and Moore 1991; Moore and Speh 1993).

Several lines of evidence indicate that the activity of the GABAergic system is under circadian control. GABA content in the SCN varies over the course of the circadian cycle showing a peak at CT16 that can be entrained by the daily photoperiod and persists under conditions of constant darkness (Aguilar-Roblero, Verduzco-Carbajal et al. 1993). GABA turnover rate also shows a peak at CT16 in several non-SCN areas of the central nervous system of Syrian hamsters (Kanterewicz, Rosenstein et al. 1995). $GABA_A$ receptor sites in the brain show circadian rhythms both in number and affinity (Wirz-Justice 1987) with a nocturnal peak in high affinity binding sites (Acuña-Castroviejo, Rosenstein et al. 1986) which can be abolished by ablation of the SCN (Kafka, Marangos et al. 1985). Finally, a mid to late night peak of radioactive ^{3}H-GABA release was observed in hamster preoptic-medial basal hypothalamic explants (Yannielli, Kanterewicz et al. 1996).

A role has been suggested for GABA in the synchronisation of circadian patterns of electrical activity between individual cells of the SCN. Electrophysiological recordings from SCN cells in dissociated cell culture show that individual cells display diverse patterns in spontaneous electrical activity (Welsh, Logothetis et al. 1995). Application of GABA to dissociated SCN cells induces phase-dependant phase shifts in the firing rhythm of individual cells and daily application of GABA induces synchronisation of rhythms between the cells (Liu and Reppert 2000). Cellular communication via gap junctions has been shown in cell culture through dye coupling and this can be reduced by application the

$GABA_A$ receptor agonist 3-hydroxy-5-aminoethylisoxazole hydrobromide (muscimol) to the culture medium (Shinohara, Hiruma et al. 2000).

1.10 GABA Receptor Subtypes

The effects of GABA are mediated though several receptor subtypes. $GABA_A$ and $GABA_C$ receptors are both ionotropic receptors associated with Cl^- channels. $GABA_A$ receptors have been identified within the SCN using autoradiographic (Francois-Bellan, Segu et al. 1989) as well as electrophysiological techniques (Liou, Shibata et al. 1990; Mason, Biello et al. 1991), while $GABA_C$ receptors were not found in the SCN using northern analysis techniques (O'Hara, Andretic et al. 1995). The third receptor subtype, the $GABA_B$ receptor, is a G-protein coupled metabotropic receptor (Bormann 2000). This is known to function presynaptically both as autoreceptor and heteroreceptor, as well as postsynaptically. Presynaptically, the $GABA_B$ receptor inhibits the release of neurotransmitter through suppression of Ca^{2+} channels, while postsynaptically it functions to inhibit neuronal firing, mediated by coupling to inwardly rectifying K^+ channels. It is also recognised for inducing the inhibition of adenylyl cyclase activity, a cellular second messenger (Kerr and Ong 1995).

High affinity of the agonist 4-amino-3-[4-chlorophenyl]-butanoic acid (baclofen) for $GABA_B$ receptors has been recognised for some time. However, there is differential selective antagonism with the currently available antagonist ligands, indicating the presence of different $GABA_B$ receptor subtypes (Jones, Tamm et al. 2000). Two genes are known that express two different receptors, $GABA_{B1}$ and $GABA_{B2}$ (Kaupmann, Huggel et al. 1997; Jones, Borowsky et al. 1998; White, Wise et al. 1998). When expressed individually in mammalian cell lines or *Xenopus* oocytes, these were found to have similar antagonist binding properties to

native $GABA_B$ receptors but have a much lower affinity for agonists. When exposed to agonist, neither is able to produce a cellular response, such as the opening of potassium channels (Kaupmann, Huggel et al. 1997). When expressed alone $GABA_{B1}$ subunits were also found to be dispersed within the cell cytoplasm and did not associate at the cell surface (Couve, Moss et al. 2000).

Evidence that colocalisation of both receptor subtypes is required to produce a functional $GABA_B$ receptor comes from several sources. The distribution patterns for the expression of mRNAs of each subtype show substantial areas of overlap within the brain (Jones, Borowsky et al. 1998; Durkin, Gunwaldsen et al. 1999; Lu, Ghasemzadeh et al. 1999). Co-expression studies illustrate that the pharmacology of the combined protein unit is equivalent to that of native receptors and produces the expected responses from agonist or antagonist stimulation. Immunoprecipitation studies show the subunits associate as heterodimers and that they are concentrated on the plasma membrane (Jones, Borowsky et al. 1998; Kaupmann, Malitschek et al. 1998; White, Wise et al. 1998). The functional importance of each of the individual subunits in this heterodimer remains to be discovered but it appears that there must be both a $GABA_{B1}$ and $GABA_{B2}$ subunit present to produce a fully functional $GABA_B$ receptor. Several splice variants of each of the two $GABA_B$ genes are known (Couve, Moss et al. 2000). Different combinations of these variants may lead to pharmacological and functional differences due to substituted residues for ligand binding and G protein coupling (Bischoff, Leonhard et al. 1999; Charles, Evans et al. 2001). This may explain the apparent differences in baclofen effects between pre- and postsynaptic sites. In cerebellum for example, splice variant $GABA_{B1a}$ localisation is in agreement with a presynaptic role while that for another splice variant, $GABA_{B1b}$ favours a postsynaptic function (Kaupmann, Schuler et al. 1998; Fritschy, Meskenaite et al. 1999).

1.11 $GABA_B$ within the Circadian System

In situ hybridisation for both $GABA_{B1}$ (Lu, Ghasemzadeh et al. 1999) and $GABA_{B2}$ (Durkin, Gunwaldsen et al. 1999) mRNAs shows high expression of both subtypes within the SCN and the raphe nuclei of the rat. Immunohistochemical analysis of the $GABA_{B1}$ protein confirms this distribution while showing, by comparison with the distribution of GAD immunoreactivity, that this subunit is not expressed on GABAergic neurones. Some evidence was found however for expression of this receptor on GABAergic interneurones in some areas of the rat brain (Margeta-Mitrovic, Mitrovic et al. 1999).

Several lines of evidence indicate that the stimulation of $GABA_B$ receptors results in modulation of the phase shifting effects of light. *In vitro,* baclofen strongly attenuates optic nerve evoked SCN field potentials (Shibata, Liou et al. 1986; Jiang, Allen et al. 1995) as well as the frequency of spontaneous miniature excitatory post-synaptic potentials (Jiang, Allen et al. 1995). The $GABA_B$ antagonist 3-Amino-2-(4-chlorophenyl) propylphosphonic acid (phaclofen) dose dependently increases SCN field potential amplitudes *in vitro* (Gannon, Cato et al. 1995). Systemically administered baclofen has been reported to attenuate both light-induced phase advances and delays of the running activity rhythm in hamsters (Ralph and Menaker 1989), as well as light-induced expression of c-fos (Colwell, Kaufman et al. 1993). Microinjection of baclofen directly to the SCN reduces both the phase delaying and advancing effects of light (Gillespie, Mintz et al. 1997) as well as reducing the amount of c-fos immunoreactivity within the SCN (Gillespie, Van Der Beek et al. 1999). The $GABA_B$ antagonist 3-amino-propyl (diethoxymethyl)phosphinic acid (CGP 35348) increases the phase delaying effect of light at CT14, while having no effect on phase advances to light at

CT19 (Gillespie, Mintz et al. 1997), or on induction of c-fos protein at either time point (Gillespie, Van Der Beek et al. 1999).

Stimulation of GABAergic transmission via $GABA_B$ receptors can also have a direct effect on the clock. *In vitro* work indicates that application of baclofen to the SCN slice results in phase advances of the rhythm of electrical activity of SCN cells at zeitgeber time (ZT) 6 and small delays at ZT22 (Biggs and Prosser 1998), where ZT12 is the time of lights off in the animal colony under a light-dark cycle. Administration of TTX blocks both these phase advances and delays suggesting that the $GABA_B$ receptors mediating these phase shifts must be located presynaptically at least one neurone away from the pacemaker cells (Bergeron, Danielson et al. 1999). Application by microinjection of baclofen directly to the SCN also phase advances the clock at CT6 (Smith, Turek et al. 1990). The ability of $GABA_B$ receptors to phase advance the clock during the day, indicates that they may be involved in the mediation of non-photic input to the SCN, since this is a time when non-photic stimuli also phase advance the pacemaker.

1.12 Interactions between $GABA_B$ and Serotonin

There is evidence for interaction between GABAergic and serotonergic transmissions within the SCN. Double-labelling techniques using [^{3}H]5-HT uptake radioautography combined with immunohistochemistry for GAD found a close interconnection between these two afferent systems (Bosler 1989). No axonal varicosities showed double labelling for both transmitter types, indicating separate neuronal populations and 41% of the 5-HT terminals were directly apposed, on a post-synaptic target, to at least one GAD-immunoreactive profile. It is also known that administration of $GABA_B$ but not $GABA_A$ agonists

increases the release of serotonin within the SCN *in vitro* (Francois-Bellan, Hery et al. 1987) as well as *in vivo* (Francois-Bellan, Hery et al. 1988).

$GABA_B$ receptors are also known to be present within the raphe nuclei (Lu, Ghasemzadeh et al. 1999; Margeta-Mitrovic, Mitrovic et al. 1999). One study utilised *in situ* hybridisation to examine coexpression of mRNAs for GAD, the $GABA_{B1}$ subunit and the 5-HT transporter (Abellan, Adell et al. 2000). It was found that most 5-HT neurones in both nuclei expressed the $GABA_{B1}$ transcript while very few cells contained the transcript for GAD. This is confirmed in both nuclei by double labelling immunohistochemistry for $GABA_B$ receptors and 5-HT (Varga, Sik et al. 2002). This study found colocalisation in virtually all serotonergic cells. Electron-microscopic examination also revealed that staining for $GABA_B$ receptors appeared on proximal dendrites and cell bodies, never on afferent axons.

The effects of $GABA_B$ receptor stimulation in the raphe nuclei have been investigated using microdialysis and electrophysiological techniques (Tao, Ma et al. 1996; Abellan, Adell et al. 2000; Abellan, Jolas et al. 2000). It was found that both presynaptic and postsynaptic effects of $GABA_B$ receptor stimulation could be found within the raphe. Higher concentrations of baclofen are required to stimulate the postsynaptic effects and $GABA_B$ antagonists fail to fully block the effects of baclofen. This would indicate that the different effects of baclofen might be mediated by different populations of $GABA_B$ receptor subtypes at pre and postsynaptic sites. Microdialysis also showed that there is a time of day effect of baclofen administration to the DR. Local administration of the drug results in a reduction of released 5-HT during lights-out conditions (Tao, Ma et al. 1996) while having the opposite effect during lights-on conditions (Abellan, Jolas

et al. 2000). Local injection of baclofen to the MR results in an increase of released 5-HT (Abellan, Adell et al. 2000)

1.13 Molecular Components of the Circadian Clock

Details of how circadian rhythms are generated in mammals at the molecular level come mainly from studies in mice. The mechanism for generating rhythms over a 24-hour period depends upon interwoven positive and negative transcriptional/translational feedback loops of specific clock genes within cells of the SCN (Figure 1.3). These genes include the period (*Per1*, *Per2* and *Per3*), cryptochrome (*Cry1* and *Cry2*), *Clock* and *Bmal1* (brain and muscle Arnt-like) genes. In animals with targeted disruption of either *Per1* or *Per2* but not *Per3* gene, locomotor activity rhythms are severely disrupted (Bae, Jin et al. 2001), and mice deficient in both *Per1* and *Per2* do not express circadian rhythms (Zheng, Albrecht et al. 2001). The *Per01* mutant of *Drosophila*, which is arrhythmic due to a lack of endogenous PER, can have rhythmicity restored by the introduction of mammalian *Per1* or *Per2* genes (Shigeyoshi, Meyer-Bernstein et al. 2002). A key role is also played by *Cry1*, *Cry2* and *Bmal1* since *Cry1/Cry2* double knockout mice (Okamura, Miyake et al. 1999; Van der Horst, Muijtjens et al. 1999) and *Bmal1* knockout mice (Bunger, Wilsbacher et al. 2000) show the immediate loss of a behavioural rhythm in constant darkness.

A positive loop is formed by heterodimers of the CLOCK and BMAL1 proteins which bind to E-boxes located in the regulatory region of *Per* and *Cry* genes and initiate their transcription (Gekakis, Staknis et al. 1998; Hogenesch, Gu et al. 1998; Yamaguchi, Mitsui et al. 2000; Lee, Etchegaray et al. 2001; Travnickova-Bendova, Cermakian et al. 2002; Etchegaray, Lee et al. 2003). Accumulation of *Per* mRNA peaks in the SCN during the subjective day, while translation to PER1 and PER2

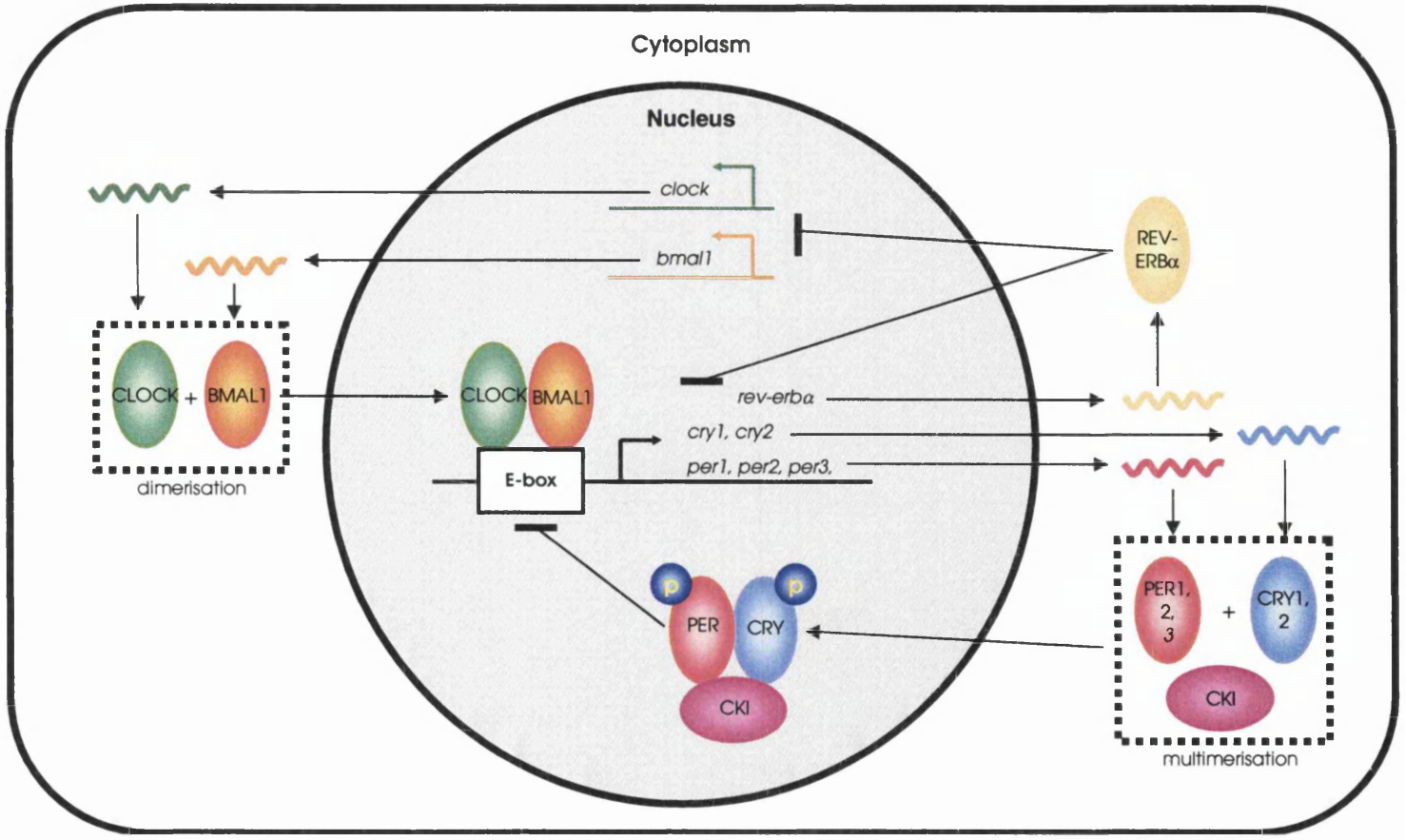


Figure 1.3. Molecular components of the core circadian clock. Transcription of *clock* and *bmal1*genes leads to build up of mRNA in the cytoplasm of SCN cells. On translation, the protein products CLOCK and BMAL1 dimerise and translocate to the nucleus. Here they stimulate transcription of *per, cry* and *rev-erbα* genes by interacting with an E-box on the promoter of these genes. Subsequent to translation in the cytoplasm, different combinations of the PER and CRY proteins form complexes that are phosphorylated by casein kinase. These complexes enter the nucleus where they inhibit their own production through interaction with the CLOCK/BMAL1 heterodimers. The transcription factor REV-ERBα enters the nucleus where it inhibits transcription of the *clock, bmal1* and *cry1* genes.

proteins occurs in the cytoplasm where concentrations peak during the middle of the night (Albrecht, Sun et al. 1997; Shearman, Zylka et al. 1997; Tei, Okamura et al. 1997; Zylka, Shearman et al. 1998). A negative loop is then formed by the translocation of these proteins to the nucleus where they form stable complexes with CRY1 or CRY2 and suppress the transcription of *Per1* and *Per2* genes by binding to the CLOCK/BMAL1 heterodimer (Kume, Zylka et al. 1999). (Figure 1.3).

