Circadian rhythms in the rat: antidepressant drugs and a model of depression

Birmingham, Karen M.

Award date:
1995

Awarding institution:
University of Bath

CIRCADIAN RHYTHMS IN THE RAT: ANTIDEPRESSANT DRUGS AND A MODEL OF DEPRESSION

Submitted by Karen M. Birmingham
for the degree of PhD.
of the University of Bath, 1995.

Karen M. Birmingham

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I wish to thank my supervisor at the University of Bath, Dr. Peter Redfern.

I also wish to thank Dr. Brian Jones, my supervisor at SmithKline Beecham Pharmaceuticals, Harlow, Essex, who authorised my lengthy stay with the company, and where the work presented in this thesis was carried out.

Several researchers at the Harlow site contributed to my scientific development. In particular I would like to express my deep and sincere gratitude to Dr. Fiona Sidey, for her unfailing energy and enthusiasm, her scientific and technical advise, her support, stamina, encouragement and friendship throughout the past four years.

I wish to thank Dr. Jim Hagan for having taught me the brain lesioning technique, and having advised me on many aspects of the Chronic Mild Stress procedure. I also greatly appreciate the endless wisdom, advice and humour he provided during my time with the company.

I thank Jeffrey Jerman for his untiring practical help in the biochemical uptake assays; and Dr. Sara Patel for her advice and practical teaching in the histological analysis.

I thank all those mentioned above and also Jeanette Sweeney, Claire Roberts and Dr. Gary Price, for their continued friendship and support during my research, and I hope that each will derive some satisfaction from seeing this work in its completed version.

I would like to thank Dr. Martin DeVries, formerly of the Department of Physiology, University of Leiden, The Netherlands, for his friendship and our many discussions on the basic concepts of circadian rhythmicity.

Finally, and most importantly, I thank my fiancé, Dr. Peter Stephen Haddock, for his love, endless scientific, computing, financial and emotional support during my thesis study. I thank him for his determination to see me succeed and his belief in me, and without him this thesis would never have been submitted.

CONTENTS

SUMMARY

The involvement of the circadian system in clinical depression is a matter of contention. The aim of this thesis was to contribute to, and advance the knowledge surrounding this issue. To this end, three main areas were chosen for exploration. Firstly, the development of a localised cell body lesion of the IGL was attempted. However, selective lesioning of this structure using excitatory amino acids from three different classes proved impractical. From these studies it was concluded that quisqualate, kainate and ibotenate could provide no improvement on existing lesioning techniques, and offer no clearer definition of the role of the IGL within the circadian system.

Secondly, the ability of the antidepressant drugs paroxetine, imipramine and clorgyline, to affect circadian rhythms was addressed. The novel feature of this study was that levels of brain monoamine uptake activity, due to antidepressant treatment, were measured *ex vivo*, in an attempt to correlate circadian effects with quantifiable changes in neurochemistry. Measurement of circadian wheel-running activity in the rat demonstrated that significant alterations in the uptake activites of noradrenaline and serotonin had no effect on the period of the free-running rhythm, the length of activity time, or the phase shift response to a light pulse. As such, the study argues against a role for antidepressant efficacy via the circadian system.

Finally, a novel approach to explore the relationship between circadian rhythmicity and behavioural depression was made. In these studies running-wheel activity was measured in rats previously subjected to the Chronic Mild Stress (CMS) animal model of depression. CMS-treated animals were shown to exhibit a decreased preference for sucrose over water (representative of anhedonia), a decreased gain in body weight and a deficit in locomotor activity in a novel environment compared to control animals. Circadian rhythm analysis revealed CMS treatment to significantly affect both the free-running and 12:12 hour light/dark entrained locomotor activity rhythm in a number of ways, and in a manner which was not attributable to the handling and lighting conditions employed in the regime. The results of this third study support the hypothesis that abnormal circadian rhythms are implicated in depressive illness, and also add weight to the two-oscillator theory of circadian locomotor activity.

CHAPTER I

Introduction

And God said: "Let there be light." And there was light. And God saw the light, that it was good, and God divided the light from the darkness. And God called the light Day, and the darkness He called Night. And there was evening and there was morning, one day. Genesis I, 3-5.

Since the beginning of time on earth a cycle of light and darkness has existed, and organisms have adapted to this cycle to derive maximum benefit from the environment. A major adaptation is an alternating pattern of sleep and wake, tailored to the light/dark cycle for each animal, whether they be nocturnal, diurnal or crepuscular. Adaptations which are less obvious are the timed variations in hormonal release, biochemical reactions and physiological processes which occur within an organism and which are aimed at optimising the survival of the organism within its environment.

The purpose of this chapter is to provide an introductory overview of the system which integrates biological functioning with the time of day - the circadian system. The aim is to describe how the physiological, pharmacological and behavioural events which possess a circadian rhythm are under the control of an endogenous clock, and how disruption of the circadian system may be linked to some illnesses.

BIOLOGICAL RHYTHMS

An event which recurs at a regular interval within a biological system is described as a **biological rhythm**, whilst the time interval at which this rhythm recurs is called the **period** of the rhythm. Rhythms of the body with a period of approximately 24 hours are described as **circadian** (*from the Latin*: circa = about; diem = day). Functions which oscillate more frequently and have a period less than 24 hours, such as breathing and heart beat, are known as ultradian rhythms. Some rhythms reccur perhaps only once every 28 days (menstruation) in which case they are termed circalunar, or maybe once per year (hibernation) and are termed circannual. The focus of this thesis is circadian rhythms.

CIRCADIAN RHYTHMS

Virtually every biochemical, physiological, hormonal and pharmacological event undergoes variation during the course of a 24 hour day. Plasma growth hormone, prolactin, thyrotrophin stimulating hormone and luteinizing hormone are all elevated in humans during sleep at night (Allan & Czeisler, 1994). Body temperature drops at night-time and peaks in the afternoon or early evening (Refinetti & Menaker, 1991). Blood flow to organs follows a circadian pattern (Houben et al., 1994) and the number of red and white blood cells in the circulation varies throughout the course of the day (Bridges et al., 1992). There is a nocturnal fall in heart rate and blood pressure (Purcell et al., 1992). These are just some examples of processes which have a circadian rhythm and illustrate the concept that homeostasis, far from being a state in which conditions are held stable by preventing them from fluctuating, actually derives its stability from a constantly changing internal milieu.

The explanation for continued and predictable biological rhythmicity is that the body possesses an innate time-keeping system - **a biological clock**, also known as an oscillator or pacemaker. This clock, which controls the timing of circadian rhythms, is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. There are two essential features to circadian rhythms: 1) that they are 'free-running' in constant conditions and 2) that they can be entrained by a time cue or zeitgeber (from the *German*: time giver). *In vivo*, the only way to measure the activity of the SCN is to monitor overt rhythms which are driven by the clock.

1) FREE-RUNNING RHYTHMS

The fact that circadian rhythms exhibit a constant frequency generated internally within the organism, and are not merely responding to fluctuations in the external environment, can be demonstrated by monitoring the rhythms of an organism housed under conditions where all factors which could provide a time cue such as lighting, temperature, humidity, noise and food availability remain constant. The rhythms displayed under these conditions without time cues are said to be 'free-running', and their period is

called **tau** (τ). Tau is thought to reflect the actual period of the biological clock unaffected by any other influences. Each species shows a characteristic free-running period, with the periods of the individual animals normally distributed around the species mean. Most species have free-running periods displaced somewhat from exactly 24 hours. This is not because evolution failed to produce a sufficiently precise clock. In fact, selection pressure has produced clocks deviating from 24 hours because stable rhythm entrainment to the natural light/dark (LD) cycle becomes more difficult the closer an animal's natural period is to 24 (Pittendrigh & Daan, 1976a).

Perhaps the first documented evidence of endogenous rhythm generation was that by the French astronomer Jean Jacques de Mairan in 1729, who noticed that the leaves of the *Mimosa* plant continued to open during the daytime and close during the nighttime when placed out of the range of sunlight. Similar rhythms have since been demonstrated to persist across animal species. In nocturnal animals, the free-running rhythm will usually consist of regularly spaced bouts of rest (sleep), activity (wake), feeding, grooming, and so on, and the times of each of these can be predicted by viewing a number of sequential cycles of behaviour.

Experiments in humans housed in a time-free environment were first carried out in the late 1970's and early 80's and involved volunteers living in isolation chambers (Wever, 1979). Subjects were allowed to choose when to perform activities such as eating or sleeping, but were given no information about time from television, radio, clocks or social interaction. Following an initial few days disturbance, the subjects maintained a routine centred around a sleep/wake cycle of approximately 25 hours. Considering that the hamster has an activity rhythm closer to 23 hours, this illustrates the fact that the free-running period is species specific. Tau is also genetically determined, a property demonstrated in drosophila *melanogaster* by Konopka & Benzer (1971). These workers showed that genetic mutations gave rise to drosophila with different periods of the activity/rest cycle. So, although inter-, and sometimes intra-, species differences are common, in one individual the period of the endogenous rhythm remains remarkably constant. This predictability is made use of in circadian rhythm experiments.

2) ENTRAINMENT OF RHYTHMS

An internal clock is of little value if it dictates that the animal is awake when its prey are asleep and vice versa. Thus, the advantage of the biological clock is that it can be entrained to the external environment and in turn, it can synchronise the rhythms it controls with the external environment. So, if an animal is removed from constant, free-running conditions and exposed to a 24 hour LD cycle, it becomes entrained with a periodicity of exactly 24 hours. The advantage of this "**entrainment**" is that it allows the animal to anticipate and adapt to events it encounters on a daily basis. Entrainment is brought about by a collection of phase shifts.

Phase shifts

The time of the internal biological clock relative to the external environment is known as its **phase**, and like all clocks, the time of the internal clock can be adjusted. The phase is altered by an appropriate stimulus impinging on the circadian system at a certain time, and the result is known as a phase shift. If the stimulus is light from the light/dark cycle, and the overt rhythm measured is activity, then light may cause either a phase delay (so that activity starts later) or phase advance (activity starts earlier) depending on the point of the free-running rhythm at which the light is given (Daan & Pittendrigh, 1976b). For a nocturnal animal, light pulses during the inactive/rest period (subjective day) do not alter the activity rhythm, whereas pulses during the early part of the dark period (subjective night) cause a delay in phase, and light pulses during the latter part of the subjective night cause phase advances. This theory is borne out by an animal in its natural environment. If a nocturnal animal enters its burrow too late after dawn, it is exposed to light at the end of its activity period; this will result in a phase advance of the circadian pacemaker, such that the animal will rise earlier the next evening and complete its activity within the dark phase. A similar entrainment process would occur if the animal began its activity whilst it was still light. The light pulse at the beginning of the active period would cause a phase delay of the next cycle, thus guaranteeing that the animal begins its activity later the next evening and in darkness. The exact relationship between the time that stimuli are applied and the resultant change in rhythm can be defined graphically as a **phase response curve**, or **PRC**.

THE PRC

The main features of the PRC, with phase delays in the early subjective night and phase advances in late subjective night, are similar in all species whether they are single-celled algae or primates and whether they are nocturnally or diurnally active. Although the LD cycle is the primary zeitgeber for the majority of mammals (Meijer, 1991), a broad range of diverse stimuli have now been shown to phase shift rhythms and a corresponding number of PRCs for these stimuli have been recorded (Turek, 1987). For example, a nocturnal animal maintained in constant light and given pulses of darkness, will generate a 'dark PRC', which is different to the 'light PRC', with advances occuring in the rest period and phase delays in the active period (Boulos & Rusak, 1982a). A similar PRC is obtained with stimuli such as cage changing and wheel-running, which do not involve the LD cycle at all (non-photic PRC) (Reebs & Mrosovsky, 1989a; Wickland & Turek, 1991).

Hence, for entrainment to occur, a zeitgeber must reset the phase in each cycle of an otherwise free-running circadian pacemaker by an amount that corrects for the difference between the period of the time cue and that of the pacemaker (Pittendrigh, 1981). Entrainment is therefore a collection of phase shifts.

Figure 1. Phase response curve to light for locomotor activity in the male Wistar rat.