The above core feedback loop is very stable and accurately maintains a 24 h rhythm. There are other complementary molecular loops assisting this core feedback loop at the gene-transcription level. Another feedback loop is formed when the CLOCK/BMAL1 heterodimer activates a gene encoding the orphan nuclear receptor REV-ERBα. This is a transcription factor that functions to repress the *Bmal1*, *Clock* and *Cry1* genes (Onishi, Yamaguchi et al. 2002; Preitner, Damiola et al. 2002; Ueda, Chen et al. 2002; Etchegaray, Lee et al. 2003). Two other transcription factors thought to be involved in the molecular clockwork are DEC1 and DEC2. These have been shown to be expressed in the SCN and inhibit the activity of the CLOCK/BMAL1 dimers either through association with them or through competition with the E-box elements of the target genes (Honma, Kawamoto et al. 2002).

Post-translational mechanisms are involved in regulating levels of clock proteins both spatially and temporally through phosphorylation, degradation and nuclear translocation. The casein kinases (CKI) δ and ε have been shown to phosphorylate PER1, PER2, CRY1, CRY2 and BMAL1 (Keesler, Camacho et al. 2000; Lowrey, Shimomura et al. 2000; Camacho, Cilio et al. 2001; Eide, Vielhaber et al. 2002). Phosphorylation by CKI serves both to allow transport of the clock gene proteins into the nucleus as well as allowing their eventual degradation. The PER2 protein usually shuttles between the cytoplasm and the nucleus and is

easily degraded by ubiquitination and the proteasome pathway (Yagita, Tamanini et al. 2002). It is also known that the ubiquitination of PER proteins is inhibited in the presence of CRY proteins and the PER proteins appear to be more fragile if they do not dimerize with CRY proteins. It has also been recently demonstrated that the CRY protein, which is a strong suppressor of *Per1* transcription, can be ubiquitinated when PER proteins are absent (Yagita, Tamanini et al. 2002). The decrease of PER in the nucleus due to proteasome-dependent degradation causes destabilisation of CRY, and the decrease in CRY will lead to the re-starting of *Per1* and *Per2* gene transcription.

1.14 Entrainment

Entrainment of the molecular clock to both photic and non-photic stimuli involves transcriptional mechanisms that alter *Per* gene expression in the SCN. Light pulses lead to increased levels of *Per1* and *Per2* in the SCN (Albrecht, Sun et al. 1997; Shearman, Zylka et al. 1997; Shigeyoshi, Taguchi et al. 1997) while intracerebroventricular injections of antisense oligodeoxynucleotides to *Per1* inhibit light-induced phase delays in mice (Akiyama, Kouzu et al. 1999). Mice with a mutated *Per1* gene exhibit altered light induced phase advances, while *Per2* mutant mice display impaired light-induced phase delays (Albrecht, Zheng et al. 2001). Entrainment by non-photic cues has been shown to be as a result of reduction of *Per* expression within cells of the SCN. Both NPY and novel wheel running activity can decrease *Per1* and *Per2* mRNA levels in vivo (Maywood, Mrosovsky et al. 1999; Maywood, Okamura et al. 2002) and in vitro (Fukuhara, Brewer et al. 2001). Systemic injections of 8-OH-DPAT and short-acting benzodiazepines suppress *Per1* and *Per2* levels in the SCN of hamsters (Horikawa, Yokota et al. 2000; Yokota, Horikawa et al. 2000).

Outwith the laboratory environment it is unlikely that photic and non-photic stimuli occur in isolation from each other and it has been shown that there is interaction between the two. Light pulses given shortly after a non-photic stimulus reduce the phase advances that would otherwise have been produced. This occurs when the non-photic stimulus is running in a novel wheel (Mrosovsky 1991), injection of NPY (Biello 1995), or injection of 5-HT agonists (Penev, Zee et al. 1997). The opposite has also been found with phase shifts to light being attenuated by activity (Ralph and Mrosovsky 1992), novel wheel running (Mistlberger and Antle 1998), injection of NPY (Weber and Rea 1997) or injection of 5-HT (Weber and Rea 1997). It has also been shown that the reduction in levels of PER expression produced by novel wheel running can be attenuated by a light pulse given immediately following the non-photic pulse (Maywood and Mrosovsky 2001).

1.15 Output pathways of the SCN

The core circadian clock regulates the temporal variation of numerous behavioural and physiological processes. The oscillations of gene expression in the molecular clock must be translated into a form that will impart rhythmicity to these other processes. Rats whose SCN neuronal firing has been inhibited with the sodium channel blocker TTX display arrhythmic behaviour patterns and rhythmicity is restored at the previous circadian phase on removal of TTX treatment (Schwartz, Gross et al. 1987; Schwartz 1991). A similar result is achieved using individual SCN neurons treated with TTX *in vitro* (Welsh, Logothetis et al. 1995) suggesting that the rhythmic electrical activity of SCN cells is an output of the clock and not a component. The neuropeptide arginine vasopressin (AVP) has been shown to augment the magnitude of the electrical activity rhythm in the SCN through a receptor-mediated excitation of SCN neurons (Mihai,

Coculescu et al. 1994). Expression of AVP is directly regulated by CLOCK/BMAL1 heterodimers acting on an E-box within its promoter region and its transcription is negatively regulated by the same molecules that negatively regulate the core clockwork (Jin, Shearman et al. 1999; Kume, Zylka et al. 1999). Mice with mutations in the *Per2* gene show loss of the rhythmic expression of the AVP gene in the SCN indicating circadian control of AVP gene expression within the SCN (Albrecht, Zheng et al. 2001).

Another mechanism by which the clock controls downstream events is to use the protein products of clock-controlled genes that are regulated by the core feedback loops. One such protein is D-element binding protein (DBP) that is expressed with a circadian period (Wuarin and Schibler 1990) and which has been shown to be regulated by CLOCK/BMAL1 heterodimers (Ripperger, Shearman et al. 2000). DBP is a transcription factor that can bind to the promoter of the *Per1* gene and positively influence *Per1* transcription (Yamaguchi, Mitsui et al. 2000) indicating the clock can react to its own target genes and thus sense the physiological state of the organism. A number of other genes are controlled by DBP including *Cyp2a4* and *Cyp2a5* the protein products of which are involved in the metabolism of the sex hormones testosterone and estradiol (Lavery and Schibler 1993; Lavery, Lopez-Molina et al. 1999). A mutation in the *Dbp* gene shortens circadian period and affects circadian sleep consolidation and rhythmic electroencephalogram activity (Franken, Lopez-Molina et al. 2000).

It has been shown that diffusible, secreted molecules from the SCN can control rodent activity rhythms. Hamsters carrying the tau mutation, which shortens the circadian period, were given bilateral lesions of the SCN. Subsequent to this, a wild-type SCN was implanted within a semi-permeable polymetric capsule in the

third ventricle of the lesioned animals. The capsule prevents neural outgrowths from the implanted SCN while still allowing diffusion of humoral signals. It was found after several weeks that the locomotor rhythm displayed by the implanted animals was that of the wild-type phenotype (Silver, LeSauter et al. 1996). The peptide, transforming growth factor α (TGFα), is expressed in a circadian fashion in the SCN of hamster and completely blocks wheel-running activity when infused into the third ventricle. The receptor for TGFα is the epidermal growth factor receptor and this is expressed in the subparaventricular zone, a major target region of the SCN. Animals with mutations in this receptor display increased wheel running activity during the day and an imprecise onset of nocturnal activity (Kramer, Yang et al. 2001). Another candidate clock output signal is prokineticin 2 (PK2) that is expressed in the SCN in a circadian fashion. Its transcription is activated by CLOCK/BMAL1 via an E-box in the PK2 promoter and inhibited by the PER and CRY proteins. The circadian pattern of PK2 expression is shifted in response to light resetting of the clock and wheel running activity is suppressed when the protein is infused into the third ventricle during the night. The receptor for PK2 is present in the SCN and in many target regions of SCN efferents (Cheng, Bullock et al. 2002).

1.16 3,4-methylenedioxymethamphetamine (MDMA)

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA), also known as ecstasy is known to have both acute and chronic toxic effects in animals and humans. First synthesised and patented in 1914 for use as an appetite suppressant it has become increasingly popular as a recreational drug. It is used primarily by young people in large dance settings, or 'raves' but is also known to be used in smaller social gatherings. Users report that after about 20 minutes the drug begins to produce euphoria, feelings of intimacy towards

others, heightened arousal, increased self-confidence and increased sensory sensitivity (Morgan 2000). Acute adverse physiological symptoms reported include tachycardia, bruxism, trismus, pupilary dilation, gait instability, nausea, suppressed appetite and hyperthermia (Morgan 2000). Users report subsequent to 24 to 48 hours of these effects, there follows a period characterised by muscle aches, fatigue, depression, irritability, difficulty in concentrating and headache (Morgan 2000). The most adverse acute effects of MDMA appear to be related to hyperthermia. This is typically accompanied by a number of clinical problems including seizures, intravascular coagulation, rhabdomyolysis, renal and liver impairment (Gowing, Henry-Edwards et al. 2002). The toxicity of the drug can be enhanced under certain conditions such as those present at a rave. A combination of the direct effects of MDMA, high ambient temperature, sustained physical activity and inadequate fluid replacement all impair temperature regulation and can even lead to death (Gowing, Henry-Edwards et al. 2002).

MDMA has been shown to cause long-term neurotoxicity in animals. Studies indicate that the drug is toxic to serotonergic systems and to a lesser extent dopaminergic systems (Burgess, O'Donohoe et al. 2000). A single dose of MDMA (40 mg/kg) in rats results in decreased levels of both 5-HT and 5-HIAA within three days and which lasts for up to eight weeks (Commins, Vosmer et al. 1987). After multiple doses (5-20 mg/kg, twice daily over 4 days), the decreases last from 6 to 12 months (Battaglia, Yeh et al. 1988). These reductions vary regionally within the brain with severe reductions in the neocortex, striatum and hippocampus, while smaller decreases were found in the brainstem and hypothalamus (Stone, Stahl et al. 1986; Battaglia, Yeh et al. 1987). The number of 5-HT uptake transporter sites was found to be reduced in rat brain after administration of MDMA from studies using both autoradiography (Battaglia, Yeh et al. 1987) and synaptosomal

preparations (Commins, Vosmer et al. 1987; Schmidt 1987). These results are consistent with the degeneration of presynaptic terminal areas.

Immunohistochemical techniques demonstrate that MDMA administration results in loss of fine serotonergic axons throughout the forebrain of the rat (O'Hearn, Battaglia et al. 1988), with the areas of fibre reduction being similar to those for the loss of transporter sites (Battaglia, Yeh et al. 1987). Fibres which suffer the loss of terminal areas are of the fine fibre type that originate in the DR while the thick beaded axons of the MR are spared from damage (Molliver, Berger et al. 1990). The cell bodies within the raphe remain immunoreactive indicating a lack of degeneration (O'Hearn, Battaglia et al. 1988). The sparing of cell bodies from damage suggests the potential for regeneration of serotonergic projections and this does occur in both rodents and non-human primates, but the pattern of regeneration is generally abnormal (Fischer, Hatzidimitriou et al. 1995).

The effects of MDMA on circadian timing have been addressed in two previous studies. One *in vitro* experiment found that pre-treatment with MDMA resulted in a reduced ability of cells of the SCN to phase shift to the 5-HT agonist 8-OH-DPAT (Biello and Dafters 2001). *In vivo* the ability of the SCN to phase shift to both light and 8-OH-DPAT was shown to be significantly reduced subsequent to MDMA administration (Colbron, Jones et al. 2002).

1.17 The Study

It is known that 5-HT or its agonists can phase advance the circadian pacemaker at ZT6 *in vitro* (Prosser, Miller et al. 1990). Likewise, stimulation of $GABA_B$ receptors during the day can phase shift the pacemaker both *in vitro* (Biggs and Prosser 1998) and *in vivo* (Smith, Turek et al. 1990). It is also known that the stimulation of

$GABA_B$ receptors during the subjective day increases the release of newly synthesised 5-HT within the SCN area (Francois-Bellan, Hery et al. 1987). It may be that the baclofen induced phase shifts are brought about via the increased release of 5-HT within the SCN.

The following experiments were carried out to investigate whether the *in vivo* stimulation of $GABA_B$ receptors at CT6 results in a phase advance shift in the circadian pacemaker as seen *in vitro*. The involvement of 5-HT in the baclofen induced phase shifts was investigated by lesioning of serotonergic input to the SCN using the neurotoxin MDMA. Finally, the anatomical effects of MDMA treatment were investigated in both the SCN and the raphe nuclei using immunohistochemical techniques.

2 Materials and Methods

2.1 General Procedures

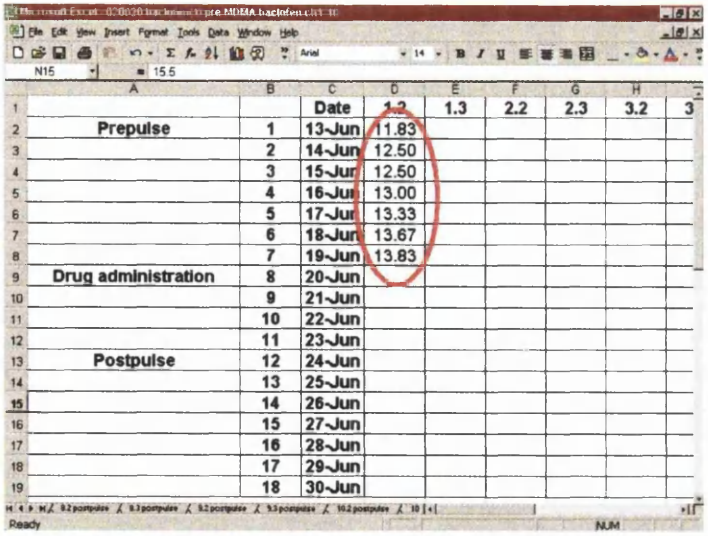
All work was carried out in accordance with local codes of practice and within the framework of the Animals (Scientific Procedures) Act of 1986. Adult male Syrian hamsters (*Mesocricetus auratus*), 100-130g, (Harlan Sprague-Dawley, Oxon, UK). Animals were housed individually in polypropylene cages (13 x 9 x 8 cm) with food and water available *ad libitum*. Cages were fitted with a 16 cm running wheel attached to a micro switch which was monitored continuously by Dataquest Pro-Data software (Data Sciences Inc., Roseville, MN, USA), with wheel running activity gathered in 10-minute bins. Animals were initially acclimatised for a minimum period of 5 days at 22 ± 2°C and light:dark cycle of 12:12 (LD 12:12). Subsequent to this, constant environmental conditions (DD) were established by switching off the animal room lights. Alternatively, the animals were transferred to a ventilated, light-tight chamber, measuring 174 x 87 x 56 cm with the lights turned off. Both room and cabinet were illuminated by dim red light (14-18 lux) to enable daily husbandry practices to be carried out. This level and wavelength of illumination has been shown to be ineffective in resetting the phase of the endogenous clock of hamsters (Biello 1995). All animals were left in DD for a minimum of 7 days to establish free-running conditions within the clock before any drug treatments were given.

2.2 Behavioural Analysis

2.2.1 Activity Onset

Activity onset (CT12) was taken as the first period of activity closest to the time of lights out from the most recent LD cycle. For the purposes of statistical analysis, CT12 was defined as the first 10-minute bin of greater than 50 wheel revolutions followed by a subsequent bin of greater than 50 revolutions within a 30-minute period. Activity onset for the day of drug treatment was calculated by forward regression of the line of best fit for the activity onsets of the 7 days prior to the treatment. Any animals with less than five data points for the regression calculation were excluded from the analysis.

To calculate the regression line, the times for CT12 were taken from the raw data files generated by the Dataquest software. Each of these was converted to a decimal figure by expressing minutes past the hour as a fraction of 60 and was then entered onto a Microsoft Excel template (Figure 2.1). Excel then automatically calculated the line of best fit and produced a graph for these data points (Figure 2.2) along with the equation of the line in the form of $y = mx+c$. The time of activity onset for the day of drug administration was calculated by substituting 8 (for the 8th day) for 'x' into the equation of the regression line and converting back from the decimal. The time of drug administration (CT6) was 6 hours before the predicted onset of activity.



			Date	1.2	1.3	2.2	2.3	3.2	3
	Prepulse	1	13-Jun	11.83					
		2	14-Jun	12.50					
		3	15-Jun	12.50					
		4	16-Jun	13.00					
		5	17-Jun	13.33					
		6	18-Jun	13.67					
		7	19-Jun	13.83					
	Drug administration	8	20-Jun						
		9	21-Jun						
		10	22-Jun						
		11	23-Jun						
	Postpulse	12	24-Jun						
		13	25-Jun						
		14	26-Jun						
		15	27-Jun						
		16	28-Jun						
		17	29-Jun						
		18	30-Jun						

Figure 2.1 Forward regression of activity onset to day of drug treatment

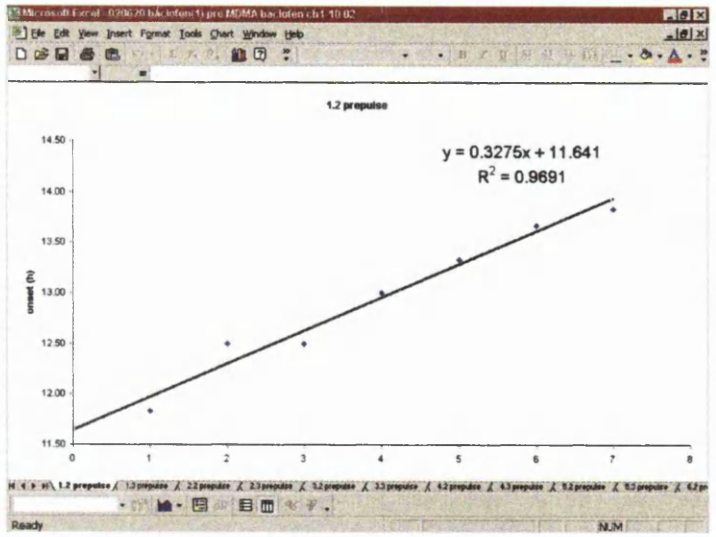
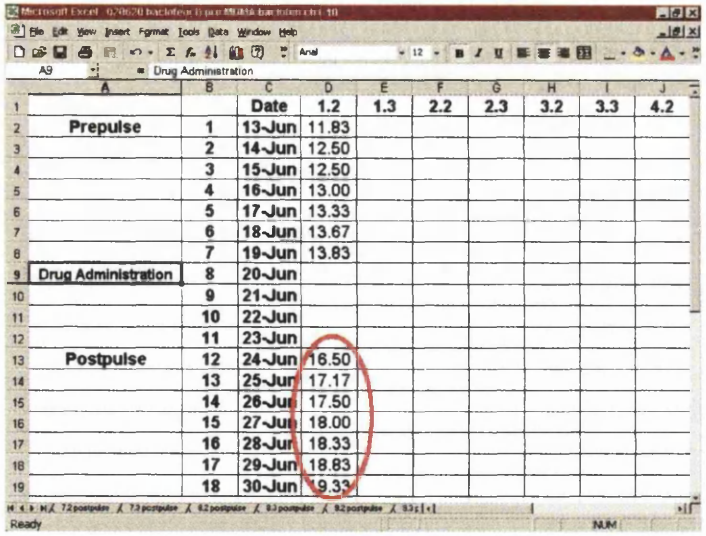
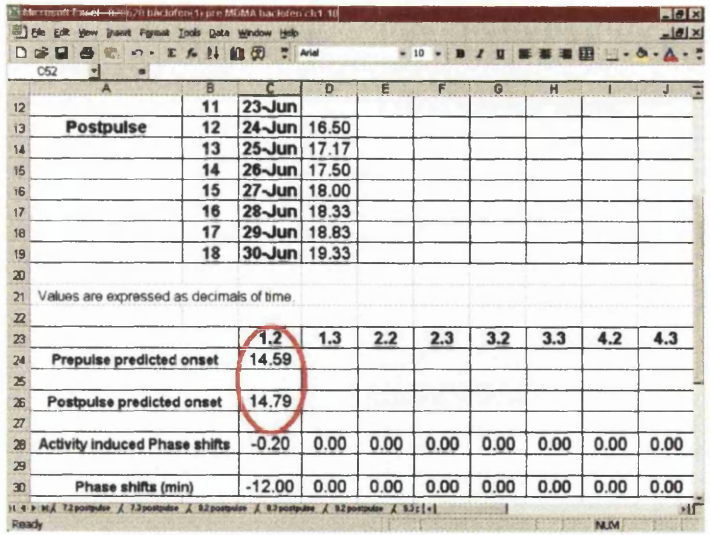


Figure 2.2 Regression line through activity onsets prior to drug treatment



			Date	1.2	1.3	2.2	2.3	3.2	3.3	4.2
	Prepulse	1	13-Jun	11.83						
		2	14-Jun	12.50						
		3	15-Jun	12.50						
		4	16-Jun	13.00						
		5	17-Jun	13.33						
		6	18-Jun	13.67						
		7	19-Jun	13.83						
	Drug Administration	8	20-Jun							
		9	21-Jun							
		10	22-Jun							
		11	23-Jun							
	Postpulse	12	24-Jun	16.50						
		13	25-Jun	17.17						
		14	26-Jun	17.50						
		15	27-Jun	18.00						
		16	28-Jun	18.33						
		17	29-Jun	18.83						
		18	30-Jun	19.33						

Figure 2.3 Backward regression of activity onset to day of drug treatment



				1.2	1.3	2.2	2.3	3.2	3.3	4.2	4.3
		11	23-Jun								
	Postpulse	12	24-Jun	16.50							
		13	25-Jun	17.17							
		14	26-Jun	17.50							
		15	27-Jun	18.00							
		16	28-Jun	18.33							
		17	29-Jun	18.83							
		18	30-Jun	19.33							
	Values are expressed as decimals of time.										
	Prepulse predicted onset			14.59							
	Postpulse predicted onset			14.79							
	Activity induced Phase shifts			-0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Phase shifts (min)			-12.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Figure 2.4 Calculation of phase shifts

2.2.2 Phase Shifts in Activity Onset

Post-drug treatment activity onsets were calculated by backward regression of a line of best fit for the activity onsets of 7 days post-treatment. The initial 3 days post-treatment were excluded from the regression calculations to allow for transient effects. Any animals with less than five data points for the regression calculation were excluded from the analysis. The method of calculation was by a similar method to that used for the calculation of activity onset. The values for CT12 were taken, converted to decimals and entered onto the Excel template (Figure 2.3) to produce a similar graph to that described above. This time however, the value of 10 was entered for 'x' into the calculated equation of best fit. The resulting value for activity onset was entered into the template. Phase shifts were calculated as the difference between pre-treatment and post-treatment activity onsets (Figure 2.4).

2.2.3 Changes in τ

The values of τ were taken directly from the regression lines produced in Excel for calculating activity onset. The value of τ is simply the gradient of these lines i.e. the 'm' in $y = mx + c$. Changes in τ subsequent to administration of baclofen were compared between animals before and after MDMA treatment.

2.2.4 Changes in Activity Offset

Activity offset was defined as the time point at which the 98th percentile of the total activity for the circadian day is reached. This has been empirically determined to be the least variable indicator of activity offset (Meyer-Bernstein and Morin 1996). Total daily activity was taken to be all wheel running activity

that fell between the times of activity onset for two consecutive days. The time of the data bin in which 98% of this figure fell, was then taken as the time of activity offset. The time of activity offset for all seven days prior to the day of drug treatment were then determined. These times were then converted to decimal and entered into the Excel template as described above. The template then calculated the time for activity offset on the day of drug treatment by forward regression. The times for activity offset for seven days post-drug treatment were then calculated by backwards regression. The three days immediately following drug treatment were excluded from this calculation to allow for transient shifts. Any animals for which there were less than five data points for either of the regression calculations were excluded from the calculations. Differences in activity offset were taken as the difference between the results of the two regression calculations.

2.2.5 Alpha Activity Period

The alpha activity period was taken as the time, in minutes, between the times of activity onset and activity offset. The mean figure for each animal was used in analysis.

2.2.6 Total Activity

Total daily activity was taken to be all wheel running activity that fell between the times of activity onset for two consecutive days. The mean figure for each animal was used in analysis.

2.3 Drug treatments

After 7 days in DD, each animal (n=16) received an intraperitoneal (i.p.) injection of R(+)-baclofen hydrochloride (10 mg/kg in 0.9% saline; Sigma, Poole, UK) at CT 6. Animals remained in DD for 10 days before receiving an i.p. injection of 0.9% saline at CT6. Following a further period of 10 days in DD, all animals were transferred into a cycle of LD 14:10. Subsequent to 7 days under the LD cycle, all animals received a subcutaneous injection of 3,4-methylenedioxymethamphetamine (MDMA; kindly donated by the National Institute of Health, USA) in 0.9% saline, from 3 to 4 hours prior to lights off. This drug was administered in increasing doses of 10 mg/kg, 15 mg/kg and 20 mg/kg over three consecutive days. Animals remained in LD 14:10 for a further 12 days before being returned to DD. After 7 days in DD a second i.p. injection of baclofen was administered at CT6 followed by 10 days in DD and a final injection of saline at CT6. All injections with the exception of those of MDMA were carried out under dim red light (14-18 lux).

2.4 Immunohistochemistry

2.4.1 Monoclonal Antibody to Serotonin

The antibody used in this study was purchased from Accurate Chemical and Scientific Corporation, Westbury, New York, U.S.A. (Product number YMC1019; Rat anti-serotonin; Cell line YC5/45). This monoclonal antibody was secreted by a hybridoma formed by the fusion of a Y3-Ag-1.2.3 rat myeloma cell with a spleen cell from a male COB Wistar rat which had been immunised against a conjugate of 5-HT and bovine serum albumin (Cuello and Milstein 1981). The hybridoma secretes specific heavy chains and both specific and non-specific light chains

(Milstein, Wright et al. 1983). The antibody belongs to the rat immunoglobulin class IgG (Milstein, Wright et al. 1983).

The antibody specifically recognises the formaldehyde conjugate of 5-HT. Without paraformaldehyde treatment, it cross-reacts in liquid phase with dopamine and tryptamine in haemagglutination tests. It does not react with catecholamine-containing neurones in formaldehyde fixed preparations. The antibody reacts with 5-HT in neurones and recognises 5-HTergic sites in fixed tissue sections (Consolazione, Milstein et al. 1981). The antibody has previously been used successfully in anatomical studies of the circadian system (Morin and Blanchard 1991; Morin, Blanchard et al. 1992; Meyer-Bernstein, Blanchard et al. 1997; Meyer-Bernstein and Morin 1998).

2.4.2 Source of Tissue

Due to time constraints brought about by a change in direction of the project, the analysis of the effects of MDMA on the SCN was taken from animals used in a previous study (Colbron, Jones et al. 2002). This looked at effects of the serotonin agonist 8-OH-DPAT and light both pre- and post-MDMA treatment. There are no known residual effects from this agonist and the MDMA treatments used were identical to those used in the current experiment. All immunohistochemical procedures were carried out by this author. All animals (n=14) were prepared for immunohistochemistry within 4 weeks of the final treatment as detailed below. All tissue for anatomical analysis of the raphe came from the animals used in the current experiment (DR, n=12; MR, n=13). These were processed for immunohistochemistry, as detailed below, between 10 and 14 days after the final injection of saline.

2.4.3 Protocol

All animals were deeply anaesthetised by i.p. injection of sodium pentobarbitone (Dunnwood, Aberdeen, U.K.) and perfused transcardially with physiological saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Each brain was removed and post-fixed overnight in 4% paraformaldehyde before being cryoprotected through a series of sucrose solutions in 0.1M phosphate buffer, ending with 30% sucrose. Brains were then frozen and sections of 30 µm were cut in the coronal plane and collected in 0.01M phosphate buffered saline (PBS).

A combination of retrograde, anterograde and dual immunofluorescence techniques has shown that serotonergic innervation of the SCN originates in the MR, while that of the IGL originates in the DR (Meyer-Bernstein and Morin 1996). Injection of retrograde tracers to the SCN showed an equal distribution of serotonergic cells throughout the rostrocaudal extent of the MR. A similar distribution throughout the DR was found when retrograde tracers were injected into the IGL. Application of anterograde tracers to the MR demonstrates that there is a substantial serotonergic fibre plexus concentrated in the medial and ventral regions of the SCN. Therefore all sections through the SCN and every third section cut through the aforementioned areas of the raphe were collected. A stereotaxic atlas of the hamster brain (Morin and Wood 2001) was used as a guide to locate the relevant stereotaxic coordinates of the brain areas during cutting on the cryostat.

See appendix 1 for a detailed protocol of the methods used in this study and appendix 2 for the theory behind the chemistry of immunohistochemistry.

2.5 Anatomical Analysis

2.5.1 SCN

Photomicrographs of stained tissue were obtained on 400 ASA Kodak camera film using a single lens reflex camera (K-1000, Pentax) attached to a microscope (Galen II, Leica). These were digitised using a flatbed scanner (Hewlett Packard, Scanjet 4470c) to greyscale 75 dpi and image size of 640x480 pixels. ROD values for the SCN were obtained using 'Scion Image' software calibrated to a greyscale density step tablet (Q-13, Kodak). For each animal brain, 4 to 6 sections were selected from equivalent areas of the SCN using the shape of surrounding structures as guide. The optical density of a control section from each brain, which received no primary antibody, was subtracted from the OD value of each section of the same brain. The mean figure for OD of each brain was used in the statistical analysis. See appendix 3 for the theory of densitometry.

2.5.2 Raphe

Photomicrographs of the raphe were obtained and digitised as detailed above. The images were opened in Adobe Photoshop and a grid of 16x8 was used as a guide in cell counts. Cell counts were made in quadrats from left to right and from top to bottom. Nuclei that crossed a grid line were included in the count of the first quadrat in which they appeared. Nuclei that overlapped and were difficult to distinguish as separate cells were counted as one cell.

2.6 Statistical Analysis

Repeated measures ANOVA were used to analyse phase shifts in activity onset and activity offset, changes in the alpha activity period and changes in total activity. Changes in τ were analysed using t-tests. Differences in anatomical data between MDMA treated animals and untreated animals were calculated using a Mann-Whitney U-test. The statistical software used in all cases was Graphpad Instat® software.

3 Results

3.1 Behavioural Analysis

Four animals were excluded from the analysis of phase shift in activity onset for not meeting the minimum criteria set. There was a significant difference in the size of phase shifts in activity onset between baclofen and saline before treatment with MDMA (Fig 3.1 & 3.2) but not after ($F(3,11) = 7.865$, $p<0.001$). There was also a significant difference between pre-MDMA baclofen and post-MDMA baclofen (Fig 3.2 & 3.3).

For half of the animals, the size of τ appeared to change after baclofen injection, which could distort the size of mean phase shifts (Fig 3.4). However when analysed, the differences were insignificant both before (Fig 3.5) and after (Fig 3.6) MDMA.

Four animals were excluded from the analysis of phase shift in activity offset for not meeting the minimum criteria set. Analysis of activity offset showed no significance between any of the drug treatment protocols (Fig 3.7). No significant differences were found between alpha activity period and total activity before and after baclofen administration. The data were pooled for the final analysis of MDMA treatment but still produced non-significant results (Fig 3.8 & 3.9).

Baclofen had a strong sedative effect on all treated animals on the day of injection, which delayed the onset of activity by several hours (Fig 3.10). The delay between the predicted onset for the day of injection and the actual onset was calculated for each animal. A t-test was used to determine whether MDMA

treatment changed the magnitude of the sedation induced by baclofen. No significance was found (Fig 3.11).

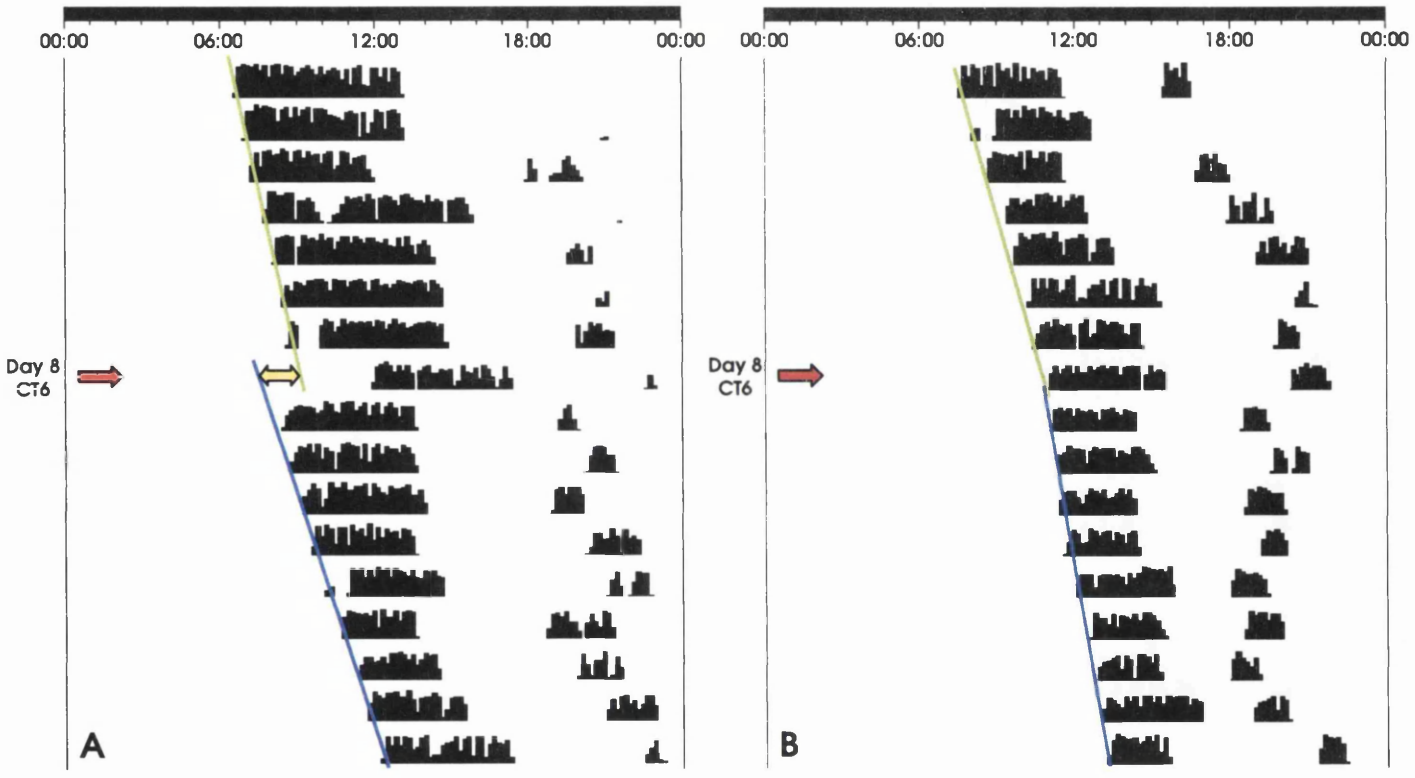


Figure 3.1 Baclofen phase advances activity rhythms when compared to controls. Representative actograms showing changes in activity levels over time. The scale at the top represents the time of day. Individual vertical black bars represent activity levels per 10-minute bin of recording with successive bins plotted side by side. Data for activity on subsequent days are plotted from top to bottom. The red block arrow indicates the day of drug administration. **A.** Animal treated with baclofen at CT6, showing a phase advance of 78.6 minutes in activity onset. The lines are approximate representations of the regression lines through points of activity onset, green for forward regression and blue for backward. The yellow double block arrow indicates the phase advance in activity onset. **B.** Saline injection at CT6 for the same animal.