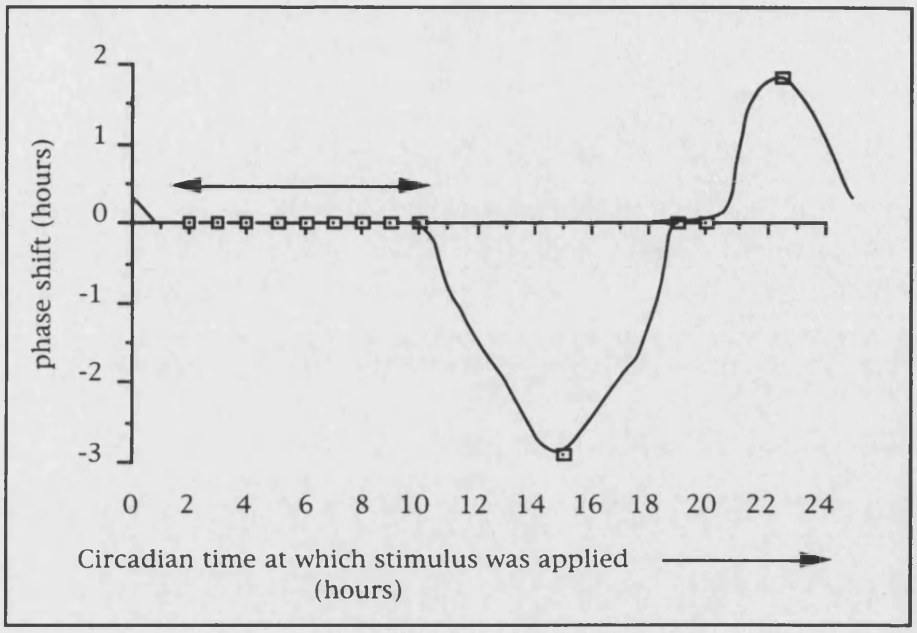


Figure 1 represents the PRC to light of locomotor activity in the male albino Wistar rat. The x axis is the circadian time of stimulus application (light pulse for 30 minutes duration at 300 lux); the y axis is the shift in phase of locomotor activity in hours. In constant dark conditions, with other factors such as temperature, food availability, noise and so on also constant, a light pulse can cause the activity rhythm to begin earlier (positive y axis) or later (negative y axis) on the next cycle. A light pulse given in the rest period (indicated by the double-headed arrow), does not cause a shift in the rhythm. The PRC is redrawn from Honma et al., (1985).

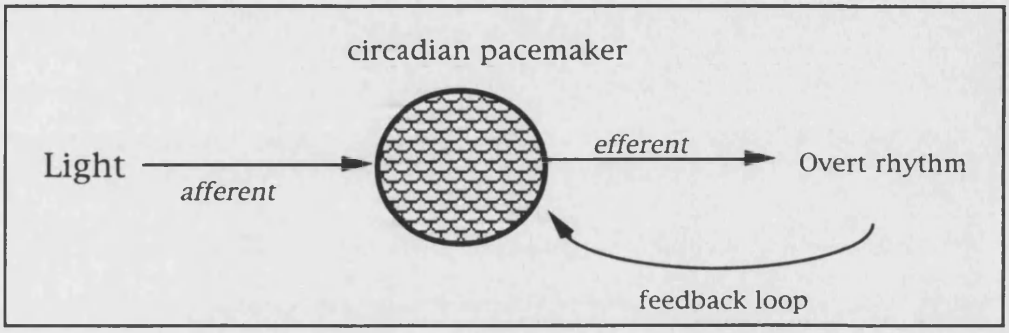
Immediate and steady-state phase shifts

If a zeitgeber that is entraining a circadian rhythm is abruptly phase-shifted by several hours, there will be several cycles of transients while the circadian rhythm resynchronizes with the new phase of the time cue (Pittendrigh & Daan, 1976a). For example, if a stimulus is administered at a circadian time which results in a phase advance of the rhythm, there are two ways in which the amount of advance phase shift can be measured. Either immediately on the next cycle of the rhythm, or when the rhythm has reached steady-state, meaning that it has regained stability following a series of phase advance shifts known as transients. Consequently, two types of PRC can be established for a given stimulus, the immediate PRC and the steady-state PRC.

WHAT ARE THE COMPONENTS OF THE INTERNAL TIMING SYSTEM?

How does the clock receive zeitgeber information and act to change rhythm parameters? The physiological system responsible for rhythm generation can be discussed in three parts: 1) an input pathway by which environmental signals are transmitted to 2) the clock, which is coupled to the processes it commands by 3) an output pathway.

Figure 2. Essential components of the circadian system



The above diagram illustrates the basic components of the mammalian circadian system. Light is received and processed via the retina (Remé et al., 1991), before being transmitted to the SCN via afferent pathways. The SCN then acts on the information it receives concerning the status of the environment to alter rhythms under its control via efferent pathways. In addition, it is becoming apparent that information from rhythms altered by the SCN may feed back to the SCN allowing further rhythm modification (Edgar et al., 1991; Edgar & Dement, 1991).

HOW IS PHOTIC INFORMATION TRANSMITTED TO THE SCN?: AFFERENT PATHWAYS

Following retinal conversion of light into neuronal signals, the SCN recieves photic information via three pathways, one direct (retinohypothalamic tract) and two indirect (geniculohypothalamic and raphe-hypothalamic tracts). The latter two reach the SCN after connecting with other brain nuclei, which are the intergeniculate leaflet and raphe nuclei, respectively. The pathways are illustrated in Figure 3.

1) THE RETINOHYPOTHALAMIC TRACT (RHT)

The RHT is a direct neuronal projection from the retina to the SCN which is of primary importance in synchronizing rhythms with the LD cycle and which alone is sufficient for the process of entrainment to occur (Pickard et al., 1987; Johnson et al., 1988b). This connection has been demonstrated to exist in a number of different mammals using an intravitreous injection of tritiated amino acids injected into the eye (Moore & Lenn, 1972; Moore, 1973). Furthermore, a species difference of this projection has been

highlighted between the rat and the hamster. Following injection of the anterograde tracer horseradish peroxidase (HRP), the RHT of the hamster is shown to terminate rostrally and dorsally to the SCN borders. Terminals of the RHT in the rat are mostly located in the ventral and lateral SCN, while the medial and dorsal SCN are only sparsely innervated (Pickard, 1982).

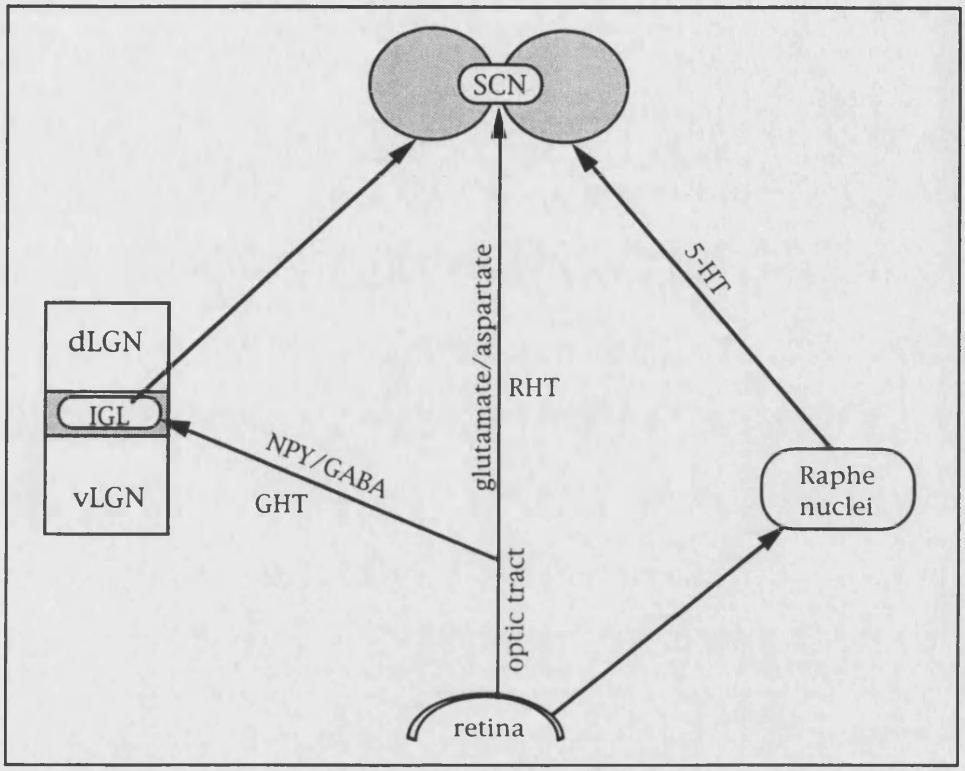


Figure 3. Schematic overview of the three pathways involved inransmitting light/dark information from the eye to the SCN. dLGN=dorsal lateral geniculate nucleus; vLGN=ventral lateral geniculate nucleus; IGL=intergeniculate leaflet; RHT=retinohypothalamic tract; GHT=geniculohypothalamic tract; NPY=neuropeptide Y; GABA=gamma aminobutyric acid; 5-HT=5-hydroxytryptamine/serotonin.

Furthermore, although the main RHT projection is to the SCN in both these species (Johnson et al., 1988a; Youngstrom, 1991; Levine et al., 1991), an even more extensive terminal field has been demonstrated in both species. HRP conjugated to cholera toxin shows RHT projections to terminate in the preoptic and anterior hypothalamic areas. The functional significance of these connections is yet to be elucidated (Levine et al., 1991). Although the endogenous transmitter of the RHT has not yet been identified, recent experiments strongly favour an excitatory amino acid (EAA) as the candidate, namely glutamate or aspartate. Glutamate immunoreactivity has been localized in retinal ganglion cells (Montero, 1990) and in the SCN (Gannon & Rea, 1993). Retinal illumination and glutamate application to the SCN both have excitatory effects on the firing rates of the majority of SCN cells (Meijer et al., 1986; 1989; Nishino & Koizumi, 1977).

Radiolabelled glutamate and aspartate are released following optic nerve stimulation in *in vitro* SCN brain slices (Liou et al., 1986). In addition, both n-methyl-d-aspartate (NMDA) and non-selective NMDA receptor-types may be involved in the EAA transmission process (Kim & Dudek, 1991). *In vivo* administration of the competitive NMDA receptor antagonist CPP (3(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid), and the non-NMDA receptor antagonist, DNQX (6,7-dinitroquinoxaline-2,3-dione), prevents the phase-shifting effects of light pulses (Colwell & Menaker, 1992). Whilst such experiments indicate that glutamate or aspartate are likely to be involved in RHT transmission of photic information to the SCN, storage of either EAA in RHT terminals has not yet been demonstrated directly. Furthermore, data contradictory to glutamate being the neurotransmitter of the RHT cannot be ignored. Stimulation of the optic nerve *in vitro* produces phase shifts similar to those produced by light pulses (Shibata & Moore, 1993), whereas *in vivo* microinjection of glutamate into the SCN produces phase shifts similar to those produced by dark pulses (Meijer et al., 1988). However, it is possible that these differences in phase shift pattern are due to injection of exogenous glutamate failing to mimic exactly the neurophysiological release of the transmitter.

2) THE GENICULOHYPOTHALAMIC TRACT (GHT)

The GHT is an indirect pathway by which light information reaches the SCN. Retinal fibres pass to the lateral geniculate nucleus of the thalamus which comprises the dorsal lateral geniculate nucleus (dLGN), the ventral lateral geniculate nucleus (vLGN) and the intergeniculate leaflet (IGL). Predominantly within the IGL, but also within the vLGN, the retinal terminals form direct contacts with neurons that colocalise NPY (Harrington et al., 1985) and γ-aminobutyric acid (GABA) (Moore & Speh, 1993). It is from here that the bundle of fibres known as the GHT projects bilaterally to the SCN (Morin et al., 1992). At least some of the retinal fibres innervating the IGL/vLGN are collaterals of RHT fibres (Pickard 1985), a factor which has made elucidation of the role of the GHT in circadian rhythms difficult, since the GHT cannot be physically isolated from the RHT. The situation is further complicated at the endpoint of the pathway by the overlap of terminals of the GHT with those of the RHT in the SCN (Van den Pol & Tsujimoto, 1985).

So what is the role of the GHT in circadian rhythm control? Some studies have shown the entrainment process of rhythms not to be affected by lesions of the GHT, suggesting that the GHT has no role in entrainment (Dark & Asdourian, 1975; Pickard et al., 1987). However, some studies suggest otherwise. IGL and vLGN lesioned animals reentrain more slowly compared to intact animals (Johnson et al., 1989) and have a different magnitude of light-induced phase advance (Harrington & Rusak, 1986). The phase angle of entrainment is altered in lesioned animals (Pickard, 1989). Also, when hamsters are exposed to constant light conditions, the period of locomotor activity

lengthens and splitting of rhythms occurs (Pittendrigh & Daan, 1976e). Both of the latter phenomena are prevented by IGL ablation or GHT lesioning (Pickard et al., 1987; Harrington & Rusak, 1988). Finally there are reports that lesioning the IGL results in a clearer offset of the activity rhythm in golden hamsters (DeVries, 1994).

The major neurotransmitter of the GHT has been identified as neuropeptide Y (NPY) (Card & Moore, 1989). Further physiological proof that NPY is the neurotranmsitter is that *in vivo* injections of NPY into the SCN area of awake, active hamsters produces the same PRC as does electrical stimulation of the GHT (Albers &Ferris, 1984; Rusak et al., 1989; Johnson et al., 1989). This type of PRC also resembles that of the dark pulse PRC (Boulos & Rusak., 1982a). Since the same pattern of phase shifts is seen following social interaction (Mrosovsky, 1988), and that IGL lesions block phase shifts involving activity (Biello et al., 1991), the IGL/GHT pathway is strongly implicated in non-photic circadian function. This pathway is further discussed in Chapter III.