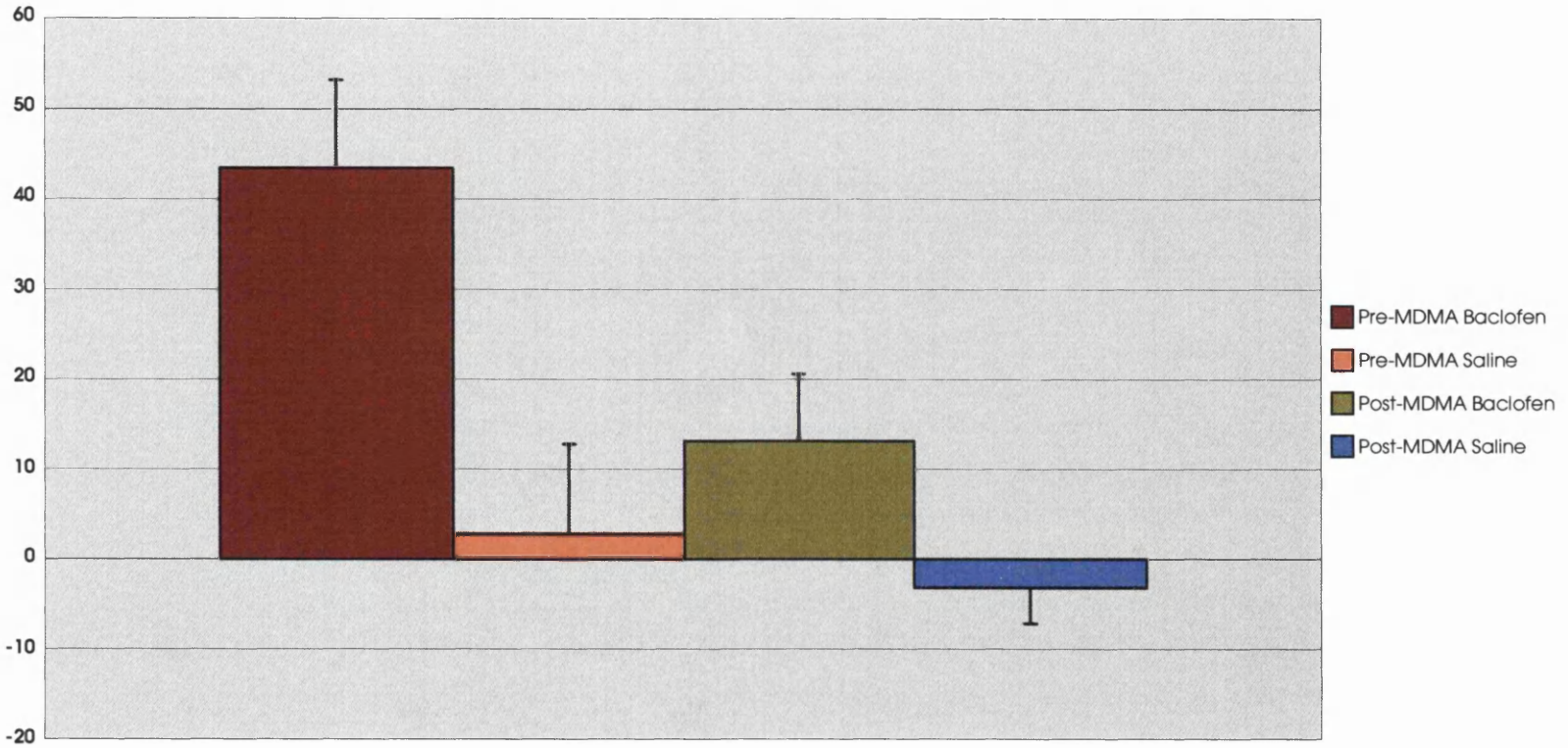


Figure 3.2 Mean Phase Shifts in Activity Onset. A significant difference was found between baclofen and saline prior to treatment with MDMA. The difference between pre-MDMA baclofen and post-MDMA baclofen was also found to be significant, $F(3,11) = 7.865$, $p<0.001$. Bars represent mean ± SEM.

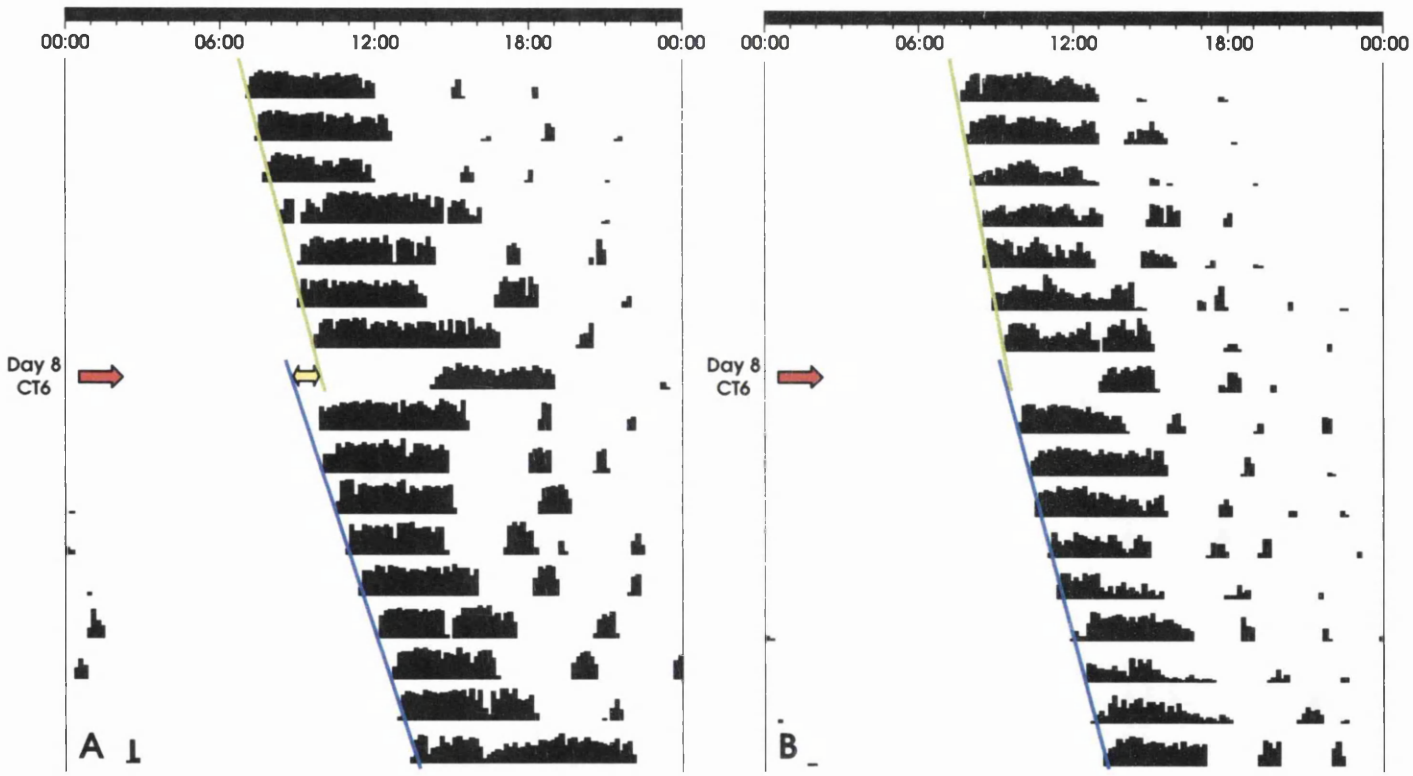


Figure 3.3 Phase advance shifts to baclofen are blocked by treatment with MDMA. A. Actogram for an animal showing a phase advance to baclofen of 73.8 minutes at CT6. **B**. Actogram for the same animal showing that the phase advance to baclofen has been blocked after MDMA treatment.

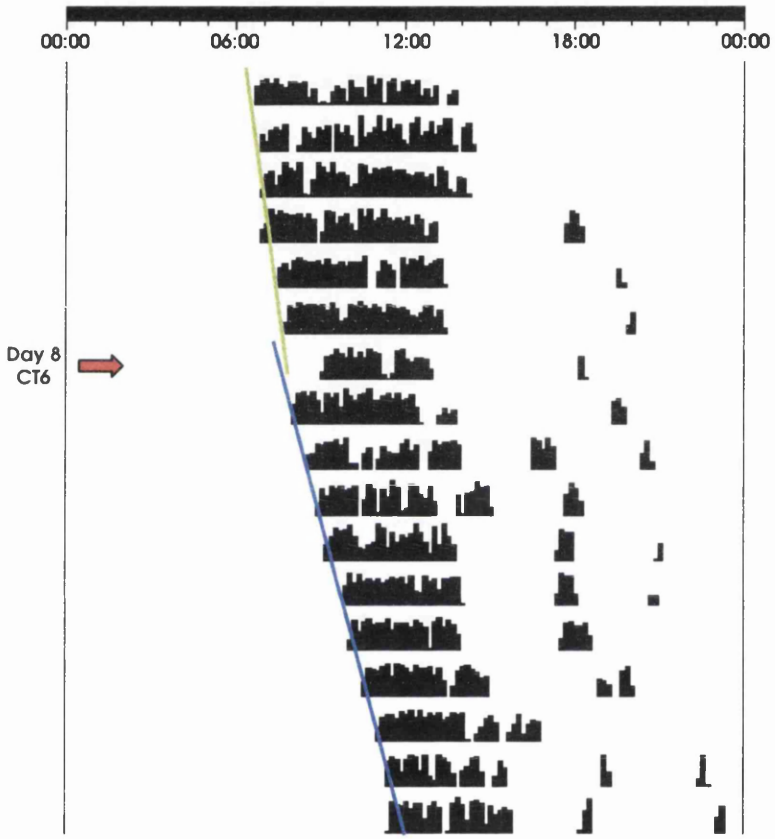


Figure 3.4 Changes in τ subsequent to baclofen treatment. Representative actogram illustrating changes in τ after administration of baclofen. The gradients of the two lines appear different, although this was found not to be significant when analysed. The red block arrow represents the day of drug administration.

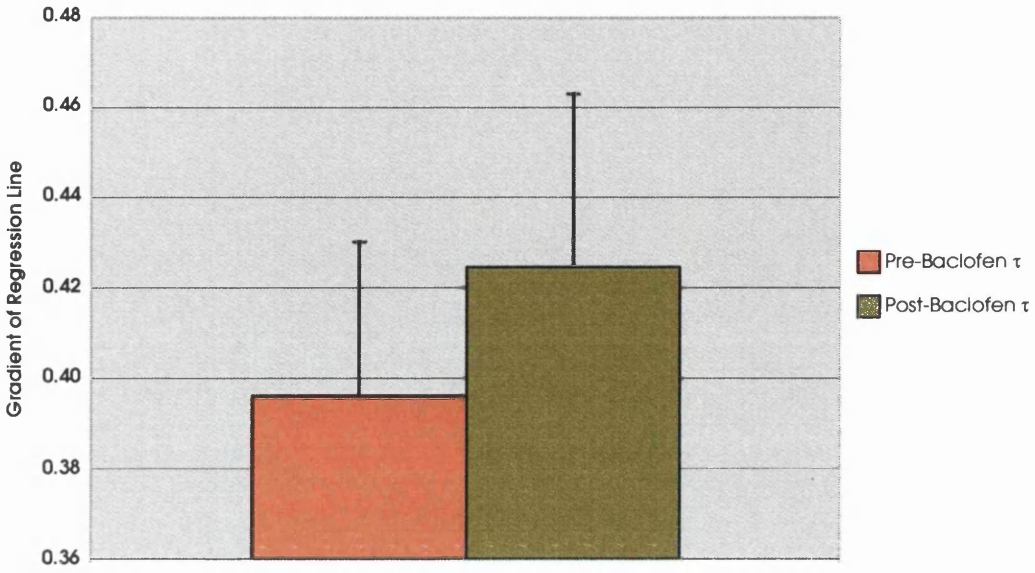


Figure 3.5 Effect of baclofen on τ prior to treatment with MDMA. There was no effect of baclofen on τ when analysed by paired t-test.

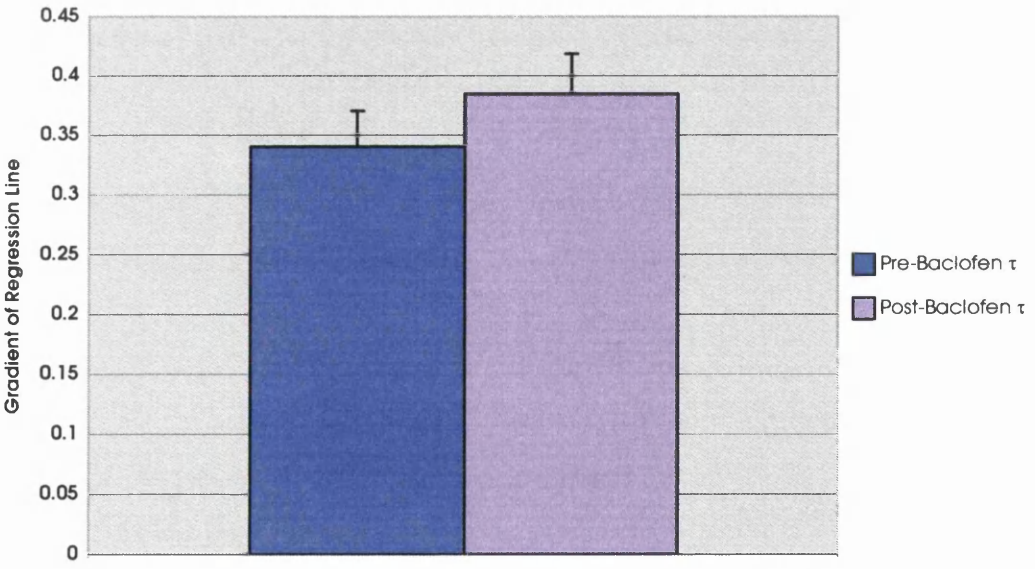


Figure 3.6 Effect of baclofen on τ subsequent to treatment with MDMA. Again there was no difference when analysed by paired t-test.

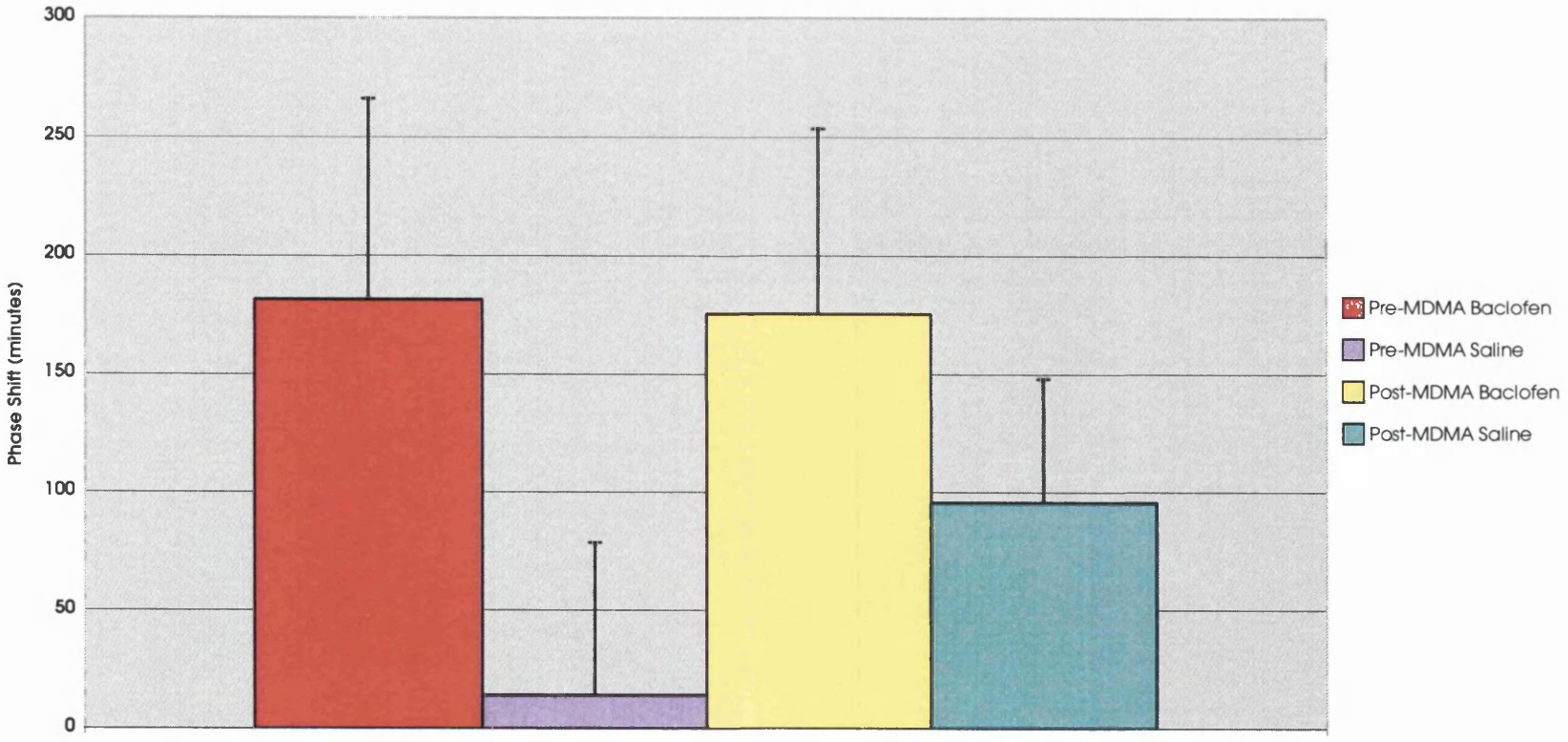


Figure 3.7 Phase shifts in activity offset. Analysis of phase shifts in activity offset revealed no significant differences between any of the treatments. Bars represent mean ± SEM.

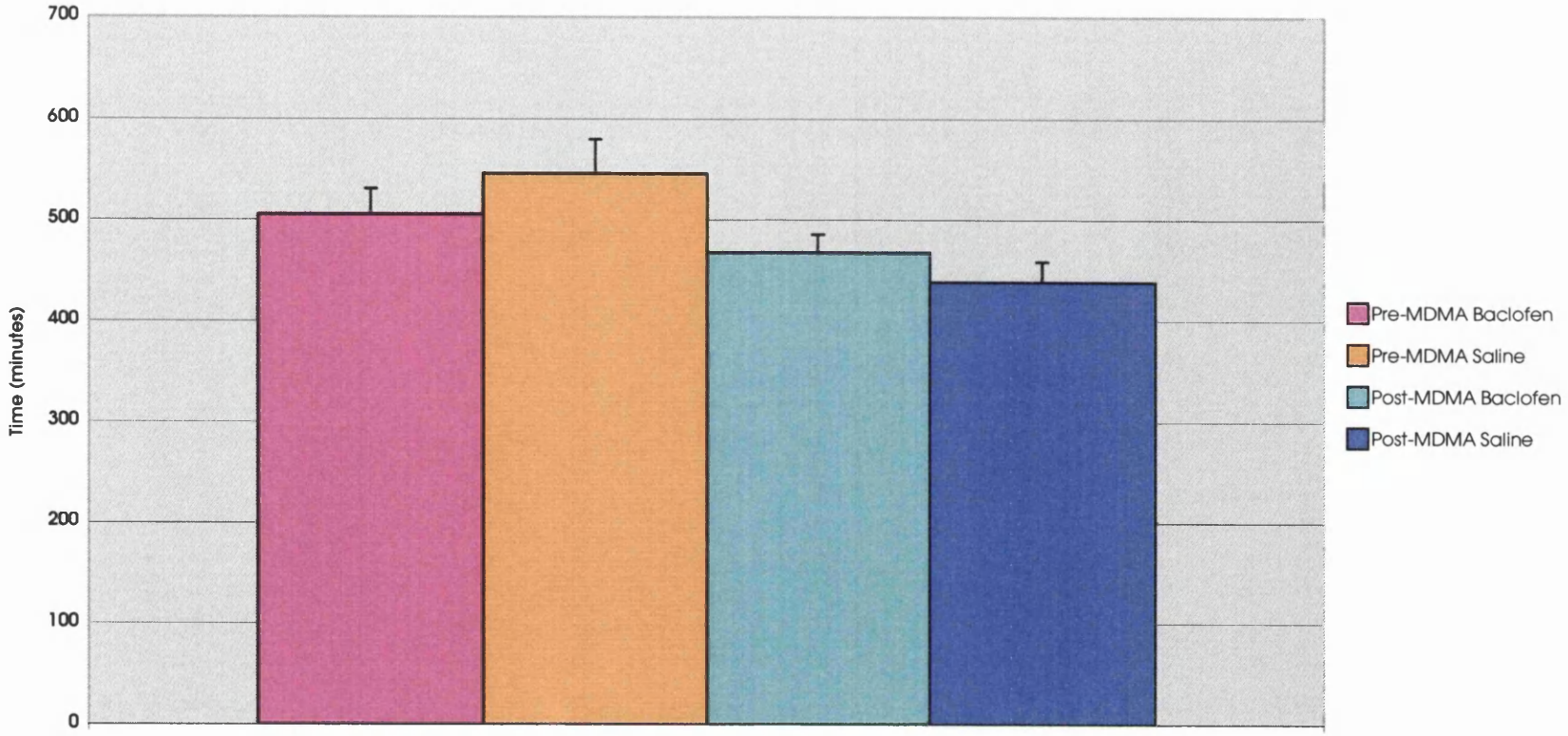


Figure 3.8 Alpha activity period. Analysis of the alpha activity period, the time difference between activity onset and offset, revealed no difference between treatments.

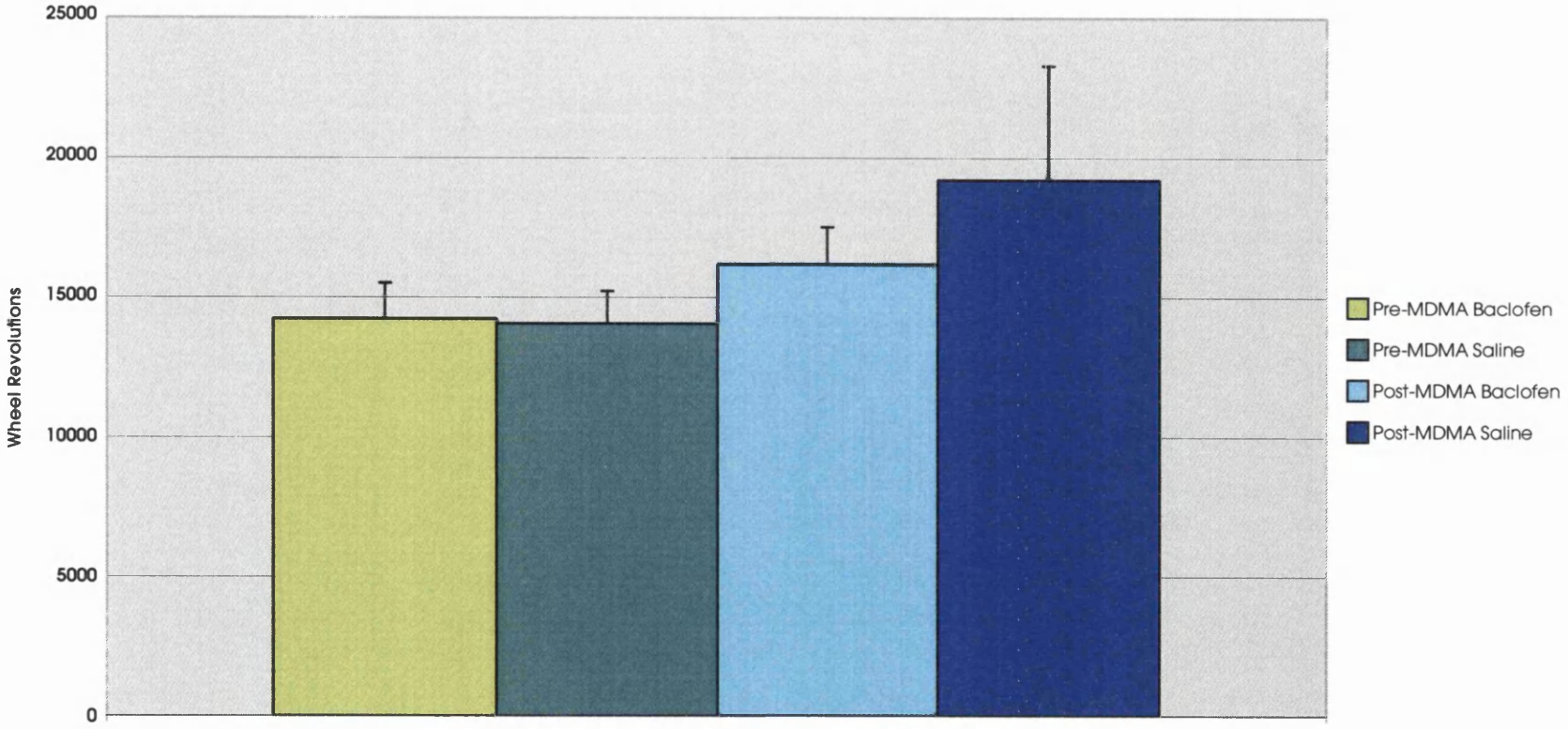


Figure 3.9 Total wheel running activity. No significant differences were found between treatments.

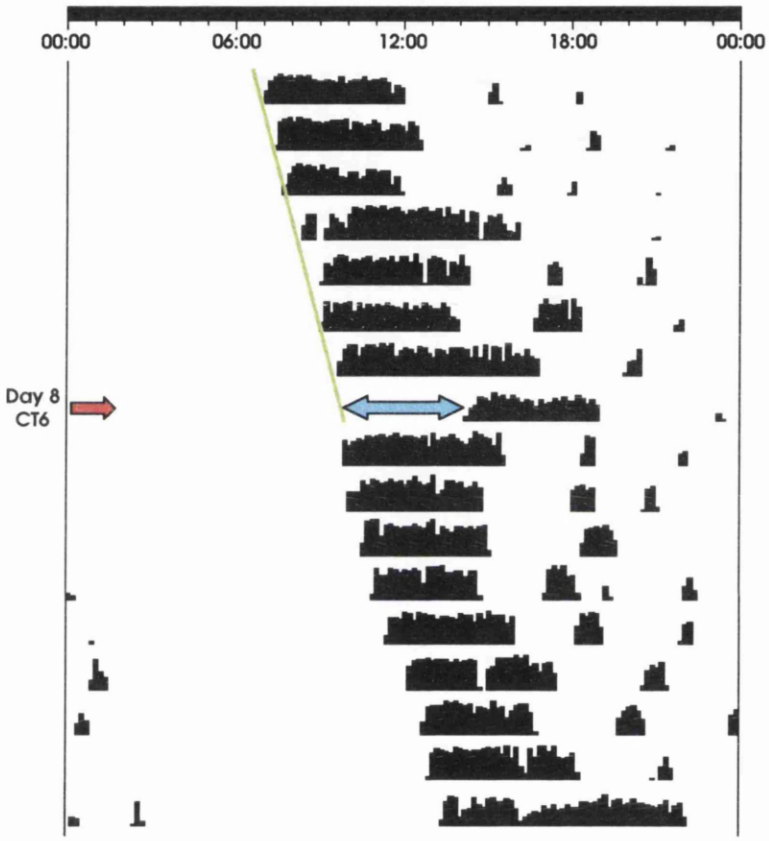


Figure 3.10 Actogram illustrating the sedative effect of baclofen. An actogram illustrating a delay in the activity onset in an animal treated with baclofen, prior to MDMA administration. All animals showed this effect subsequent to baclofen treatment. The green line approximates the forward regression line through the activity onsets of the seven days prior to drug treatment. The red block arrow indicates the day of drug administration. The blue block arrow shows the 256-minute delay in activity onset subsequent to the drug administration.

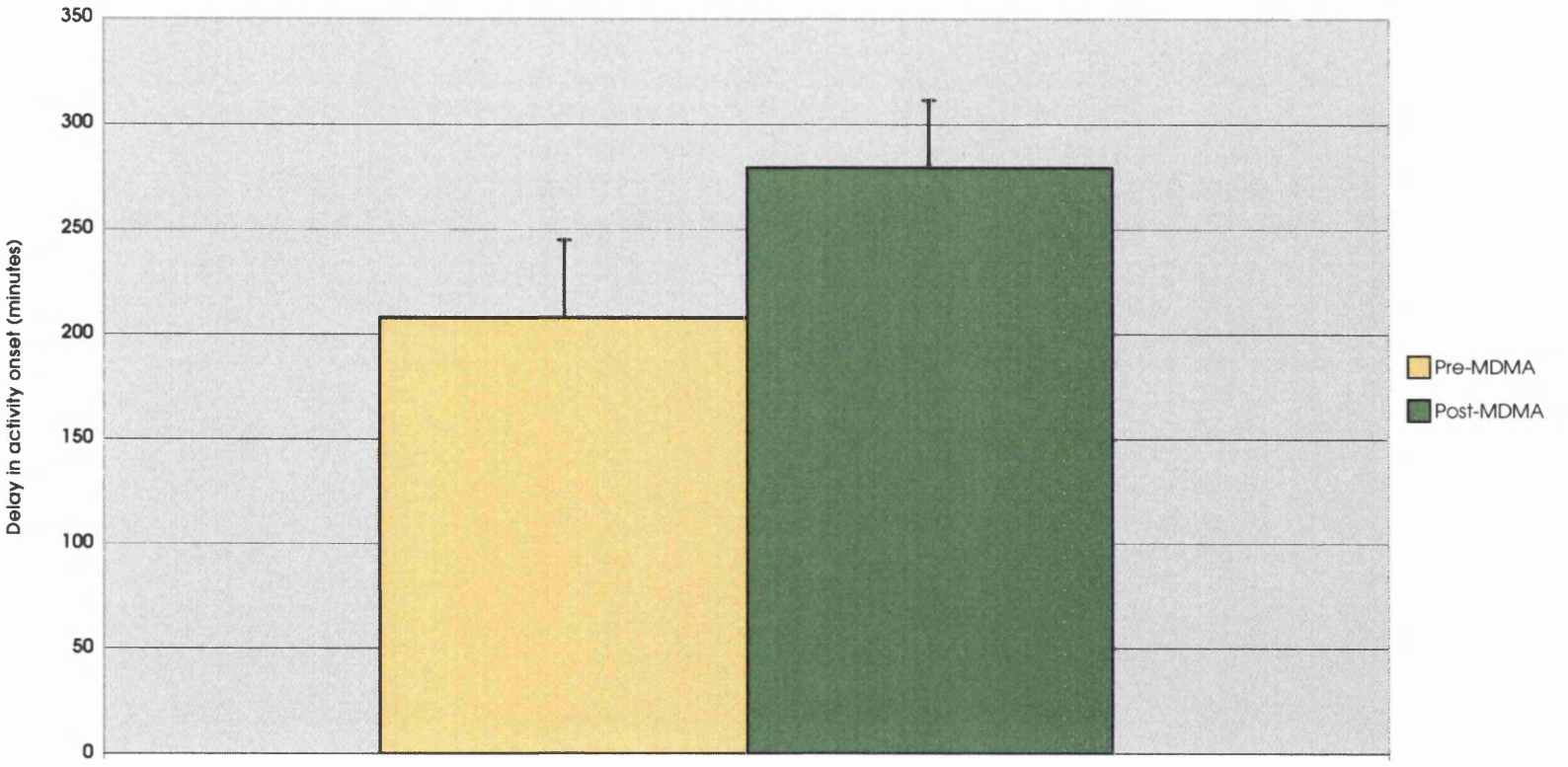


Figure 3.11 Graph of the sedative effect of baclofen. Analysis showed there was no significant difference between delays before or after MDMA treatment.

3.2 Anatomical analysis

3.2.1 SCN

The optical density in the SCN of animals treated with MDMA (0.11 ± 0.005; Mean ± SEM) was significantly reduced when compared to untreated controls (0.50 ± 0.161; $U = 4$, $P<0.05$; Figure 3.12 and 3.13).

3.2.2 Raphe

No significant difference in the numbers of immunopositive cell nuclei was found subsequent to treatment with MDMA in either the dorsal (Figures 3.14 and 3.16) or median (Figures 3.15 and 3.17) raphe nuclei.

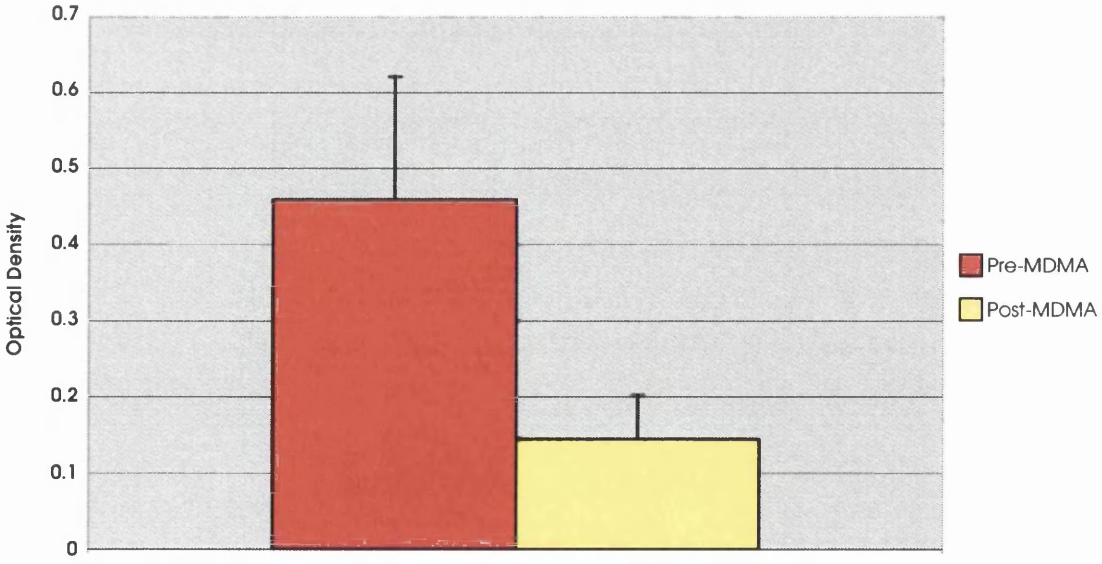


Figure 3.12 Optical densities of SCN brain tissue stained with antibody against 5-HT.
The density of immunohistological staining for 5-HT was significantly reduced in brains treated with MDMA.