3) THE RAPHE-HYPOTHALAMIC TRACT (RAHT)

Like the GHT, the raphehypothalamic tract (RaHT) is an indirect pathway from the retina to the SCN. Tracer studies have demonstrated a direct retinal innervation of the mid-brain raphe nuclei of both rat and cat (Foote et al., 1978; Shen & Semba, 1994), from which the RaHT passes to the SCN. The transmitter of the RaHT is serotonin (5-HT) (Moore et al., 1978), and this pathway is responsible for the high 5-HT content of the SCN (Steinbush, 1981). Although daily variations in extracellular 5-HT content of the SCN do exist, (with highest levels of 5-HIAA occuring after lights-off), they are not thought to be circadian in nature (Glass et al., 1993). In fact, the rhythmic change in the 5-HT content in the raphe has been shown to be directly influenced by environmental illumination (Cagampang et al., 1993). What is the role of the RaHT in circadian system? 5-HT has been shown *in vivo* (Edgar et al., 1993) and *in vitro* (Prosser et al., 1993) to phase shift circadian rhythms of neuronal activity in the SCN. Destruction of serotonergic cells of the midbrain raphe by intracerebroventricular injection of 5,7-dihydroxy tryptamine (5,7-DHT), causes changes in the circadian rhythm of hamster wheel-running activity, resulting in earlier onsets and later offsets with a prolonged activity phase under LD conditions (Smale et al., 1990). 5,7-DHT destruction has also been shown to cause an alteration in the phase angle of entrainment, to change the PRC to light, and to lengthen the circadian period in LL. In total, these studies suggest that the role of the RaHT is to modulate rhythms and not to influence the clock directly (Morin & Blanchard, 1991). Anatomically, the majority of 5-HT afferents have been shown to terminate in the ventrolateral SCN where they overlap with the terminal field of the RHT (Card & Moore, 1984). Pharmacologically, *in vivo* application of 5-HT agonists to the SCN suppresses the concentration of extracellular glutamate in that region (Glass et al., 1993). Both pieces of evidence contribute to the idea that the RaHT may modulate the effects of the RHT transmitter at the level of the SCN. 5-HT_7

receptors may be those mediating serotonin-induced phase shifts in the SCN (Lovenberg et al., 1993). In conclusion, although the SCN has a very high density of 5-HT, this transmitter and the RaHT pathway have no clearly defined circadian role as yet.

THE SUPRACHIASMATIC NUCLEUS

It has been well documented over the past two decades that the suprachiasmatic nucleus (SCN) of the anterior hypothalamus serves as a mammalian body clock, which drives circadian rhythms (Meijer & Rietveld, 1989; Morin, 1994). In 1972, two research groups independently discovered that lesioning the suprachiasmatic nucleus of the anterior hypothalamus in the rat resulted in loss of the circadian rhythms of corticosterone secretion (Moore & Eichler, 1972), and locomotor and drinking behaviour (Stephan & Zucker, 1972). Other circadian rhythms were subsequently found to be under the control of this same nucleus: pineal melatonin (Klein & Moore, 1979); food intake (Boulos et al., 1980), pituitary prolactin (Bethea & Neill, 1980; Kawakami et al., 1980, Yogev & Terkel, 1980); body temperature (Eastman et al., 1984), and the sleep/wake cycle (Eastman et al., 1984; Ibuka et al., 1977). Recovery of circadian rhythmicity in these behaviours following lesions of the SCN did not occur, even if the lesion was performed in neonatal rats (Mosko and Moore, 1978).

Whilst lesion studies indicate that the SCN is essential for the expression of circadian rhythms, they do not prove that the pacemaker itself is located in the SCN, since SCN destruction may have resulted in damage to other systems such as a link from the SCN to a second oscillator. To conclude that the SCN itself is the pacemaker, it must be shown that the SCN is capable of independently generating a rhythm. Perhaps the first evidence for this came from *in vivo* rat studies by Inouye and Kawamura, (1979; 1982). These workers isolated the SCN from the rest of the brain by knife cuts in freely moving rats, producing a 'hypothalamic island'. They found that rhythmicity in multi-unit activity was lost in all brain areas except the SCN indicating that this area could not only maintain a rhythm when isolated, but was also responsible for inducing rhythmicity in the regions outside the cut. Further confirmation that the SCN is the site responsible for rhythm generation came from the *in vitro* work of Prosser & Gillette (1989). Using a hypothalamic brain slice preparation, these workers showed that SCN neuronal cell firing was found to be highest during the subjective day, and that this rhythm persisted for at least three successive cycles.

Unequivocal evidence that the SCN is the origin of circadian rhythms was provided by *in vivo* transplantation experiments. Rats and hamsters made arrhythmic, as measured by locomotor activity, following an SCN lesion did not recover rhythmicity. However, when either whole tissue grafts or dispersed cell suspensions of foetal SCN were injected into the area of the third ventricle and optic chiasm of the lesioned animal, circadian rhythmicity was restored (Sawaki et al., 1984; Lehman et al., 1987 & Silver et al., 1990). More importantly, the period of the newly established rhythm was always

that of the donor and not of the host. This latter point was eloquently demonstrated by Ralph et al., (1990), who found that transplantation of foetal SCN from a *tau* mutant hamster donor into the SCN area of the previously-lesioned, wild-type host, resulted in restored rhythmicity which always exhibited the period of the *tau* mutant donor.

MORPHOLOGY OF THE SCN

Studies in the rat show the SCN to be small (approximately 8,000 neurons), paired nuclei, situated bilaterally around the third ventricle above the dorsal border of the optic chiasm, and to have an elongated shape. The nuclei have an estimated volume of 0.068 mm^3, with a mean diameter measuring approximately 8 μm dorsomedially and 9.5 μm laterally (Van den pol, 1980). In the rostrocaudal direction, the nuclei are approximately 600-650 μm in length, 300 μm at the broadest lateromedial plane and 600 μm at the highest dorsoventral plane (Lydic et al., 1982; Card & Moore, 1984). Each of the SCN nuclei can function as an independent pacemaker, since the ablation of one nucleus does not alter circadian function. In fact, circadian function is not lost until at least 75% of both nuclei are destroyed (Van den Pol & Powley, 1979; Mosko & Moore, 1978). Moreover, based on results of experiments such as those of Schwartz et al., (1987), each cell of the SCN may be an individual oscillator, and all are normally coupled to form a network that functions as a single pacemaker. Schwartz and co-workers (1987), infused the sodium channel blocker tetrodotoxin (TTX) into the SCN of freely moving rats for 14 days, and found that this produced locomotor arrhythmia and an inability to entrain. Upon cessation of TTX infusion, rhythmicity and entrainment returned with the same phase and period as prior to infusion, indicating that the cells of the SCN were not destroyed and were still able to keep time, but that sodium action potential communication between the cells of the SCN is necessary for coherent rhythm generation.

The rat SCN has been well characterized immunohistochemically and at least 25 neuroactive substances or synthetic enzymes are associated with the nuclei. Transmitters include acetylcholine, vasopressin, somatostatin, gastrin-releasing peptide, vasoactive intestinal peptide, neuropeptide Y, substance P, gamma amino butyric acid, cholecystokinin and prolactin. It is not an aim of this thesis to review these and information can be found in: Card & Moore (1984; 1982) and Van den Pol & Tsujimoto (1985).

MULTIPLE OSCILLATORS

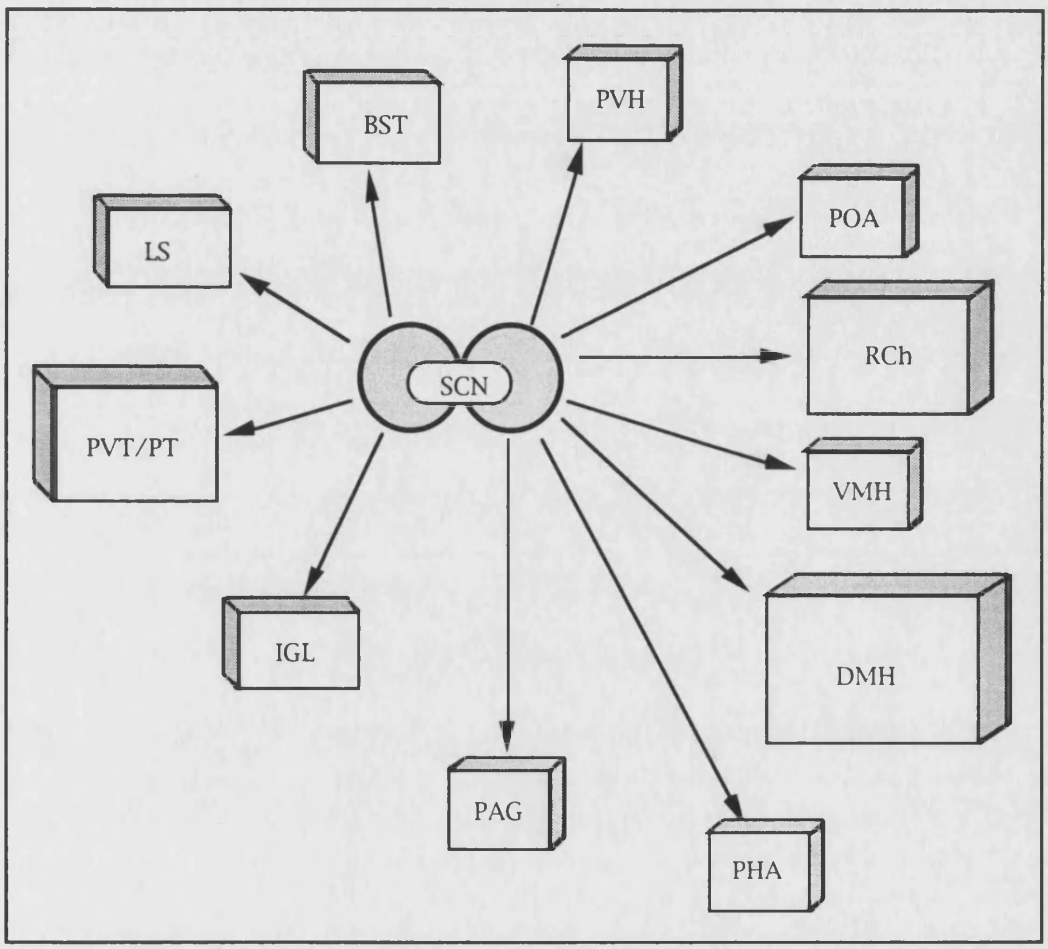
The diversity of rhythms eliminated by ablation of the SCN indicate at least a generalised contribution of the SCN to all rhythms. However, some data suggest that the SCN is not the only pacemaker. The presence of an extra-suprachiasmatic oscillator has been suggested by restricted-feeding experiments in which SCN-ablated rats (Mistleberger, 1990; Stephan, 1986) and hamsters (Mistleberger, 1993), anticipate daily feeding times. More recent evidence suggests that this feeding-entrainable pacemaker itself consists of more than one oscillator (Stephan, 1992). Despite areas such as the lateral geniculate nucleus and the retrochiasmatic area being proposed as the location of extra-SCN oscillators (Johnson et al., 1988b), no structure apart from the SCN has as yet been identified as having the required oscillatory properties.

The SCN itself may comprise more than one pacemaker. In humans maintained under constant conditions, the circadian cycles of body temperature and sleep/wake have been shown to break away from one another and run with different free-running periods. In this condition (known as spontaneous internal desynchronisation) the body temperature established a period of 24.8 hours whilst the sleep/wake cycle demonstrated a period of 33 hours (Wever, 1979). Since one oscillator cannot generate two different periods simultaneously, it seems that each rhythm is under the control of a different pacemaker. Although this break-away of the body temperature rhythm has not been demonstrated in rodents (Eastman & Rechtschaffen, 1983; Honma & Hiroshige, 1978), ample evidence of more than one oscillator in animals does exist. Hamsters maintained in constant light conditions of greater than 100-200 lux for more than 40 days display a splitting of the locomotor activity rhythm into two separate bands (Pittendrigh & Daan, 1976e). Based on similar observations, these researchers described the pacemaker in rodents as consisting of two separate oscillators coupled to each other in a stable phase relationship. Theoretically, one of these oscillators controls the evening onset of the rhythm, whilst the other controls the morning offset of the same rhythm. Practical evidence that has been suggested to support this theory includes the phenomenon of splitting, and characteristics of the rat pineal N-acetyltransferase (NAT) rhythm (Illnerova, 1991). These issues are discussed in full in Chapter V. From a practical point of view, dissociation of rhythms has important survival consequences. Tightly linked behaviours would be deleterious in situations of changing food supply and predation. Thus, a more rational role of the SCN would be to coordinate rhythms into a temporally dependent, entrainable circadian pattern that can be over-ridden when the situation demands.

HOW IS INFORMATION TRANSMITTED BY THE SCN?: EFFERENT PATHWAYS

Food and water intake, body temperature, the sleep/wake cycle, secretion of adrenal corticosterone and pituitary prolactin are all examples of functions that are believed to be under *neuronal* control by the SCN, since surgical isolation of this area results in arrhythmicity of these rhythms (Eskes & Rusak, 1985; Honma et al., 1984).

Figure 4. Efferent projections of the suprachiasmatic nucleus.