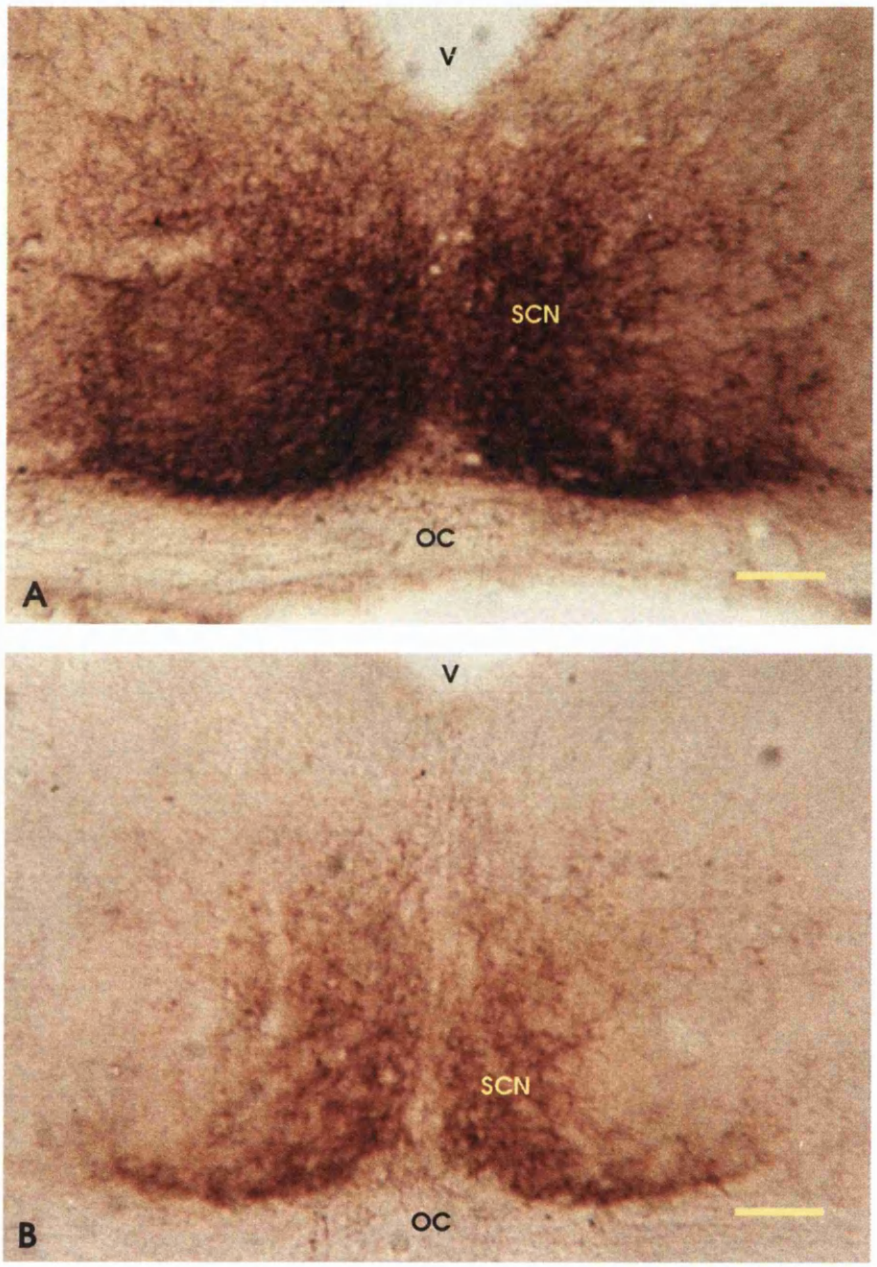


Figure 3.13 Photomicrographs of coronal sections through the SCN stained with antibody against 5-HT. A = Control brain. **B** = MDMA treated brain. The bilateral nuclei of the SCN can be seen lying above the optic chiasm and below the third ventricle. The brown colour is the end result of the immunohistochemical staining of 5-HT fibres. A reduction in density can be seen in tissue from the brain treated with MDMA. V = third ventricle; OC = optic chiasm; SCN = suprachiasmatic nucleus; x10 magnification, scale bar = 50 μm

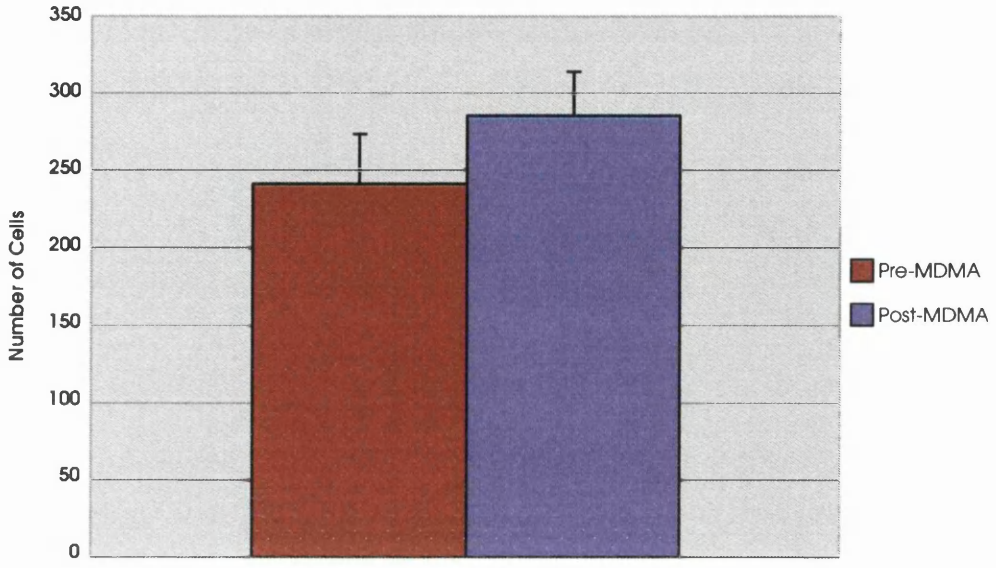


Figure 3.14 Cell counts of 5-HT immunopositive cell bodies within the dorsal raphe nucleus. No difference was found between control brains and MDMA treated brains.

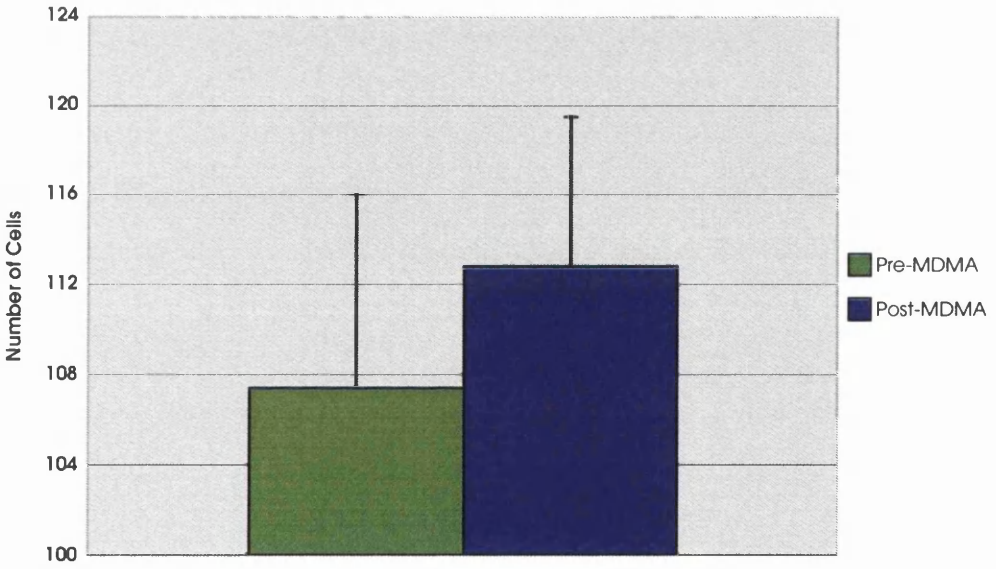


Figure 3.15 Cell counts of 5-HT immunopositive cell bodies within the median raphe nucleus. No difference was found between control brains and MDMA treated brains.

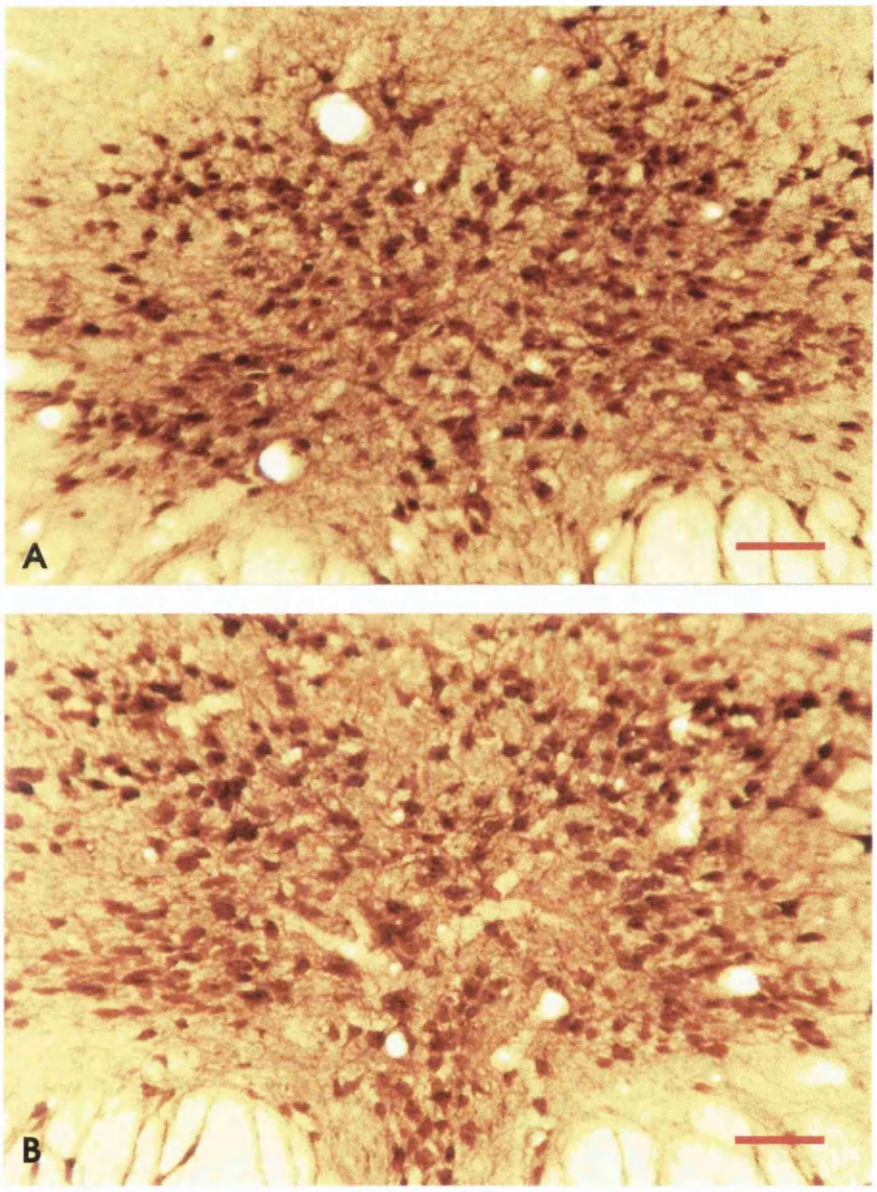


Figure 3.16 Photomicrographs of coronal sections through the dorsal raphe nucleus stained with antibody against 5-HT. A = control brain. B = MDMA treated brain. x10 magnification, scale bar = 50 μm.

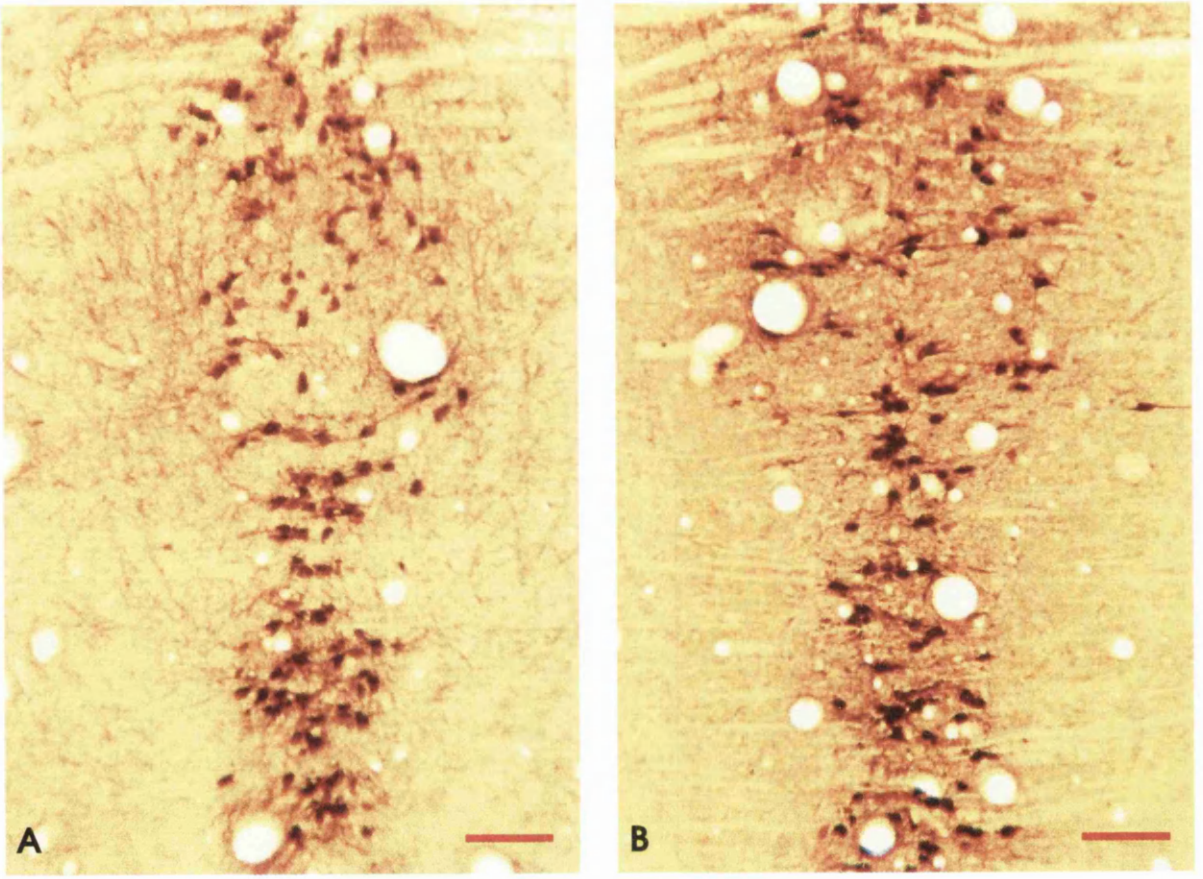


Figure 3.17 Photomicrographs of coronal sections through the median raphe nucleus stained with antibody against 5-HT. A = control brain. B = MDMA treated brain. x10 magnification, scale bar = 50 um.

4 Discussion

4.1 Phase Shifting Effect of Baclofen at CT6

Systemic administration of 10 mg/kg of R(+)-baclofen hydrochloride to Syrian hamsters at CT6 results in a phase advance shift in the onset of activity rhythms that is significantly different from the phase shift induced by control injections of saline administered at the same time. The phase and direction of these baclofen shifts are similar to those of shifts induced by other non-photic stimuli, indicating that $GABA_B$ receptors may be involved, either directly or indirectly in the mediation of phase shifts of this type.

This result is in agreement with previous work demonstrating baclofen-induced phase advance shifts of SCN neuronal activity *in vitro* at ZT6 (Biggs and Prosser 1998) and of activity rhythms *in vivo* at CT6 (Smith, Turek et al. 1990). The phase shifts from the *in vivo* study were of a similar magnitude to those observed here despite the fact that the lighting conditions were different (LL as opposed to DD). The phase advances *in vitro* were larger (97.8 ± 24 minutes) than those resulting from this study (43.4 ± 9.8 minutes). The differences seen between these two types of study may be due to the loss off inhibitory afferent input to the pacemaker in the SCN slice preparation resulting in a greater magnitude of phase shift.

An alternative explanation for the difference in the magnitude of phase advances may be due to the sedative effect of baclofen. On the days when baclofen was used as the phase shifting stimulus, there was a mean delay in the onset of activity of 207.8 ± 37.3 minutes. There was no difference subsequent to MDMA administration (279.6 ± 31.8 minutes), which would indicate that the

mechanism for this effect does not involve 5-HT. It has been previously shown that restriction of activity can act as a phase shifting stimulus in itself (Van Reeth, Hinch et al. 1991). It was found that under LL conditions, a three-hour period of immobilisation at CT12 resulted in a phase delay of 51 ± 9 minutes. Since baclofen administration appeared to restrict all activity onsets by similar period, it may be that phase advances to baclofen at CT6 might have been larger but have been counterbalanced by a phase delay from immobilisation due to the drug. It may also have been that the animals were still active subsequent to drug administration but unable to engage in wheel-running activity. This could have been clarified through the use of infrared movement detectors placed around the animals' cages.

4.2 The SCN as the Site of Action for Baclofen

Systemically administered baclofen has the potential to induce phase advances by acting at any site within the circadian system where $GABA_B$ receptors are located. $GABA_B$ receptors are known to be present within the SCN both from studies utilising *in situ* hybridisation (Lu, Ghasemzadeh et al. 1999) and immunohistochemistry (Margeta-Mitrovic, Mitrovic et al. 1999). The *in situ* hybridisation study would indicate that $GABA_B$ receptors are expressed by SCN cells and a direct, postsynaptic effect of baclofen has previously been shown. One *in vitro* study (Jiang, Allen et al. 1995) found that baclofen caused an outward current in SCN cells that was not blocked by TTX. Since TTX prevents the propagation of sodium-dependant action potentials this would indicate that the baclofen was acting directly on pacemakers cells. The outward current produced was associated with an increase in membrane conductance consistent with an increase in potassium conductance and resulted in inhibition of SCN cells. An *in vivo* study investigating photic signalling at CT13.5 also found

evidence for a direct effect of baclofen on SCN cells (Mintz, Jasnow et al. 2002). It was found that phase shifts to microinjection of a glutamate agonist were significantly reduced by baclofen and that this reduction was also unaffected by TTX. It may be that the phase advances observed in this study, at CT6 a time when the electrical activity of SCN cells is high, are brought about by postsynaptic inhibition by baclofen. The mechanism for this could be hyperpolarisation and so a reduction in the electrical activity of SCN cells resulting from the opening of inwardly rectifying potassium channels.