Key:
SCN, suprachiasmatic nucleus; BST, bed nuclei of the stria terminalis; LS, lateral septal nucleus; PVT/PT, paraventricular nucleus of the thalamus, paratanial nucleus; IGL, intergeniculate leaflet; AHA anterior hypothalamic area; PVH, paraventricular nucleus of the hypothalamus, MPO and POA, preoptic area nuclei; RCh, retrociasmatic area; VMH, ventromedial nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; ZI, zona incerta; PHA, posterior hypothalamic area; PAG, periaqueductal gray. (Redrawn from Watts, 1991).

However, elucidation of the efferent processes of the SCN responsible for generating rhythms in these funtions has been hampered by certain physical factors. The diameter of some of the fibres originating from the SCN are amongst the smallest in the brain

(Van den Pol 1980), making the traditional histological procedure of Golgi staining to reveal neuronal tracts virtually impossible. The position of the SCN means that the optic chiasm, the medial corticohypothalamic tract, the tuberoinfundibular tract and the supraoptic decussations are all close-by, making lesioning experiments to determine efferent projections uninterpretable. Recently, knowledge of the anatomical control of rhythms has improved with the use of the tracer plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHA-L: Gerfen & Sawachenko, 1984; see Figure 4), which can be detected by fluorescent antibody means following its injection into the SCN. Despite the advent of PHA-L, current data still do not provide an explanation of how SCN efferents are involved in mediating the circadian component of any one rhythm in particular.

What is known so far is that efferent projections appear to be highly localised and limited to parts of the hypothalamus and midline thalamus, with smaller projections into the lateral septal nucleus and periaqueductal gray (Van den Pol, 1980). The SCN has no long ascending or descending tracts, instead all connections are localised to the hypothalamus. Densest of all efferent connections is the intrahypothalamic projection to the subparaventricular zone, accounting for approximately $^3/_4$ of all SCN neural output (Watts, 1991). Like the SCN, the subparaventricular zone appears to be able to influence the limbic areas of the medial forebrain bundle, a structure which determines the level of arousal and attention for the expression of motivated behaviour. The SCN and subparaventricular nuclei achieve this via projections to the lateral septal and paraventricular nuclei; the former makes connections with the hypothalamus and ultimately with the hippocampus, whilst the latter projects to the hippocampus, amygdala and nucleus accumbens. Therefore, rhythmic circadian processes may be brought about by polysynaptic neuronal connections, with the first part of this relay system being the short intrahypothalamic SCN efferents. A second intrahypothalamic SCN projection of importance is that to the preoptic region, via which the SCN may be able to influence physiological processes such as fluid balance, reproduction, sleep and thermoregulation (Swanson, 1987). In the rat, but not the hamster, the SCN has been demonstrated to connect with the IGL of the thalamus (Morin et al., 1992). Inter-SCN connections appear to be more numerous in the hamster than the rat, although the neurotransmitters in these two pathways is still not known (Orpen & Steiner, 1994).

In addition to these neuronal connections, the SCN is postulated to produce diffusible signals that reach nearby hypothalamic sites and the cerebrospinal fluid (Silver & LeSauter, 1993); however, the nature of these humoral substances remains unknown. The work of Majzoube et al., (1991) suggests that these signals may be neuropeptides, since studies on vasopressin-containing neurons of the SCN indicate that cells of the SCN can release peptides into the extracellular fluid and the CSF.

SCN transplanation experiments have the potential to yield vital information on the neural efferents or diffusible subtances required for circadian transmission. In some instances, these experiments have shown the restoration of rhythmicity with very few

neuronal efferents being established from the graft SCN to the host tissue (Lehman et al., 1987; 1991), adding weight to the humoral theory of circadian control.
To summarise; present data indicate that the SCN has minimal efferent processes. Given the wide and varied range of rhythms which exhibit circadian control, it is still unclear how the small number of efferent projections characterised to date is sufficient to produce such far-reaching rhythmicity.

DEPRESSIVE DISORDER: SYMPTOMS, FACTS AND FIGURES

Depression is one of the affective disorders. The main feature of this group of diseases is an alteration of mood to such a degree as to prohibit functioning in every day life, and in the case of major depression, this mood change may ultimately be life-threatening in the form of suicide. Clinical depression is generally diagnosed according to the guidelines such as those set out in the American Psychiatric Association's Diagnostics and Statistics Manual (1987). This requires that certain symptoms be present during the same two-week period and represent a change from previous functioning. One of the symptoms must either be depressed mood or the loss of interest in pleasurable activities (anhedonia). In conjunction with this, at least five of the symptoms listed below must also be present on a daily basis. Furthermore, it must be established that the patient has no underlying organic disease and is not schizophrenic.

Diagnostic criteria for major depressive episode:

- significant weight loss or weight gain
- insomnia or hypersomnia
- psychomotor agitation or retardation
- fatigue or loss of energy
- diminished ability to think or concentrate
- feelings of worthlessness or innapropriate guilt
- recurrent thoughts of death or suicide

According to the American Psychiatric Association (1987), depression is the most common mental illness, affecting up to 14 million of the American population each year. The condition develops in 25% of females and in 10% of males during their lifetimes and as many as 70% of suicide victims have been shown retrospectively to have suffered from depression (Scrip Report, 1992).

BIOCHEMICAL THEORIES OF DEPRESSION

Noradrenaline (NA) and serotonin (5-HT)

Perhaps the best established biochemical theory to account for depressive disorder is the monoamine hypothesis of depression. Proposed by Schildkraut in 1965, this theory was based largely on clinical observations. Hypertensive patients treated with reserpine, (a drug known to block the accumulation of monoamines into synaptic vesicles, thereby reducing the amount available for synaptic transmission), were seen to exhibit depressive symptoms. Also, the anti-tuberculosis drug iproniazid caused mood-elevating effects in patients, and was subsequently shown to be an inhibitor of monoamine oxidase. The idea that there was a monoamine insufficiency at critical synapses in the brain gained further support by the association between the clinically effective antidepressants of the time (monoamine oxidase inhibitors and tricyclic antidepressants) and their known acute biochemical effect - the increase in noradrenergic transmission. However, the longer time required for therapeutic efficacy compared to biochemical action, and the development of antidepressant drugs with a different mode of action (such as mianserin and iprindole), were amongst the anomalies that challenged this hypothesis. Subsequently, this early hypothesis based on a presynaptic deficiency in neuronal function was superceded in popularity by biochemical theories concentrating on altered postsynaptic function. The focus of the postsynaptic hypothesis was the knowledge that most, if not all, clinically effective antidepressant drugs (including electroconvulsive therapy), desensitize noradrenergic transmission following their chronic administration. They achieve this either by down-regulating the density of β-receptors or by de-amplifying the cyclic AMP-generating system or both, regardless of the class of antidepressant (Baker & Greenshaw, 1989; Heninger & Charney, 1987; Sulser, 1986). However, this latest hypothesis is still under question and β-adrenoceptor down-regulation may not be a pre-requisite of antidepressant activity, since many newer medications appear not to have this effect (Okada & Tokumitsu, 1994).

In the same way that the emphasis of a fault in monoamine transmission changed from the pre- to the post-synaptic region, the emphasis on the neurotransmitter responsible changed from noradrenaline to 5-HT. As with noradrenaline, a role for 5-HT in depression was supported by the biochemical action of clinically effective drugs such as the serotonin selective reuptake inhibitors (SSRIs). Furthermore, evidence of down-regulation of 5-HT_2 receptors has also been shown following long-term antidepressant treatment (Baker & Greenshaw 1989; Heninger & Charney, 1987). Antidepressant research in many pharmaceutical companies is currently aimed at targeting more than one of the 5-HT receptor types at the synaptic cleft. Indeed, the next generation of antidepressant drugs is likely to be a group of compounds which affect multiple 5-HT sites around the synaptic cleft.

Pathological data can be found to support an abnormality in both the noradrenaline/5-HT monoamine systems. Elevated β-adrenoceptor (Mann et al., 1986) and 5-HT_2 receptor (Stahl, 1994) numbers have been observed in suicide victims, and the cerebrospinal fluid (CSF) levels of the noradrenaline/5-HT metabolites 3-methoxy-4-hydroxyphenylglycol (MHPG) (DeLeon-Jones et al., 1975) and 5-hydroxyindole acetic acid (5HIAA) (Traskman-Bendz et al., 1984), respectively, have been shown to be decreased in depressed patients. Some researchers believe that these two monoamine systems are functionally integrated at the neuronal level and favour a "Serotonin/Noradrenaline Link" hypothesis to account for the pathophysiology and pharmacotherapy of affective disorders (Sulser & Sanders-Bush, 1987).

Alternative neurotransmitter hypotheses

Some studies report low CSF levels of the dopamine metabolite, homovanillic acid (HVA) in depressed patients with a history of suicide attempts (Roy, 1994). In addition, some antidepressants cause presynatpic subsensitivity at the dopamine receptor as well as postsynaptic supersensitivity (Baker & Greenshaw 1989; Heninger & Charney, 1987), mechanisms which may be able to account for depressive symptoms such as anhedonia (Willner, 1983). However, whilst the clinically effective antidepressant drugs nomifensine and buproprion selectively block dopamine reuptake (Willner, 1983), compounds which are known to be more efficient at blocking reuptake, such as cocaine and amphetamine, are not effective antidepressants.

Another neurotransmitter recently proposed to be implicated in the pathogenesis of depressive illness is GABA. A range of antidepressant compounds have been shown to upregulate GABA receptors (Lloyd et al., 1985 & 1987), and CSF levels of GABA have been shown to be decreased in depressed patients (Gerner & Hare, 1981). In addition, GABA agonists such as alprazolam have been shown to demonstrate antidepressant effects, for example by reversing immobility in the 'forced swim' test (Flugy et al., 1992). This data adds weight to the hypothesis that the GABA/benzodiazepine/chloride complex is implicated in the action of antidepressants.

In conclusion, although patients afflicted by depression have been successfully treated with antidepressant drugs for many years, and 80-90% of sufferers respond to treatment, there is still no consensus on the physiological mechanisms responsible for depression or the pharmacological action of antidepressants. So, is depression linked to deviations in presynaptic and postsynaptic neurotransmitter function or is it due to a defect in another regulatory system such as the circadian system?

IS DEPRESSION A CIRCADIAN DISORDER?

Like any other physiological regulatory system, the circadian system can malfunction. When it does, the temporal coordination of psychological, behavioural, physiological, biochemical and hormonal rhythms, essential for health, is disturbed and illness may result. There is now a large body of evidence indicating that at least some forms of depression may involve a disruption of normal circadian rhythmicity (Van Cauter & Turek, 1986; Moore-Ede et al., 1982). The symptoms of depressive disorder which implicate disturbances of rhythms include the following:

1. Sleep disturbances - these are inextricably linked with depressive illness, with insomnia and early morning awakening being most characteristic (Gillin et al 1979). A reduced latency to the first REM episode in sleep is also reported (Kupfer, 1976), and this has been suggested to predict a favourable response to antidepressant medication (Rush et al., 1989).
2. Abnormal circadian patterns of hormone secretions, such as cortisol and prolactin (Linkowski et al., 1987).
3. Mood fluctuation in depressed patients, referred to as diurnal variation (DV).
4. The seasonal onset of certain types of depression suggests a chronobiological component in depressive disorder (Wehr & Rosenthal, 1989).

SUPPORT FOR A CIRCADIAN THEORY OF DEPRESSION

One form of depression which has an undeniable rhythmic component is that of seasonal affective disorder (SAD), which is characterized by recurrent depressive episodes in autumn and winter (the peak incidence occurring in December). Vulnerability to reduced environmental light (the dominant zeitgeber for mammalian circadian rhythms) is hypothesized to be the main precipitating factor of winter depression (Oren et al., 1994), and as much as 5% of the general population is affected by SAD (Schwartz, 1993). In contrast to typical endogenous depression, SAD is characterised by excessive fatigue and increased sleep, increased appetite in the form of carbohydrate craving and weight gain. The nature of the rhythm disturbance underlying SAD is a subject of controversy. A popular theory which has persisted is that SAD is due to a phase delay of the sleep/wake cycle with respect to the pacemaker (Sack et al., 1990); however, it has also been proposed that this disorder is due to an abnormal phase angle between the sleep/wake rhythm and the core body temperature rhythm (Kern & Lewy, 1990). Both the onset of melatonin secretion and the core body temperature rhythm have been shown to be phase delayed in SAD patients compared to controls (Dahl et al., 1993), although based on measurements of 6-sulphatoxymelatonin Wirz-Justice et al., (1993) argue against a phase-delay hypothesis for SAD.

In addition to displaying symptoms which can be described in terms of the biological clock and rhythms it controls, this form of depressive disorder has an even stronger link with circadian rhythm theories in that SAD has been shown to be ameliorated by bright light therapy (Lewy, et al., 1987; Lack & Wright 1993). Exposure to light greater than 2500 lux for more than 1 hour per day, reverses the symptoms of SAD within 3-4 days (Blehar & Rosenthal, 1989), and light therapy can be considered the most successful clinical application of circadian rhythm concepts in psychiatry to date. Light therapy has been used to support the idea that phase-delayed circadian rhythms underlie SAD, since bright light has been shown to advance body temperature and melatonin secretion in patients (Dahl et al., 1993). As with the nature of the underlying rhythm disorder, the time of day (morning or evening), when bright light therapy should be administered to SAD patients is also controversial (Wirz-Justice & Anderson, 1990; Wirz-Justice et al., 1993; Lafer et al., 1994). This issue should eventually be resolved when the full PRC to light pulses is established for humans (Minors et al., 1991).