It is also thought that baclofen can have a presynaptic effect in the SCN. The phase advances in spontaneous electrical activity induced by baclofen at ZT6 *in vitro* were blocked by TTX suggesting a presynaptic action (Bergeron, Danielson et al. 1999). Application of baclofen *in vitro* can inhibit the release of glutamate through activation of $GABA_B$ heteroreceptors on terminals of the retinohypothalamic tract (Jiang, Allen et al. 1995). It may be that baclofen phase advances are induced by disinhibition of inhibitory afferents to the SCN. $GABA_B$ receptors may be present on afferents from the GHT. The effect of baclofen on these receptors would be to inhibit the release of GABA and/or NPY and so remove the inhibitory effect of these neurotransmitters on cells of the SCN. This mechanism seems unlikely however, since coexpression of $GABA_{B1}$ receptors and GAD was not found by immunohistochemistry (Margeta-Mitrovic, Mitrovic et al. 1999). Evidence from cultured SCN neurones indicates that activation of $GABA_B$ receptors by baclofen results in a strong presynaptic inhibition of GABA release from these cells (Chen and van den Pol 1998). Thus it may be that baclofen activates autoreceptors on GABAergic interneurones within the SCN resulting in the reduction of neurotransmitter release and ultimately in a phase advance.

Thus baclofen may result in phase advances of hamster activity rhythms by acting at the level of the SCN. This may be either by direct inhibition of SCN cells or by a reduction in transmitter release from afferent pathways or possibly a combination of both. Since *in situ* hybridisation and immunohistochemical studies have found evidence for the presence of $GABA_B$ receptors throughout the circadian system, it seems likely that baclofen could act at any of these sites. It may be that the observed phase shift at CT6 is the net result of stimulation of $GABA_B$ receptors at both pre- and postsynaptic sites.

4.3 The Raphe Nuclei as the Site of Action for Baclofen

Both *in situ* hybridisation (Lu, Ghasemzadeh et al. 1999) and immunohistochemical techniques (Margeta-Mitrovic, Mitrovic et al. 1999) have indicated that $GABA_B$ receptors are also present within the raphe. Combined immunohistological staining for both 5-HT and $GABA_B$ receptors indicates that all serotonergic cells in both raphe nuclei express $GABA_{B1}$ receptor subunits (Varga, Sik et al. 2002). This study found at both light and electron microscope level that the subcellular location of $GABA_B$ receptors was on proximal dendrites and cell bodies. This would indicate that systemically administered baclofen could act on these $GABA_B$ receptors to inhibit the firing of raphe cells causing reduction of both 5-HT metabolism and its release in projection areas. The 5-HTergic cells of the MR project to the SCN while those of the DR project to the IGL. Administration of baclofen at CT6 could result in changes in 5-HT levels in these areas and so lead to a phase shift.

The effects of $GABA_B$ receptor stimulation by baclofen has been studied in both raphe nuclei by a combination of microdialysis and electrophysiological techniques (Tao, Ma et al. 1996; Abellan, Adell et al. 2000; Abellan, Jolas et al.

2000). From these studies, it would appear that the net effect of systemically administered baclofen on 5-HT neurones in the raphe results from a balance between direct postsynaptic inhibition and indirect presynaptic disinhibition. Lower doses of baclofen were found to have a preferential action at presynaptic $GABA_B$ receptor sites while the $GABA_B$ antagonist phaclofen cannot fully block the effects of higher doses of baclofen (Abellan, Jolas et al. 2000). These observations would suggest the presence at the different cellular locations, of alternate $GABA_B$ receptor subtypes with varying affinities for agonist and antagonist.

The dose of drug used in these experiments was 10 mg/kg of baclofen hydrochloride. A dose response curve for R(+)baclofen has been previously reported (Ralph and Menaker 1989). The maximal inhibition of phase shifts to light by R(+)baclofen is achieved at a dose of 15 mg/kg. The baclofen hydrochloride used in these experiments is a more water-soluble version of baclofen and has been shown to produce a significant reduction in light-induced c-fos production in cells of the SCN at 10 mg/kg (Crosio, Cermakian et al. 2000). Thus systemic administration of baclofen at the lower dose used of 10 mg/kg may act preferentially at presynaptic $GABA_B$ autoreceptors on inhibitory GABAergic neurones that synapse on raphe 5-HT neurones. GABAergic input to the raphe is high during the day (Nitz and Siegel 1997), so baclofen would reduce the GABAergic inhibition on 5-HT neurones resulting in an increase of serotonin metabolism and release. This would be in agreement with the *in vitro* work which demonstrated an increase in the release of newly synthesised 5-HT in the SCN subsequent to baclofen administration during the subjective day (Francois-Bellan, Hery et al. 1987). This increase in serotonin release from the MR-SCN pathway may be involved in the phase advances observed.

If low doses of baclofen show preferential action at presynaptic $GABA_B$ receptors, then the MR serotonergic terminal zone in the SCN may be another site of drug action. Stimulation of $GABA_B$ autoreceptors on these afferents would result in a reduction in 5-HT release and disinhibition of SCN cells possibly leading to a phase shift. MDMA was administered by i.p. injection in increasing doses over three consecutive days. This is a protocol known to be neurotoxic to serotonergic neurones resulting in degeneration of terminal areas in rodents (O'Hearn, Battaglia et al. 1988). This can be seen in our study as a significant reduction in the optical density of the serotonergic terminal field within the SCN. If 5-HT is involved in mediation of baclofen phase advances, then it would be expected that a reduction of the terminal 5-HT field would affect the 5-HT being released within the SCN and so affect the size of the baclofen-induced phase advances. The phase advances to baclofen subsequent to MDMA lesions were indeed significantly smaller. Thus it seems likely that a presynaptic action of baclofen on 5-HTergic cells from the MR that synapse on the SCN may be involved in phase shifts to this agent at CT6.

It may be possible that the phase shifts to baclofen at CT6 are mediated by the 5-HTergic innervation of the IGL. The IGL is known to be involved in the mediation of non-photic phase shifts at CT6 through release of NPY (Biello, Janik et al. 1994) and/or GABA (Morin and Blanchard 2001) onto the SCN. If $GABA_B$ receptors are present on the 5-HTergic pathway from the DR to the IGL, then these may also be involved in mediation of non-photic phase shifting. Activation of these receptors would result in a reduction in 5-HT released onto the IGL. This would remove an inhibitory influence on the IGL and so result in an increase in the amount of NPY and/or GABA released onto the SCN from the GHT that may lead to a phase shift.

There also appears to be differences in the effect of baclofen at different times of day. Local administration of baclofen through microdialysis probes to the DR during the lights-off period results in a reduction in extracellular 5-HT both in the DR and a projection area (Tao, Ma et al. 1996). However, local administration in the DR (Abellan, Jolas et al. 2000) or MR (Abellan, Adell et al. 2000) during the lights-on period results in an increase in extracellular 5-HT. It is known from *in vivo* microdialysis experiments that extracellular 5-HT levels in the SCN area are at their lowest at CT6 under DD conditions (Dudley, DiNardo et al. 1998). Thus it may be that systemically administered baclofen at this time increases the release of 5-HT which ultimately leads to a phase advance shift of the activity rhythms.

Some authors have speculated that there is a reciprocal 5-HTergic pathway between the DR and MR. Electrical stimulation of either the MR or DR at CT6 both resulted in significant phase shifts in activity rhythms (Meyer-Bernstein and Morin 1999). Microdialysis measurement has shown that electrical stimulation of the DR produces an equivalent release of 5-HT within the SCN area as that produced by stimulation of the MR (Dudley, Dinardo et al. 1999). Systemic administration of the non-specific 5-HT antagonist metergoline blocked the release of 5-HT subsequent to DR stimulation but had no effect on the release subsequent to MR stimulation (Dudley, Dinardo et al. 1999). Microinjection of the 5-HT_{1A} autoreceptor agonist 8-OH-DPAT or the antagonist WAY 100635 directly to the MR resulted in inhibition and stimulation respectively of 5-HT release within the SCN. Similar microinjections to the DR had no effect on 5-HT release within the SCN (Dudley, Dinardo et al. 1999). $GABA_B$ receptors, if present on this pathway would be an additional substrate through which phase shifts to baclofen may be mediated.

Thus there are a number of possible sites for the actions of systemically administered baclofen on the raphe nuclei during the subjective day. The drug

may be acting presynaptically to inhibit neurotransmitter release onto cells of either nucleus. The action may be directly on receptors of raphe cells and so inhibit cell firing and metabolism. Alternatively, $GABA_B$ heteroreceptors on terminals of the MR to SCN pathway may be affecting transmitter release in this nucleus. It may be a combination of all of these, however the strongest possibility from the current data would be the last of these. MDMA caused a measurable decrease in 5-HTergic innervation of the SCN and this was paralleled by an attenuation of phase shifts to baclofen.

4.4 The Effect of Baclofen on the Molecular Clock

It has previously been shown that several different non-photic stimuli act both to produce phase advances in activity rhythms and to reduce production of PER within the SCN during the subjective day. These stimuli include novel wheel running (Maywood, Mrosovsky et al. 1999), injection of NPY (Maywood, Okamura et al. 2002), injection of 8-OH-DPAT (Horikawa, Yokota et al. 2000) or injection of benzodiazepines (Yokota, Horikawa et al. 2000). It may be that the phase advances produced by baclofen in these experiments are also as a result of the reduction in *Per* expression within the SCN. Expression of *Per* during the day is maximal (Maywood and Mrosovsky 2001) and a reduction in this through baclofen administration would ultimately change the pattern of clock gene expression within the SCN. This in turn would lead to changes in the expression of clock-controlled genes involved in output pathways such as TGFα, a molecule that has been implicated in the control of hamster activity rhythms (Kramer, Yang et al. 2001).

4.5 The Neurotoxic Effect of MDMA

Damage to the 5-HTergic system through the use of neurotoxins other than MDMA has been previously shown to affect various parameters of circadian rhythmicity. The neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) can destroy 5-HTergic terminals, fibres and neurones without damaging other neurotransmitter systems. Lesions induced through the use of this neurotoxin have been shown to have effects on several circadian rhythm parameters. Under the entraining conditions of a light-dark cycle, activity onset usually occurs just after lights-off for nocturnal rodents. Subsequent to lesioning by 5,7-DHT however, activity onset occurs slightly before lights-off and activity offset is usually delayed by 1.5 to 2 hours, although the total activity for the period remains the same (Morin 1999). In hamsters under constant darkness, circadian rhythmicity persists without any alteration in period, although the expanded activity phase persists (Morin and Blanchard 1991). This differs from the effects that were observed in this study where lesioning by MDMA had no effect on either the alpha activity phase or total activity. This may be due to differing sites of action of the two neurotoxins. The effect of 5,7-DHT on activity onset, offset, alpha period and total activity is similar whether the lesions are intraventricular or induced by injection directly to the MR (Morin 1999). It is possible that the effects of 5,7-DHT occur through the destruction of 5-HTergic cell bodies within the MR since the effects of this neurotoxin can occur by retrograde degeneration of nerve cells (Morin and Blanchard 1991; Morin 1992). MDMA however is known to result in the depletion of 5-HTergic axons and axon terminals with very little effect on cell bodies (Stone, Stahl et al. 1986) as confirmed by the results of this study. A difference between the effects of MDMA and 5,7-DHT has previously been shown with respect to circadian rhythmicity (Colbron, Jones et al. 2002). This study found that phase shifts to light were attenuated subsequent to MDMA treatment, an effect

opposite to the effect of 5,7-DHT that results in augmentation of phase shifts to light (Morin and Blanchard 1991).

An alternative explanation for the difference between the effects on activity of these two neurotoxins may be provided by a study that compared the effect of injecting 5,7-DHT directly into the MR with the effect of injections directly to the SCN (Meyer-Bernstein, Blanchard et al. 1997). It was found that bilateral infusion of 5,7-DHT into the SCN area resulted in different behavioural circadian parameters under a LD cycle than when the neurotoxin was diffused directly into the MR, even though both methods virtually eliminated 5-HTergic innervation of the SCN. Infusion to the MR, and so destruction of cell bodies, resulted in the usual advance in the onset of activity, delay in offset and extended activity phase. However bilateral infusion to the SCN, and so presumably destruction of terminal areas, resulted in earlier activity onset but had no effect on either offset or duration of the activity phase. The interpretation of this data at the time was that the 5-HTergic pathway from the MR to the SCN controlled the onset of activity but not any of the other circadian parameters. It was suggested that 5-HTergic innervation from the MR to other brain areas indirectly controlled the other parameters through alternate transmitter systems. A comparison can be drawn from this with the present study. MDMA caused a reduction in 5-HTergic cell terminal areas with no effect on cell bodies within the MR, a situation similar to bilateral infusion of 5,7-DHT into the SCN. MDMA had no effect on alpha period or total activity, it did however cause a change in the timing of activity onset.

The mode of administration of the neurotoxins may also contribute to the observed differences in effect. Experiments involving 5,7-DHT utilise local injection of the neurotoxin at specific stereotaxic coordinates. This results in lesions of

specific target pathways, which allows the contribution of that particular pathway in phase shifting to be assessed by subsequent experiments. MDMA is however administered systemically which results in global neurotoxicity of 5-HTergic pathways. Other areas of the brain that are rich in 5-HT may act on input pathways to the circadian system. Damage to the fibres caused by MDMA in these areas will also contribute to changes in the functioning of the circadian system.

The neurotoxic effects of MDMA would also be expected in the 5-HTergic terminal field within the IGL. It has been shown that MDMA has a greater neurotoxic effect on neurones originating from the DR than those originating from the MR, possibly due to the anatomical differences in the 5-HTergic innervation originating from these two nuclei (O'Hearn, Battaglia et al. 1988). Neurons from the MR are predominantly of a 'beaded' variety characterised by numerous rounded varicosities 2 to 3 μm in diameter while those of the DR are of a more uniform thin calibre type (Kosofsky and Molliver 1987). However, in the hamster there is no morphological difference between 5-HTergic innervation of the IGL or SCN with afferents to both areas being very fine with small fusiform terminals (Morin and Meyer-Bernstein 1999). Since damage was found to MR terminals in the SCN and that DR terminals are generally more susceptible to neurotoxic damage from MDMA, it seems probable that there would be damage to the 5-HTergic innervation of the IGL. Thus it may be that damage to 5-HTergic terminals in the IGL may be responsible for, or contribute to, the attenuation of baclofen induced phase shifts observed subsequent to MDMA administration.

4.6 Future Directions

One of the major shortcomings of the experiments used in this study is the inability to distinguish the exact site of action of systemically administered baclofen within the circadian system. One technique commonly used in circadian research which overcomes this is the use of indwelling guide cannulae implanted with the use of stereotaxic positioners. These can be aimed directly at specific areas of the brain from known stereotaxic coordinates and allow direct application of drugs to an area of interest such as the SCN or the raphe nuclei. This technique has previously been used successfully to investigate the effect of baclofen within the SCN. One study looked at the effects of baclofen on light induced delays and advances during the night (Gillespie, Mintz et al. 1997) while another looked at the direct effect of baclofen on the SCN at CT6 under constant light (Smith, Turek et al. 1990). No experiments have been carried out using this technique at CT6 under conditions of constant darkness. No study so far has looked at the effects of baclofen injected through cannulae aimed directly at either of the raphe nuclei or the IGL.

The involvement of 5-HT in systemically administered baclofen phase advances could be further examined by combination with local injection of antagonists of 5-HT receptors. There are several different 5-HT receptors that are known to be present within the circadian system. Selective antagonists are available for several of these enabling the blockade of that particular receptor. Used in combination with systemically administered baclofen would allow clearer identification of the neural pathway along which baclofen mediates its effects.

Recent evidence suggests that $GABA_B$ receptors are composed of different receptor subtypes and that different combinations of these vary in their

pharmacological properties. It has also been suggested that the different subtypes are coupled to alternate signal transduction mechanisms such as G proteins. It has been previously shown *in vitro* that pertussis toxin can discriminate between pre- and postsynaptic actions of baclofen in the rat DR (Colmers and Williams 1988). If a similar treatment was to be used *in vivo*, then it may be possible to determine the contribution of the different subtypes to baclofen phase shifts at CT6 and allow a closer determination of the exact location of these receptors.

The neurotoxic effect of MDMA on the 5-HTergic terminal field within the SCN was examined in this study. No account was taken of any similar toxicity to the 5-HTergic terminal field within the IGL. A similar immunohistochemical examination of any damage to this area would be necessary before any firm conclusion could be drawn regarding the exact location of the site of action of systemically administered baclofen.

4.7 Conclusions

Systemic administration of the $GABA_B$ agonist baclofen during the subjective day to Syrian hamsters, results in a significant phase advance shift of activity rhythms when compared to control injections of saline administered at the same time. This would indicate that $GABA_B$ receptors may have a role in the mediation of non-photic phase shifting of the circadian clock since this is a time at which other non-photic stimuli produce phase advances. This has implications for the treatment of circadian rhythm disorders in that drugs that affect neurotransmission through the $GABA_B$ receptor may have potential therapeutic value.

Further, neurotoxic damage to the 5-HTergic innervation of the SCN induced by the substituted amphetamine MDMA resulted in attenuation of the phase shifts induced by baclofen. This suggests that the 5-HTergic innervation of the SCN is involved in baclofen-induced phase advances. It has previously been shown that MDMA reduces that ability of the SCN to phase shift to a different non-photic stimulus as well as to light (Colbron, Jones et al. 2002). It is known that there is an increase in the use of MDMA as a recreational drug that may lead in the future to increasing incidences of circadian rhythm disorders.