Another condition which has lead to suggestions of a link between depressive disorder and circadian rhythms is that of shift work. In developed countries, approximately 10% of the active workforce is engaged in night/shift work. Shift workers are exposed to shifts of the environmental cues which normally synchronize human biological rhythms. Clinical intolerance to shift work has been defined by a set of medical complaints which have been likened to those in patients with endogenous depression; these include poor sleep quality, unusual irritability, malaise and feelings of inadequacy (Clancy & McVicar, 1994). In a study of ambulance workers, those which were more susceptible to intolerance to shift work displayed a greater tendency to internal desynchronisation of rhythms such as oral temperature, salivary cortisol and grip strength (Motohashi, 1992). Similarly, the symptoms of jet-lag have been likened to those in depressive disorder. With rapid travel across time-zones the phase of environmental cues is abruptly altered, and although individuals vary in their responses to travel across multiple time-zones, the symptoms commonly include sleep disruption, gastrointestinal disturbances, fatigue, headache, dizziness and loss of apetite. This general malaise is proposed by some researchers to be due to the different rates of resynchronisation of some overt circadian rhythms (Redfern, 1989).

There are also links between sleep, circadian rhythms and depressive symptomology. Most characteristic of sleep in depressive patients are the changes in REM sleep. Shortening of the time to the first REM episode and lengthening of its duration plus an increase in the amplitude of REM sleep are all reported in depressive individuals (Riemann et al., 1994). REM sleep disorders have been proposed to represent the expression of multiple circadian cycle abnormalities (Le Bon, 1992), whereas some researchers believe more specifically that a phase advance underlies the rhythm

abnormalities of sleep markers (such as REM sleep, growth hormone and cortisol secretion) in depressives (Mendlewicz, 1991). Total and partial sleep deprivation has been shown to alleviate depression, and there is a circadian variation of the propensity to relapse following daytime napping (Wiegand et al., 1993).

Finally, animal studies of the effects of antidepressant drugs on circadian rhythms go some way to supporting the circadian hypothesis of depression. The phase-delaying and period-lengthening effects of antidepressant drugs such as clorgyline (Duncan et al., 1988) and imipramine (Greco et al., 1990) are consistent with the hypothesis that disorders of period may be aetiologically related to depression (Wehr & Wirz-Justice, 1982). This issue is discussed in detail in Chapter III.

THEORIES OF CIRCADIAN RHYTHM DYSREGULATION

Since many studies in humans have shown an association between disturbances of circadian rhythms and depressive disorder, a number of different circadian theories have been proposed to account for the abnormalities in phase, period and/or amplitude of the physiological, behavioural and endocrine rhythms.

The desynchronisation hypothesis

A desynchronisation of some rhythms with respect to the 24 hour environment and to other internal rhythms was proposed to account for depressive illness by Halberg et al., (1968). As previously outlined, strong evidence to support this theory comes from the belief that the symptoms of jet-lag and shift work are due to an internal desynchronisation of rhythms with respect to the external environment and to each other (Deacon & Arendt, 1994; Motohashi, 1992). Additional evidence specific to depression has been recorded; for example, oral temperature in manic depressives was shown to be desynchronised from the 24 hour day (Kripke et al., 1978). However, more data exists to refute the desynchronisation hypothesis than to sustain it. For example, Wehr et al., (1985) and Souetre et al., (1988) both carried out studies on depressed patients and found circadian rhythms to be normal in terms of temporal organization.

The phase advance/phase delay hypotheses.

The phase advance hypothesis of major depression was first proposed by Papousek (1975). According to this hypothesis, the circadian system of depressed patients is believed to "run fast" (i.e. with a period of < 24 hours), leading to a chronic state of phase advance relative to the environment. This hypothesis can account for the early morning awakenings and early REM sleep episodes reported by some depressed patients (Wehr & Wirz-Justice, 1982). It may also account for the early acrophase of the

cortisol, prolactin and temperature rhythms seen in such individuals (Goetze & Tolle, 1987; Linkowski et al., 1987), and for the early acrophase of the noradrenaline metabolite MHPG in depressed patients (Piletz et al., 1994). Furthermore, the phase advance hypothesis is consistent with the idea that antidepressants "slow down" the biological clock (Wehr & Wirz-Justice, 1982). However, as with the internal desynchronisation hypothesis, data exists to repudiate the phase advance hypothesis (Goldenberg, 1993; Wehr et al., 1985). Some data suggest that the opposite type of rhythm disturbance, a phase delay, is responsible. Sack et al., (1985) showed antidepressant treatment to be potentiated following a phase advance of the sleep/wake cycle in depressive patients, and Lewy et al., (1987) showed that amelioration of SAD by bright light therapy was accompanied by a phase advance of the onset of melatonin secretion. Both pieces of evidence suggest that the original disorder was linked to a delayed rhythm phase. Therefore, the possibility that SAD is a problem of phase delay (Dahl et al., 1993), rather than phase advance, contributes to this type of abnormality also being associated with major depressive disorder.

The reduced amplitude hypothesis.

A reduction in rhythm amplitude is perhaps the most recent theory to emerge as an explanation of circadian dysfunction in depression. Candito et al., (1990) reported a reduced amplitude of plasma tryptophan in depressed individuals, whereas they found no evidence of an abnormal phase position of this rhythm. In fact, body temperature, plasma cortisol, thyroid stimulating hormone, noradrenaline, and melatonin are amongst those rhythms with reduced amplitude demonstrated to exist in depressed patients, which have returned to normal upon recovery from illness (Goldenberg, 1993; Goetze & Tolle, 1987; Hallonquist et al., 1986). In addition to the evidence for an antidepressant-induced increase in rhythm amplitude, Czeisler et al., (1987) have proposed that it is the amplitude-enhancing effect of light therapy which is therapeutically important. However, as with other hypotheses of circadian dysregulation, evidence exists which challenges the decreased amplitude theory (Von Zerssen et al., 1985).

Zeitgeber malfunction.

Abnormal social zeitgebers may also be the cause of some depressive symptoms. Szuba et al., (1992) highlight the incidence of 'spontaneous remission' of depressive illness on administration to psychiatric wards, and contemplate the possibility that this is due to stabilisation of the social zeitgebers on exposure to routine in such institutions.

SUMMARY

The process of circadian rhythm generation has been outlined along with what is currently known about the circuitry of the mammalian circadian system. Many aspects of this system remain to be clarified, such as the physiological role of the IGL and GHT pathway in rhythm generation. An aim of this thesis was to elucidate the role of these structures in the circadian rhythmicity in the rat using brain lesioning techniques.

In addition, the illness of depressive disorder has been discussed, and its potential aetiology in terms of biochemical and circadian theories has been presented. It is apparent that neither the biochemical tranmsitter nor the type of rhythm disturbance involved is known with certainty. Using wheel-running activity as the measured circadian rhythm, a further aim of this thesis was to ascertain whether there is a common link between the biochemical effect of antidepressants and circadian rhythm alterations. Finally, it was decided to analyse circadian rhythms within an animal model of depression since this is a little-researched area, and has the potential to yield important information on the involvement of circadian rhythms in depression.

CHAPTER II

Principles of circadian measurement

CIRCADIAN ACTIVITY MONITORING SYSTEM

The system used to monitor circadian rhythms in this series of experiments was similar to that used in a number of research labotatories worldwide (Looy & Eikelboom, 1989; Janik & Mrosovsky, 1994; Rosenwasser, 1989). The apparatus consisted of individual rodent activity wheels attached to a 24 hour continuous data acquisition unit within the 'wheel-room'; this acquisition unit was then connected to a computer containing a data analysis system external to this room. The wheel-room was light sealed and under independent lighting control. The room was entered daily at random times for food and water replenishment., and cages and wheels were changed approximately once every 4 weeks.

More specifically, the monitoring system used was the Dataquest III (version 5.0. Data Sciences Inc., Minesota, U.S.A.) hardware and software system. This comprised individual opaque plastic cages (56 x36 x18 cm) fitted with 35 cm diameter stainless steel running wheels (supplied by Mini-mitter, Sunriver, Oregon, USA). Attached to the side of each wheel was a magnet (3 x2 x0.5 cm) which triggered a pulse when it passed by a reed relay switch. Each reed relay was attached by a lead to a receiver below the cage and in turn each receiver was connected to a BCM100 consolidation matrix. The BCM100 is a switching matrix which provides power for the receivers and consolidates the signals from the receivers onto two 26 flat conductor cables leading to the computer. This system allows individual wheel revolutions to be recorded continuously in one room and the data to be stored and analysed in another room. Thirty individual wheels, each with their own receiver, were connected to four BCM's. Every 10 mins data from the BCM was downloaded to the IBM compatible personal computer in the adjacent room. Data was stored on the computer in the form of ASCII files which, when required, were transferred to the TAU data analysis program (Mini-Mitter Co. and Jonathon Schull; Oregon, U.S.A.). TAU converts the ASCII files into analyzable Chainfiles, and each Chainfile represents a single animal's continuous data from the beginning to the end of the experiment. Actograms were produced from these Chainfiles.

PRINCIPLES OF CIRCADIAN MEASUREMENT

Since the endogenous rhythm of the circadian pacemaker itself cannot be determined directly *in vivo*, its properties are studied by observing the behaviour of the overt rhythms it drives. In many circadian studies in rodents, the rhythm measured is running-wheel activity. All circadian measurements in the following experiments were made from actogram, periodogram and waveform plots of the circadian rhythm of wheel-running in male rats.

THE ACTOGRAM

An actogram is a collection of daily histograms with activity plotted in a vertical column, successive days on the y-axis and clock time on the x-axis. An example of an actogram for wheel-running produced using the TAU analysis program is given in Figure 5. Wheel revolutions are expressed as counts per 10 minute bin, and are marked in black. The bin (or cell) size can be altered to sum data into bigger or smaller aliquots of time. To clarify the picture of activity, 'biological noise' in the form of spontaneous wheel-runs can be removed by fitting a threshold to the actogram. For example, if a threshold of 50 is applied, this means that total wheel-revolutions per 10 minute bin which were less than 50 will not appear on the actogram and that 10 minute bin will be represented by a blank space. Similarly, applying limits to the actogram produces a clearer picture of activity. A limit of 50 means that all cells with greater than 50 wheel revolutions will be presented as a complete block. This prevents the TAU program from automatically scaling down all cells if one of the cells has a particularly high number of revolutions.
By convention, the same actogram is ploted side-by-side (double plotted), to allow easier visual inspection of the activity pattern. This means that the first horizontal line shows the activity for day 1 plotted continuously with that for day 2. The second horizontal line begins with day 2 data running into day 3 data etc.; therefore the abscissa represents 48 hours of real time.

PHASE MARKER

It is important to decide upon a phase marker for the rhythm being measured, since this provides a reference point to link the rhythm with the external environment. When measuring rodent locomotor activity, the onset of activity is commonly used as a phase marker (Colwell et al., 1990; DeCoursey, 1986), because onset has a small variability and is predictable from day to day. The onset of activity can be defined as a continuous 10 minute bin of wheel-running followed by at least one other such bin of continuous activity in the subsequent 30 minutes. In addition, it is preferable that an onset be preceded by at least 6 hours containing no activity (Lees et al., 1983). These are only guidelines, since in practice rat wheel-running activity is not so mechanical. The offset of activity is generally more variable than the onset (Aschoff, 1981), although it can in certain cases provide a strong enough signal for measurement (in the rat more so than in the hamster). By definition, the onset of daily activity is termed CT12 (where CT=circadian time).

CIRCADIAN TIME

In a free-running rhythm, real time of day is different from circadian time (CT), which takes its reference from the rhythm being measured. One circadian hour lasts $\tau/24$ of a real hour (where τ = the time for one complete cycle in hours). Thus any point in real time has its corresponding measurement in circadian time. If the onset of activity is taken

to occur at CT12, other circadian time points can be computed relative to this point. For example, if a treatment was to be carried out at CT18, in real time this would be 6 hours after the real time onset of activity, calculated as:

$$CT18 = (6 \times \frac{\tau}{24}) + CT12$$

For a nocturnal animal, the subjective night when the animal is active, lies between CT12-24, and is the light-sensitive period of the circadian cycle. The subjective day, when the animal is resting, lies between CT00-12 and is a period when the animal is unresponsive to light (Daan & Pittendrigh, 1976b).