Appendix 1: Immunohistochemistry Protocol

A1.1 Chemicals Used and Sources

Chemical Name	Supplier Code	Supplier Address
5HT antibody; clone YC5/45	YMC1019	Accurate Chemical & Scientific Corporation, Westbury, NY, USA
Biotinylated anti-rat IgG made in rabbit	BA-4000	Vector, Peterborough, UK
DPX mountant	44581	Sigma-Aldrich, Poole, UK
DAB substrate kit	SK-4100	Vector, Peterborough, UK
Hydrochloric acid	H/1150/PB17	Fisher Scientific, Loughborough, UK
Hydrogen peroxide	285196H	BDH, Poole, UK
Normal rabbit serum	S-5000	Vector, Peterborough, UK
Paraformaldehyde	16965-0010	Fisher Scientific, Loughborough, UK
Potassium chloride	P5405	Sigma-Aldrich, Poole, UK
Sodium bicarbonate	S5761	Sigma-Aldrich, Poole, UK
Sodium chloride	S5886	Sigma-Aldrich, Poole, UK
Sodium hydroxide	930-65	Sigma-Aldrich, Poole, UK
Sodium phosphate dibasic anhydrous	S5136	Sigma-Aldrich, Poole, UK
Sodium phosphate monobasic dihydrate	71509	Sigma-Aldrich, Poole, UK
Sucrose	S/8560/63	Fisher Scientific, Loughborough, UK
Triton® X-100	BPE151-100	Fisher Scientific, Loughborough, UK
Vectastain ABC Kit - Standard peroxidase	PK-4000	Vector, Peterborough, UK
Xylene	X/2022/17	Fisher Scientific, Loughborough, UK

A1.2 Stock Solutions

0.2M Phosphate Buffer Solution (PB):

A	sodium phosphate monobasic dihydrate	6.24 g in 200 ml double distilled water (ddH_2O)
B	sodium phosphate dibasic anhydrous	28.4 g in 1000 ml ddH_2O

- mix 190 ml of A with 810 ml of B
- check the pH is ~7.4, if necessary adjust with 1M sodium hydroxide or 1M hydrochloric acid

Perfusion Buffer:

per litre:	distilled water (ddH_2O)	950 ml
	0.2M buffer stock solution	50 ml
	sodium chloride (NaCl)	8.25 g
	potassium chloride (KCl)	0.25 g
	sodium bicarbonate ($NaHCO_3$)	0.5 g

4% Paraformaldehyde (PAM):

per litre:	ddH_2O	500 ml
	0.2M buffer stock solution	500 ml
	NaCl	8.8 g
	KCl	0.2 g
	paraformaldehyde	40 g

- heat solution to 60°C on a stirring hotplate
- stir until solution clears then filter through a double layer of filter paper

Sucrose solutions:

per 100 ml	10%	10g of sucrose in 0.2M PB
	20%	20g of sucrose in 0.2M PB
	30%	30g of sucrose in 0.2M PB

0.01M Phosphate Buffered Saline (PBS):

per litre:	ddH_2O	950 ml
	0.2M buffer stock solution	50 ml
	NaCl	8.8 g
	KCl	0.2 g

0.01M phosphate buffered saline with Triton-X (PBS+T):

per litre:	PBS	997 ml
	Triton-X	3 ml

1% Hydrogen Peroxide Solution:

per 30 ml:	PBS+T	29 ml
	30% hydrogen peroxide (H_2O_2)	1 ml

Blocking Solution:

per 10 ml:	PBS+T	9.7 ml
	normal rabbit serum (3%)	300 µl

Primary Antibody (1:100):

per 10 ml:	blocking solution	9.9 ml
	antibody – YC5/45	100 µl

Secondary antibody (1:200):

per 10 ml:	PBS+T	9.95 ml
	biotinylated rabbit anti-rat	50 µl

Avidin-biotin complex (ABC):

per 5 ml:	PBS+T	5 ml
	reagent A	1 drop
	reagent B	1 drop

- make up a minimum of 30 minutes before use

Diaminobenzidine solution:

per 5 ml:	ddH_2O	5 ml
	buffer solution	2 drops
	DAB solution	4 drops
	H_2O_2	2 drops

A1.3 Procedure

1. **Wash:**
   a. using a transfer pipette to remove the PBS solution in which the sections were collected and replace with fresh PBS solution
   b. place culture plates on the rotary shaker to agitate
   c. allow to wash for 10 minutes
   d. repeat this process for a second 10 minute wash

2. **Quenching endogenous peroxidase activity:**
   a. remove the wash solution
   b. replace with 1% H_2O_2 in PBS+T and agitate for 5 min

3. **Wash:**
   a. 2 x 10 minutes in PBS – as above

4. **Blocking non-specific background staining:**
   a. remove the wash solution
   b. apply blocking solution
   c. incubate tissue in blocking solution with agitation for 1.5 hours at room temperature

5. **Primary antibody:**
   a. keep one section as a non-specific staining control and remove blocking solution from all other sections
   b. apply primary antibody
   c. secure lids onto plates with sellotape
   d. incubate for 3 days at 4°C

6. **Wash:**

a. using a transfer pipette to remove the primary antibody and replace with PBS
b. place culture plates on the rotary shaker to agitate
c. allow to wash for 10 minutes
d. repeat this process for a second 10 minute wash

7. **Secondary antibody:**
   a. remove the wash solution
   b. apply secondary antibody using a 1 ml Gilsen pipette
   c. incubate for 90 minutes with agitation at room temperature

6. **Wash:**
   a. 2 x 10 minutes in PBS – as above

7. **ABC solution:**
   a. remove the wash solution
   b. apply ABC solution using a transfer pipette
   c. incubate for 90 minutes with agitation at room temperature

8. **Wash:**
   a. 2 x 10 minutes in PBS – as above

9. **DAB visualisation:**
   a. apply DAB solution with a transfer pipette and allow 2 to 5 minutes incubation until a suitable intensity of staining is achieved
   b. carry out this stage on one culture dish at a time to prevent over-staining

10. **Wash:**
    a. 2 x 10 minutes in PBS – as above

11. **Specimen mounting:**
    a. dehydrate through increasing concentrations of alcohol
    b. clear in xylene
    c. coverslip and mount with DPX mountant

Appendix 2: Immunohistochemical Theory

A2.1 Antibodies

Antibodies are a class of proteins, the immunoglobulins, produced by B-lymphocytes of the immune systems of animals in response to invasion of the body by foreign molecules. They form one or more Y-shaped units, which are each composed of four polypeptide chains. Each of these Y-shaped units contains two identical copies of a 'heavy' chain and two identical copies of a 'light' chain, named based on their relative molecular weights. There are five classes of antibody based on the number of Y-units and the type of heavy chain (IgG, IgM, IgA, IgD and IgE). Antibodies are produced by the immune system in response to antigens, which are high molecular weight chemicals such as proteins or polysaccharides. Smaller substances known as haptens, if these are chemically coupled to a larger carrier protein such as bovine serum albumin may also generate immune responses.

Antibodies bind to antigens at a specific site known as an epitope. This is usually one to six monosaccharide or amino acid residues on the surface of the antigen. Conformational epitopes are dependant upon a specific three-dimensional antigenic conformation such as would be formed from the interaction of two native protein subunits. Linear epitopes correspond to a simple primary sequence. The range of possible binding sites for antibodies is large with each having its own structural properties depending upon the type of molecular bonding present. The bonding between antibody and antigen is dependant upon non-covalent bonding such as hydrogen bonds, hydrophobic bonds, electrostatic forces and van der Waals forces. Immunochemical techniques rely upon the specific binding properties of an antibody for its antigen.

Antibodies are produced for use in immunochemical research by repeatedly immunising a suitable animal by a suspension of the antigen of interest. Blood serum is harvested at the peak of antibody production and the antibodies isolated. When very large antigen molecules are used for the immunisation, the resulting antibodies are said to be polyclonal. This is due to the large number of epitopes on the surface of the antigen molecule, each of which results in the production of an immunoglobulin specific for that epitope. Polyclonal antibodies have the disadvantage that they can cross-react with other epitopes on the antigen molecule. Monoclonal antibodies are homogenous populations of immunoglobulins produced by hybridomas, which are the result of the fusion of a B-lymphocyte with an immortal cell line. These antibodies all recognise the same epitope but because of this are sensitive to changes it the epitope brought about by processes such as chemical fixation of tissue samples. Monoclonal antibodies are used as the primary antibody in immunochemical research due to their high specificity and results can be highly reproducible if experimental conditions are kept constant.

The detection of antigens in biological tissue is known as immunohistochemistry (IHC). In this technique, an antibody is used to link a cellular antigen to a stain that can be observed under the microscope. There are three main stages involved in IHC, specimen preparation, antibody staining and antibody detection.

A2.2 Specimen Preparation

The cells and tissues have firstly to be preserved in a life-like manner. This is achieved by the use of fixatives, which are chemicals that stabilise the cells and

tissues and protect them from the rigors of processing and staining techniques. Tissues have differing protein content and so differing abilities to retain their structure depending upon fixation method used. The methods that are best for the preservation of tissue structure generally work by altering proteins, which may result in the masking of some epitopes and so block or impede antigen labelling. Fixatives may work by formation of cross-linkages between reactive groups in the polypeptide chains of proteins or by protein denaturation by coagulation, or a combination of the two. Requirements for fixation varies widely between tissue types, so methods used must be optimised for each in order to retain antigens and cellular structure while keeping epitope masking to a minimum.

Perfusion fixation involves the pumping of fixative through the vascular system of an intact animal. The animal is given an injection of anaesthetic and has its chest cavity opened. A needle connected to a supply of physiological saline is inserted into the left ventricle of the heart and the right atrium is cut. The saline is allowed to flow at a steady pressure and so pumps out all the blood from the body. The saline is then replaced by fixative and this is allowed to flow at a steady pressure through the body. The tissue of interest is then harvested. This method has the advantage of being quick as well as allowing the fixative good accessibility to all tissues throughout the body.

There are several methods of preparing sections of tissue sample for use in IHC. Cryostat sectioning involves the rapid freezing of the tissue sample for cutting on a cryostat. This method allows good antigen preservation and the use of many different fixatives so allowing optimisation of fixative for each antigen. The sections produced by this method can be stained free-floating and not slide mounted which allows greater surface area for incubation. This method however gives less morphological detail and resolution.

A2.3 Antibody Staining

The purpose of immunohistochemistry is to identify the presence or absence of a particular antigen within a tissue sample. There are several methods of achieving this but all involve exposing the tissue to an antibody raised against the molecule of interest. Detection of the antibody is achieved through several stages that ultimately result in attachment of a label that can be viewed under the microscope. The primary antibody may be directly labelled, or unlabelled, with detection achieved by a labelled secondary antibody. If a secondary antibody is used, it must be generated against the immunoglobulin of the animal in which the primary antibody was raised. For instance, if the primary antibody was raised in rat then the secondary antibody could be raised in rabbit against rat immunoglobulin. Antibodies bound in tissue samples can be detected by several methods. Enzyme-mediated detection involves the use of enzymes such as horseradish peroxidase or alkaline phosphatase as the label. These are then reacted with a suitable substrate that yields a precipitate. Various signal amplification techniques can be used to increase the signal to antibody ratio and so allow better visualisation of the end product.

A2.4 Avidin-Biotin Technique for Antibody Detection

Avidin is a glycoprotein that has such high affinity for the small vitamin biotin that reaction between the two molecules is essentially irreversible. Each molecule of avidin has four binding sites for biotin. Additionally, a variety of biological molecules including antibodies and enzymes can be conjugated to several molecules of biotin. Following from this, biotinylated proteins can each attach to more than one molecule of avidin. These properties have been exploited in

immunohistochemical techniques to allow the formation of macromolecular complexes between avidin and biotinylated proteins. The ABC technique employs unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (ABC). The biotin molecules on the secondary antibody react with the ABC and as a result greatly amplify the antibody signal (Figure A2.1).

An enzyme substrate is then added which results in the conversion of a colourless chromogen into a visible, coloured end product. The ABC techniques utilises the sensitive hydrogen peroxide-3,3'-diaminobenzidine (DAB) reaction which produces a brown end product which is insoluble in organic solvents. One drawback of this method is that many types of biological tissue have endogenous levels of the enzyme peroxidase present that can react in the final stage and produce high levels of non-specific staining. This has to be prevented by quenching the endogenous peroxidase activity with hydrogen peroxide before staining with the primary antibody.

Following this final stage, the tissue sections are mounted on microscope slides, dehydrated through increasing concentrations of alcohol, cleared in xylene and finally cover-slipped.

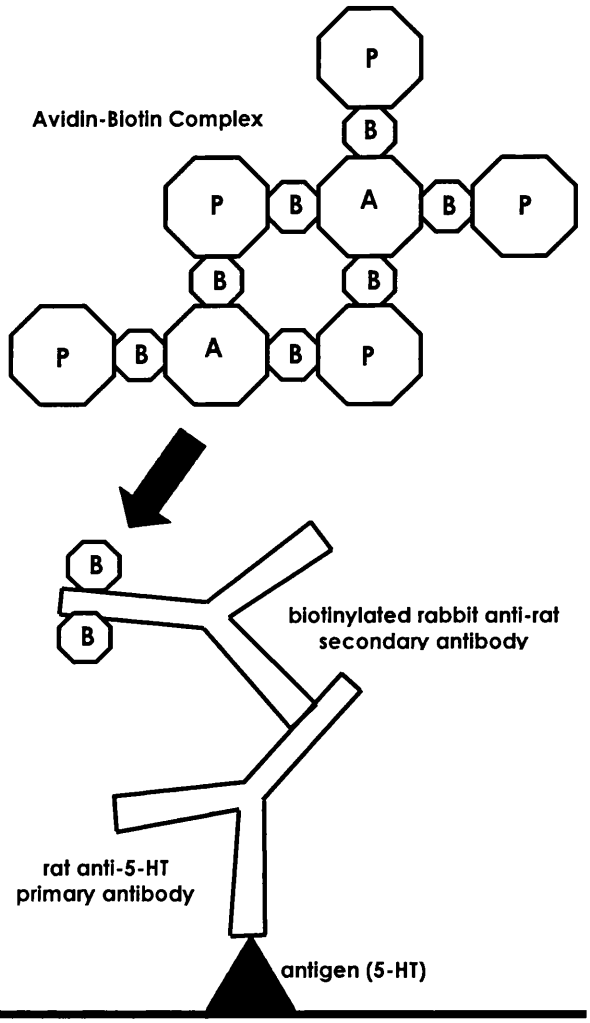


Figure A2.1. The ABC technique for immunohistochemistry as used in this study. A = avidin; B = biotin; P = peroxidase.

Appendix 3: Densitometrical Analysis

The principle of densitometry is based on the fact that radiation, including light is lost as it passes through a medium. If we have a beam of monochromatic light of radiant power P_O passing through a sample solution then absorption takes place and the radiation leaving the sample has radiant power P. Beer's Law states that for transmittance (T):

$$T = P/P_O$$

and for absorbance (A):

$$A = \log_{10} P_O/P$$

This radiation loss in a media is a function of the substance's molarity or concentration. According to Beer's law, concentration is proportional to optical density (OD). From this it is possible to use a scaling system for pixels in a photograph, which has a one to one correspondence with the concentration of a substance being studied. The logarithmic optical density scale and net integral of density values for an object in an image is the proper measure for use in quantification. So by Beer's Law, the density of a point is the log ratio of incident light upon it and transmitted light through it:

$$OD = \log_{10} P_O/P$$

There are several standard methods used to find the density of an object or a point on an image. The one used in this study uses photomicrographs of the SCN taken at X10 magnification and then digitised on a flatbed scanner. OD values

are not measured directly by this method. Pixel values in the photograph are linear with respect to T, which is the anti-log of the negative of OD:

$$T = 10^{-OD}$$

The software used, Scion Image, can be calibrated for optical density measurements using external standards. For some applications, external standards are available for known concentrations of the substance being measured, which allows actual molar concentrations to be calculated. Unfortunately there are no such standards available for 5-HT in nervous tissue and so a photographic step tablet was used (Figure A3.1).

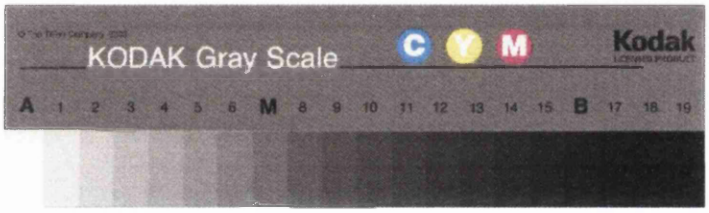


Figure A3.1 Photographic step-tablet used for calibration of densitometry software

This only allows relative optical density (ROD) to be measured rather than absolute values. The calibration procedure allows the transformation of pixel values directly from a scale that is linear with respect to T into a scale that correlates to OD or concentration. An equal area of each of the grey bands on the step tablet is measured to give a mean greyscale value for that band. These values are then matched with the corresponding known optical densities for each of the bands to produce a calibration curve (Figure A3.2). The images of sample tissue are then loaded into the software and measurements taken of the region of interest. This greyscale measurement is then converted to optical density by comparison with the calibration curve.

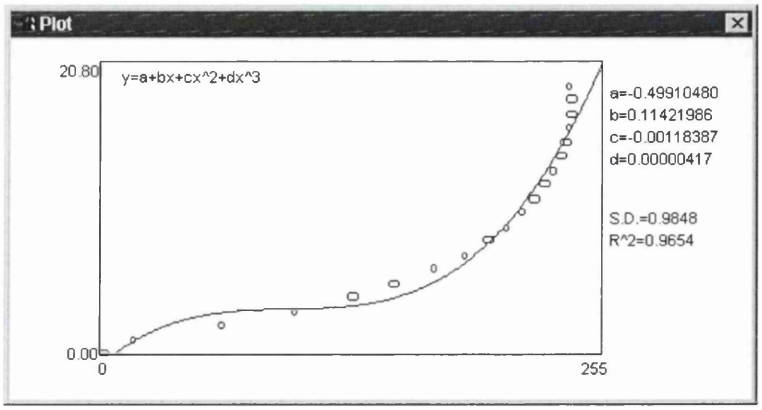


Figure A3.2 Calibration curve for optical density

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