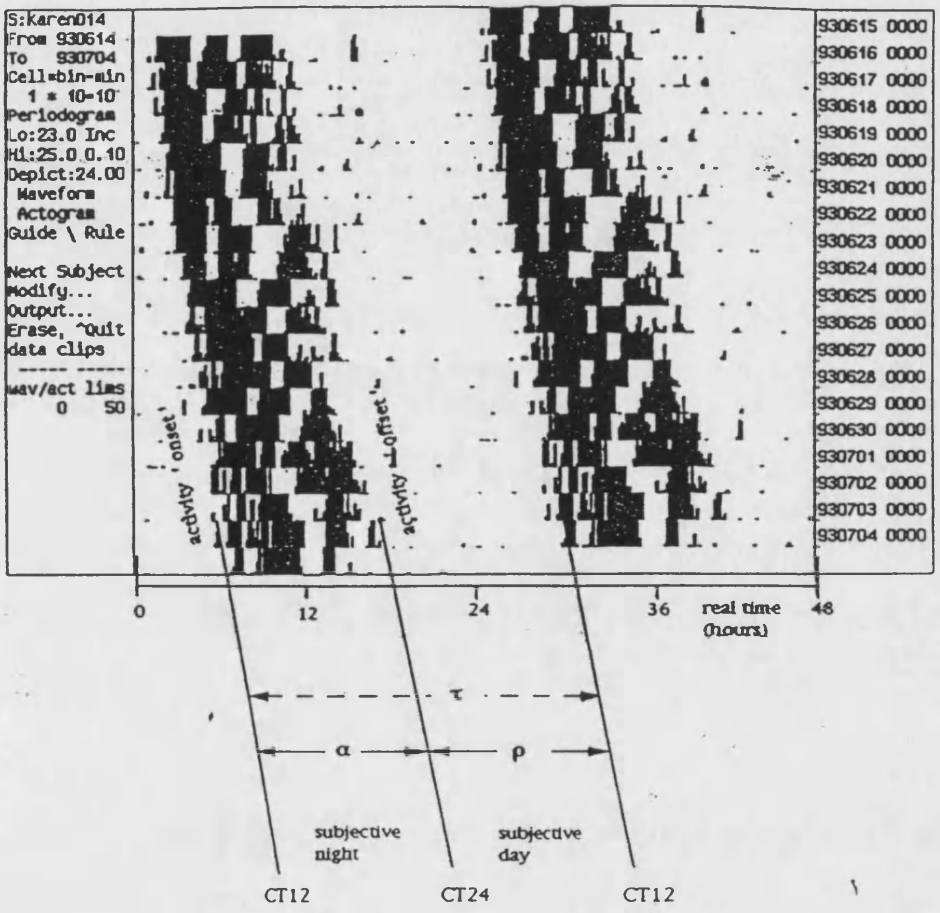


Figure 5. A double plotted actogram showing the wheel-running activity of a male Hooded Lister rat housed under constant dark conditions; where τ is the free-running period; α is the fraction of each cycle devoted to running wheel activity; ρ is the fraction of each cycle designated as the rest period. The ordinate indicates the number of days spent in constant dark conditions (DD), and the abscissa indicates the real time of day. The information given in the top left hand corner gives the identification number of the rat [S:karen014], the dates of the experiment "From and To", the bin size of 10 minutes and indicates that a limit of 50 wheel revolutions per bin size was applied to the actogram.

THE PERIODOGRAM

A periodogram is a form of spectral analysis of the activity data, and plots the period against a measure of the power of the periodic component (Whittaker & Robinson, 1964). An example of a periodogram is illsutrated in the upper half of Figure 6. The data used to generate the periodogram is the same block of data used in the actogram illustration. The periodogram provides an alternative way of estimating period compared to fitting a guide rule onscreen to the onsets of activity in the actogram. Any differences in the period value between the two methods may be due to subjective measurement by the observer in disregarding small bouts of activity which would be included in the computerised periodogram.

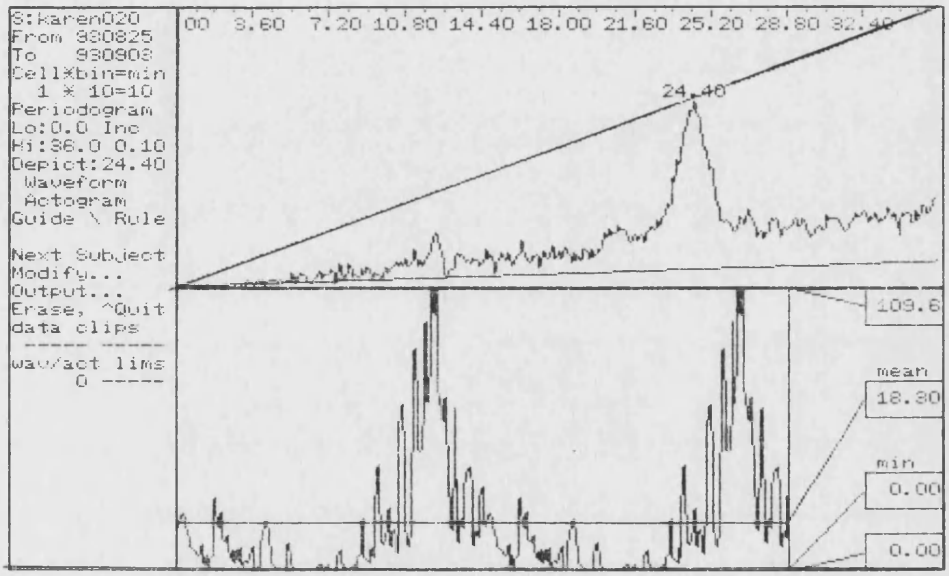


Figure 6. The upper half of the illustration displays a chi-square periodogram printout calculated by the TAU program. Each point on the vertical axis is the variance of the entire data set corresponding to that time point on the actogram. The range of periods is indicated at the top of the horizontal axis and the peak is labelled with the periodicity of the rhythm. The diagonal line represents 99% significance, whilst the line below the periodogram activity represents the 95% significance level. The lower half of the illustration displays a waveform plot calculated by the TAU program. In descending order the boxed numbers on the right hand side of the plot represent the maximum, mean and lowest number of wheel revolutions per 10 minute bin for this averaged cycle.

THE WAVEFORM PLOT

The lower half of Figure 6 shows a waveform printout for the same aliquot of data as that used in the actogram and periodogram. To obtain this plot, the actogram was first set to depict the period value for the rhythm. Each point on the waveform represents the average number of counts per corresponding cell in the actogram over the chosen

number of days. Thus, the waveform represents the activity of an average cycle for the block of data chosen.

For the series of experiments described in this thesis, the following quantitative information was derived from actograms, periodograms and waveform plots:

- τ was calculated in one of two ways. Either onscreen by the observer eye-fitting a guide rule to the activity onset/offset on the actogram, or by automatic periodogram calculation.
- The duration of activity time, α, was measured from the actogram as the interval between activity onset and offset, including any periods of inactivity/rest between these markers. Note that the value of this interval (converted to hours) was not a high precision measurement because of the poor definition in some activity offsets.
- The amount of activity per 10 minte bin in an average cycle A, was recorded from a periodogram prinout.
- The **phase angle of entrainment** was determined by ruling a line of best fit to steady-state activity onsets/offsets over a number of successive days (not less than 7 days were used), and extrapolating this line to the horizontal axis of the actogram. This gives the time of onset/offset in real hours indicating the phase relationship of the rhythm to a chosen point in the external environment.
- The amount of **phase shift** in a rhythm due to a particular perturbation was measured by eye-fitting lines to steady-state onsets both before and after the disturbance. These lines were then extrapolated and the difference between them at the point of disturbance was measured and converted into hours or minutes to give the amount of phase shift.

CHAPTER III

IGL lesioning studies

INTRODUCTION

The intergeniculate leaflet (IGL) was recognised to be a separate anatomical entity within the lateral geniculate nucleus (LGN) of the thalamus by Hickey and Spear (1976). It extends the entire length of the LGN (approximately 2mm in length in the rat) and is distinguishable along this length from adjacent dorsal and ventral geniculate nuclei (dLGN and vLGN). One IGL contains approximately 1500 neurons, all of which are reported to contain GABA (Moore & Speh, 1993; Card & Moore, 1989; Harrington et al., 1987; Meijer et al., 1984).

The IGL receives a direct retinal input (Morin et al., 1992), and influences the SCN via the geniculohypothalamic tract (GHT) (Harrington & Rusak, 1988; 1990). This innervation is bilateral, although the ipsilateral projection is more dense than the contralateral (Card & Moore, 1989). At least some of the retinal fibres innervating the IGL and vLGN are collaterals of retinohypothalamic tract (RHT) fibres (Pickard, 1985). As well as innervating the SCN, the IGL has a commisural projection to the contralateral IGL (Harrington et al., 1985; 1987; Pickard, 1985). In the rat, the neurons of the GHT contain NPY and GABA colocalised, whilst neurons projecting to the contralateral IGL contain GABA colocalised with enkephalin (Card & Moore, 1982; 1989; Moore & Speh, 1993; Takatsuji & Tohyama, 1989). However, in the hamster, the neurotransmitters in these two pathways are the same, i.e. GABA/enkephalin neurons project both to the SCN and the contralateral IGL (Morin et al., 1992). A further difference between these two species is that all NPY in the rat SCN has been shown to be provided solely by the GHT (Takasuji & Toyhama, 1989; Card & Moore, 1982), whereas there is thought to be an alternative source of NPY input to the SCN in hamsters (Harrington et al., 1985). The hamster and rat are used routinely in circadian system investigations, and results obtained are often used interchangeably. The differences highlighted above between these species mean that caution must be exercised in the interpretation of some of the data.

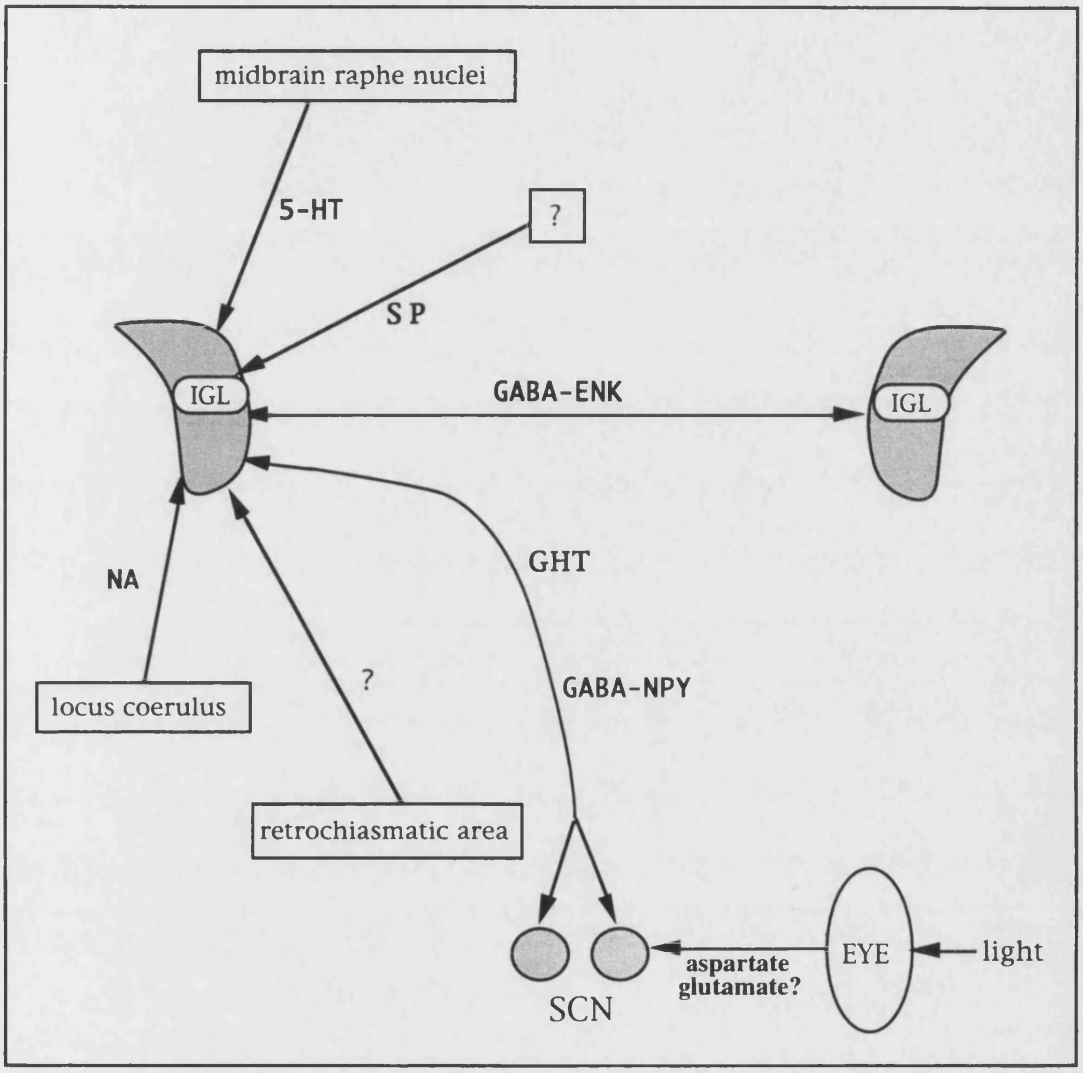
The IGL projections to the SCN are bilateral and terminate almost exclusively in the ventrolateral SCN, where they overlap with the RHT projection on VIP-containing neurons (Van den Pol, 1980; Van den Pol & Tsujimoto, 1985). The IGL receives a reciprocal input from the SCN itself and from areas surrounding the SCN (retrochiasmatic area; anterior hypothalamic area), thus providing a means of feedback from the SCN (Mistleberger et al., 1991a; 1991b). The connections of the IGL within the circadian system are illustrated in Figure 7.

PHOTIC ROLE OF THE IGL

As previously discussed, the IGL is the relay station of an indirect photic pathway (the GHT) from the retina to the SCN (Harrington et al., 1987). Whereas the RHT pathway is necessary for entrainment of rhythms (Pickard et al., 1987; Johnson et al., 1988), the GHT pathway is not thought to be necessary for photic entrainment, since destruction of this

pathway does not prevent entrainment to an LD cycle (Johnson et al., 1989; Dark & Asdourian, 1975). Although the IGL may not have a role in monitoring the abrupt changes in light and dark, it *is* thought to mediate the more subtle changes in environmental luminescence (Pickard et al., 1987).

Figure 7. Diagrammatic representation of the major connections of the IGL in the rat.



Diagrammatic representation of the major connections of the IGL. The IGL receives a serotonergic input from the midbrain raphe; a noradrenergic input from the locus coeruleus; input from the retrochiasmatic area (unknown transmitter); Enkephalin/GABAergic input from the contralateral IGL; input from the retina and Substance P input from an unknown source. The IGL projects to the contralateral IGL, and to the SCN via the GHT. NPY=neuropeptide Y; GABA=gamma amino butyric acid; NA=noradrenalin; 5-HT=serotonin; ENK=enkephalin; SP=Substance P (see Morin et al., 1992)

Destruction of the hamster IGL slows reentrainment of the activity rhythm to a phase delay, and alters the phase angle of entrainment of running wheel activity (Pickard, 1989). IGL destruction also causes hamsters to reentrain more slowly to a phase advance of the LD cycle (Johnson et al., 1989). IGL-lesioned hamsters tended to have shorter free-running periods in continuous light and were less likely to show splitting of activity

rhythms than sham-operated controls (Harrington & Rusak, 1988). Furthermore, hamsters maintained in continous light until they demonstrated split activity rhythms were more likely to re-fuse the split rhythm following IGL lesioning compared to sham-operated controls (Harrington & Rusak, 1988). Finally, the free-running activity period in continuous dark was greater than that of controls, and the lengthening of period normally seen on transfer to continous light was significantly reduced in IGL-lesioned hamsters (Pickard et al., 1987). Together, these results indicate a role for the IGL in mediating several photic effects on the circadian system.

NON-PHOTIC ROLE OF THE IGL

Non-photic PRCs exist to social interaction, cage change and novelty-induced running (Mrosovsky et al, 1989). Similar PRCs also exist in response to dark pulses administered to animals maintained in constant light conditions (Boulos & Rusak, 1982a). The phase shifts which produce both of these types of PRC are thought to occur because of the activity these stimuli induce in animals (Reebs et al., 1989; Van Reeth & Turek, 1989; Janik & Mrosovsky, 1994). Injection of NPY, (the major neurotransmitter of the GHT), into the SCN (Albers & Ferris, 1984; Rusak et al., 1989), and electrical activation of the IGL (Rusak et al., 1989), both produce a PRC similar to the dark-pulse and the cage-change PRC. Furthermore, lesioning the hamster IGL blocks dark pulse-induced phase shifts (Harrington & Rusak, 1986). Janik and Mrosovsky (1994) have suggested that IGL lesioning in some way prevents the generation of locomotor activity, and that the effects of IGL lesions on circadian rhythms are due to the loss of non-photic, rather than photic, input. Since an increase in activity is known to accelerate reentrainment to a phase advance (Mrosovsky et al., 1992; Sisk & Stephan, 1981), decreased activity could have the opposite effect and slow reentrainment. This combined evidence implicates the IGL in non-photic transmission of information to the SCN. Constant light is known to have an inhibitory effect on activity in rodents (Moore-Ede et al., 1982) and whilst the failure of IGL-ablated animals to lengthen tau when transferred to constant light conditions may arise because of the loss of a photic input to the SCN, it may also be because the hamsters are somehow prevented from responding to the increased illumination by an alteration in activity (Janik & Mrosovsky, 1994). In one of the few cases in which the amount of activity was monitored in an IGL-lesion experiment, Johnson et al., (1988) found that lesioned animals displayed a decrease in the average amount of wheel-running activity in 24 hours plus a reduced rate of reentrainment to a 6 hour phase shift.

IGL LESIONS

The involvement of the IGL in the circiutry of the mammalian circadian timing system has been investigated by lesioning the IGL with radiofrequency (Harrington & Rusak, 1986; 1987; Pickard et al., 1987) and electrolytic techniques (Dark & Asdourian, 1975; Legg &

Cowey, 1977; Janik & Mrosovsky, 1994) lesions. However, these techniques are non-selective and often result in mass destruction of the area of interest, making interpretation of results difficult. In an attempt to demonstrate IGL modulation of circadian activity in the golden hamster, Pickard et al., (1987) based their conclusions on lesions which destroyed not only the LGN (dorsal and ventral, plus IGL), but also involved damage to the fornix, optic tract, zona incerta and hippocampus. An improvement on these methods was the advent of excitotoxins as lesioning tools. The excitotoxin NMDA, selectively destroys cell bodies and leaves fibres of passage unaffected, and afferents that terminate in the area, minimally affected (Hastings et al., 1985).

Figure 8. Coronal section of the IGL at 4.30 mm rostral to bregma.

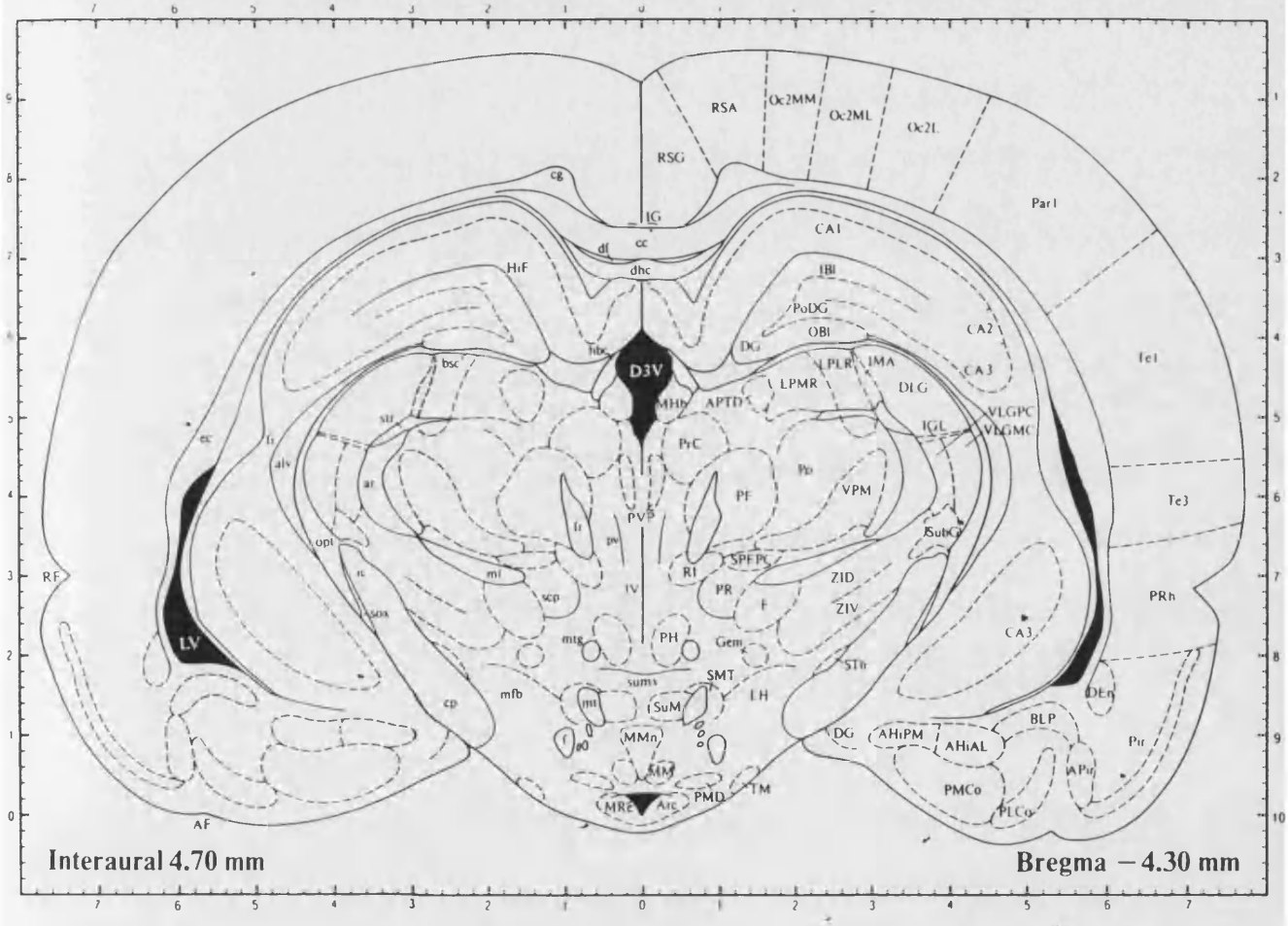


Figure 35

3V 3rd ventricle
AF amygdaloid fissure
AHiAL amygdalohip area, anterolat
AHiPM amygdalohip area, posteromed
alv alveus hippocampus
APir amygdalopiriform transition
APTD anterior pretectal nu, dorsal
ar acoustic radiation
Arc arcuate hypoth nu
BLP basolateral amygdaloid nu, post
bsc brachium superior colliculus
CA1-3 fields CA1-3 of Ammon's horn
cc corpus callosum
cg cingulum
cp cerebral peduncle, basal
D3V dorsal third ventricle
DEn dorsal endopiriform nu
df dorsal fornix
DG dentate gyrus
dhc dorsal hippocampal commissure
DLG dorsal lateral geniculate nu
ec external capsule
f fornix
F nu fields of Forel
fi fimbria hippocampus
fr fasciculus retroflexus
Gem gemini hypoth nu
hbc habenular commissure
HiF hippocampal fissure
IBl inner blade dentate gyrus
ic internal capsule
IG indusium griseum
IGl intergeniculate leaf
IMA intramedullary thal area
LH lateral hypoth area
LPLR lat post thal nu, laterorostral
LPMR lat post thal nu, mediorostral
LV lateral ventricle
mfb medial forebrain bundle
MHb medial habenular nu
ml medial lemniscus
MM medial mammillary nu, medial
MMn medial mammillary nu, median
MRe mammillary recess 3rd ventricle
mt mammillothalamic tract
mtg mammillotegmental tract
OBl outer blade dentate gyrus
Oc2L occipital Cx, area 2, lateral
Oc2ML occipital Cx, area 2, mediolat
Oc2MM occipital Cx, area 2, mediomed
opt optic tract
Par1 parietal cortex, area 1
Pf parafascicular thal nu
PH posterior hypoth area
Pir piriform cortex
PLCo posterolateral cortical amyg nu
PMCo posteromed cortical amyg nu
PMD premammillary nu, dorsal
Po posterior thal nuclear group
PoDG polymorph layer dentate gyrus
PR prerubral field
PrC precommissural nu
PRh perirhinal cortex
pv periventricular fiber system
PVP paraventricular thal nu, post
RF rhinal fissure
RI rostral interstitial nu mlf
RSA retrosplenial agranular Cx
RSG retrosplenial granular Cx
scp superior cerebellar peduncle
SMT submammillothalamic nu
sox supraoptic decussation
SPFPC subparafascicul thal nu, parvo
STh subthalamic nu
str superior thal radiation
SubG subgeniculate nu
SuM supramammillary nu
sumx supramammillary decussation
Te1 temporal cortex, area 1
Te3 temporal cortex, area 3
TM tuberomammillary nu
VLGMC vent lat genicul nu, magnocel
VLGPC vent lat genicul nu, parvocell
VPM ventral posteromedial thal nu
ZID zona incerta, dorsal
ZIV zona incerta, ventral

Unfortunately, in practice, the amount of destruction seen with NMDA is still large, extending to areas such as the dLGN and vLGN; ventral posterolateral thalamic nucleus

(VPL); reticular thalamic nuclei (Rt); stria terminalis (St); ventrolateral geniculate nucleus, magnocellular and parvocellular (VLG (mc/pc); subgeniculate nucleus (SubG); hippocampus (CA1; CA2; CA3); occipital cortex, area 2, mediolateral (Oc2ML); occipital cortex, area 2, lateral (Oc2L); parietal cortex area 1 (Par 1); polymorph layer, dentate (PoDG); intermedullary (IMA); medial geniculate nucelus ventral (MGV); marginal zone medial geniculate (MZMG); medial geniculate nucelus dorsal (MGD) (Johnson et al., 1988; 1989; Smale & Morin, 1990). Hence, in an animal lesioned with NMDA, it is difficult to attribute changes in behaviour to ablation of the IGL alone; in fact Smale and Morin (1990) attributed altered photoperiodic responses in the hamster to hippocampal damage, when the intended area for lesioning was the lateral geniculate nucleus. Figure 8. shows a coronal section 4.30 mm from bregma at the level of the IGL (Paxinos & Watson, 1986); the surrounding structures referred to above, which are often accidentally lesioned when the IGL is the target, are also shown in this Figure.

EXCITOTOXINS AS LESIONING TOOLS

Olney (1969), was amongst the first to discover that prolonged exposure of central neurons to the excitatory amino acid (EAA) glutamate, resulted in neuronal cell death. Initially the idea that a substance found in high concentrations (4mM) in the CNS could be neurotoxic was met with scepticism. However, the excitotoxic theory of neuronal damage has now been established (Choi, 1988; Olney et al., 1986; Rothman & Olney, 1987). Although the classification is constantly being reviewed, EAA receptors can be broadly divided into NMDA (NMDA and ibotenate) and non-NMDA (kainate and quisqualate) receptors (Watkins, 1984; Winn, 1991). EAA receptor density varies throughout the CNS such that different excitotoxins are selectively potent in different areas (Watkins, 1984). For example, in the nucleus basalis magnocellularis, the order of potency was kainate>NMDA=ibotenate>quisqualate, whilst quisqualate was more specific than the other neurotoxins in that it produced the least damage to surrounding areas whilst still producing the desired effect of cortical deafferentation. Kainic acid produced the most extensive damage in this structure, and also caused seizure activity in some animals (Dunnett et al., 1987).

EXCITOTOXIN MECHANISM OF ACTION

Excitotoxins bind to EAA receptors on neuronal dendrites, resulting in rapid swelling caused by depolarisation, chloride influx and water entry; slow necrosis secondary to calcium accumulation and finally cell death (Rothman & Olney, 1987; Choi, 1985). Calcium is believed to enter the cell through channels directly gated by the excitotoxins, and an increase in intracellular calcium appears to be a universal feature of excitotoxic cell death. However, different EAAs cause different amounts of calcium entry, for example calcium influx stimulated by NMDA is greater than that stimulated by kainate (MacDermott et al., 1986). Calcium-activated enzymes involved in excitotoxicity include

proteases, protein kinases, phosphatases, phospholipases and endonucleases. Their relative contribution differs according to the excitotoxin involved and the specific cell type, e.g. nitric oxide synthetase appears to contribute to NMDA toxicity but not AMPA toxicity (Moncada, et al., 1992).

It can be seen that excitotoxins are intrinsically different in the mechanism and amount of cell death that they cause. Furthermore, destruction by a particular excitotoxin differs between tissues depending on the receptor density for that excitotoxin within the particular tissue.

AIMS

The majority of IGL lesion experiments to date have been performed in hamsters, and as outlined in the Introduction to this Chapter, species differences exist in the circadian system between the hamster and rat. An aim of the present study was to clarify the role of the IGL in circadian rhythm control in the rat. In addition, evidence in the literature stresses the importance of taking into account changes in activity levels when interpreting behaviour in IGL lesioned animals. It was therefore hoped to monitor this parameter in IGL-lesioned rats. However, the initial aim of this study was to determine whether a more selective lesion of the IGL was possible in comparison with existing studies. Previously, the only excitotoxin used to lesion the IGL has been NMDA, and researchers have reached conclusions based on results of lesions which destroyed not only the IGL, but much of the surrounding tissue. The goal in this study was therefore to use different excitotoxins in the hope of producing more specific IGL lesions.

METHOD

Subjects

Male Hooded Lister rats (250-350 g body weight at the beginning of the experiment; supplied by Charles River, Margate, Kent) were group housed (6 per cage) in opaque plastic cages (45 cm long, 24 cm wide and 20 cm high) with standard rat chow (Special Diet Services, Witham, Essex), and water being freely available. Ambient temperature was 23°C and the lighting schedule was a 12:12 hour light/dark cycle (white light: lights on 07.00h, 170 lux, lights off 19.00h). All animals were weighed, singly housed and moved to the operating theatre 24 hours prior to surgery.

Anaesthesia

Animals were anaesthetized with a single i.p. injection of a Sublimaze® (fentanyl 0.6 mg/Kg) and Domitor® (medetomidine 0.03 mg/Kg). A suitable level of anaesthesia for the operation was characterised as a loss of the righting reflex, loss of the blink reflex and no

response to foot/tail pinch. A constant state of anaesthesia was maintained by further administration of a half-maximal dose of fentanyl and medetomidine if required.

Surgical Procedure

The top of the animal's head was shaved and the skin cleaned with antiseptic solution (Hibitane®). The animal was then positioned in a Kopf stereotaxic frame with ear bars preventing horizontal movement of the head and an incisor bar and nose-bar preventing vertical movement. The incisor bar was set at -3.0±0.2 mm below the intra-aural line. A saline-dampened piece of gauze was placed over the eyes and the animal was covered with a surgical drape up to the neckline. An incision (approximately 2 cm) was made along the

Table 1: Stereotaxic coordinates used for lesions of the IGL.

	AP (anterio-posterior) distance (mm)	ML (distance from midline) (mm)	DV (distance from cortical surface) (mm)
intergeniculate leaflet	4.25	4.0±1	5.5

midline of the head with a scalpel, the skull surface was exposed and the skin and membranes were held back with wound clips. The top of the skull surface was cleaned and IGL coordinates were measured from bregma using the atlas of Paxinos & Watson, (1986).

Injection Procedure

A 1mm diameter hole was drilled through the skull, and the top of the position of the duramater was measured for use in the DV calculation. A 27-gauge bevelled needle was lowered to the correct position and the injection of excitotoxin was made. All injections were of 0.5 µl over 2 mins (infusion rate of 0.25 µl/min) with a Harvard injection pump. The needle was left in position for a further 6 mins to allow diffusion away from the tip. Excitotoxins were dissolved in phosphate-buffered saline (PBS) and the pH adjusted to 7.4 by the addition of NaOH where necessary. Unilateral and bilateral lesions of the IGL were performed using three different types of excitotoxin; quisqualate (0.2M), kainate (1, 2, 5 or 10 mM) and ibotenate (10 mg/ml; Gray et al., 1993). At the end of the injection procedure, the head wound was sutured with Ethilon 6/0 thread and the animal was removed from the stereotaxic frame (Johnson et al., 1988).

Anticonvulsant administration

It was discovered (after the second animal had been injected with quisqualate), that animals experienced convulsions following injection of quisqualate into the IGL. It was therefore decided to administer the anticonvulsant drug valium® (diazepam; 1 mg/Kg i.m.)

immediately after the injection needle had been withdrawn, and before the animal was reversed from anaesthesia.

Reversal of anaesthesia

Anaesthesia was reversed within 1-2 minutes following a single i.p. injection of Nubain® (nalbuphine) and Antisedan® (atipamezole) (each at a dose of 0.02 mg/Kg). Animals were then returned to their individual cages for a 90 min observation period, during which time body temperature was maintained by warming lamps.

Sham-operated animals

For control purposes, animals were injected with 0.5µl of PBS into the IGL area instead of excitotoxin. Otherwise, the protocol of anaesthesia, IGL injection, anticonvulsant administration and anaesthetic reversal was the same as for excitotoxin injections.

Table 2: Sources of drugs and chemicals used in the lesioning procedure.

Drug/chemical	Source
quisqualic acid	Sigma Chemicals, Poole, Dorset, UK.
kainic acid	"
ibotenic acid	"
n-methyl-d-aspartate	"
medetomidine	SmithKline Beecham Animal Health Ltd., Surrey, UK.
fentanyl citrate (Sublimaze®);	Janssen Pharmaceuticals Ltd., Oxford, UK.
nalbuphine hydrochloride (Nubain®);	DuPont Pharmaceuticals Ltd., Hertfordshire, UK.
atipamezole hydrochloride (Antisedan®)	Norden Laboratories, Hertfordshire, UK.
diazepam (Valium®)	Roche Products Ltd., Hertfordshire, UK.
Ethilon 6/0 Monofilament polyamide 6 suture.	Ethicon Ltd., U.K.

HISTOLOGY

IGL lesioning was assessed in two ways. Firstly by cresyl violet cell body staining which reveals areas of gliosis where cell bodies have been destroyed (Murabe, et al., 1981). For this type of staining, sectioning on the cryostat was started approximately 0.75 cm rostral to the lesion hole and every third section (20 µm thick) was taken until approximately 0.75 cm rostral to the lesion. Slices were put directly onto a gelatin-coated slide.

Secondly, the effectiveness of the lesion in terms of IGL destruction, was determined using immunocytochemical staining for NPY in the SCN. A successful bilateral IGL lesion results in the total loss of NPY-immunoreactive staining in the SCN (Takasuji & Toyhama, 1989; Card & Moore, 1982). Unilateral lesioning would result predominantly in loss of NPY in the ipsilateral SCN, with a minor reduction in NPY on the contralateral side

(Card & Moore, 1989). For NPY staining, sectioning by cryostat was started from the tip of the frontal cortex and proceded rostrally at 50 μm sections. The change in shape of the optic tract was used as a guide for the approach of the SCN region. Slices rostral and dorsal to the SCN were collected in phosphate buffer prior to the NPY-staining procedure.

1) Cresyl violet cell body staining method

Five days following surgery, the animal was given a lethal injection of Euthatal® (sodium pentobarbitone; 100 mg/kg i.p.), guillotined, and its brain quickly removed. Following preservation in 4% phosphate buffered formalin for a minimum of 72 hours, the brain tissue was fixed onto a cryostat block with Cryo-m-bed® and rapidly frozen with Arcton gas. Every third coronal section (20 μm thick) was mounted on gelatin-coated slides and left to air dry for approximately 1 hour. Each slide containing tissue sections was treated as described in Table 3.

Table 3. Cresyl violet cell body staining in rat brain tissue sections.

Stage	Procedure
1	Immersed in 70% Industrial methylated alcohol (I.M.S.) for 5 mins
2	Immersed in distilled water for 5 mins
3	Stained with 1% cresyl violet for 15 - 20 mins
4	Dipped in and out of a solution of 70% alcohol containing 2 drops of glacial acetic acid per 350-400 mls alcohol
5	Immersed in 70% alcohol for 3 mins
6	Immersed in 90% alcohol for 3 mins
7	Immersed in 95% alcohol for 3 mins
8	Immersed in 100% alcohol for 3 mins
9	Immersed in 100% alcohol for 3 mins
10	Immersed in Histoclear for 5 mins
11	Immersed in Histoclear for 5 mins
12	Mounted in a DPX medium, coverslip put on and and left to air dry.

2) Immunocytochemical staining of rat brain slices

The soluble peroxidase-anti-peroxidase (PAP) method developed by Sternberger (1970) was used to reveal NPY-immunoreactivity. In this procedure, anti-peroxidase is combined with the peroxidase before tissue application, resulting in three peroxidase to two antibody

molecules, which enhances the sensitivity of the method. Briefly the procedure involves application of a primary antibody, raised in rabbit and specific to the antigen (NPY), to the tissue section. Secondly, anti-rabbit IgG (in this case raised in goat) is applied in excess. Since this antiserum is present in excess, only one of the two Fab (fragment antigen binding) sites attaches to the Fc (fragment crystalline) site of the primary antibody, the second Fab site remains free. In the third step, PAP complex is applied and an Fc portion of one of the rabbit IgG molecules in the complex combines with the free Fab arm of the goat anti-rabbit IgG. The peroxidase component of the complex is then visualised by enzyme histochemistry using diaminobenzidine (DAB) as the chromagen (Kowall & Beal, 1988).

Tissue preparation

To enable the immunocytochemical visualization of NPY in the rat brain, tissue was chemically fixed *in vivo* with a paraformaldehyde fixative (4% phosphate buffered formalin). The animal was administrered an overdose of Euthatal (100 mg/kg i.p.) 5 days following surgery. Upon cessation of breathing (but with the heart still beating), the abdomen and thorax was opened up to the neck and the chest cavity revealed. After penetrating the pericardium, the left ventricle was slit to allow insertion of a perfusion needle which was passed up into the aorta. To prevent blood clotting, 20 mls of 0.1M sodium phosphate buffer containing 0.1mls of 1000 unit heparin was perfused at a rate of 33 mls min^{-1} using a peristaltic pump. This was followed by perfusion of 400 mls of a fixative solution of 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4). At the start of the perfusion the blood supply to the lower limbs was occluded, and the right atrium cut to allow out-flow of the perfusate. The brain was then removed and stored in buffered paraformaldehyde solution overnight before transfer to a 15% sucrose in sodium phosphate buffer solution. The brain remained in the dehydrating (cryporotectant) sucrose solution until it sank to the bottom of the vessel, indicating loss of water from the tissue to the surrounding medium under an osmotic gradient. The brain tissue was then rapidly frozen to a cryostat block and treated as described in Table 5.

Table 4 gives the composition of the phosphate buffer used as the medium in which to collect tissue sections for NPY-staining, and to which 4% formalin solution was added for use as a fixative.

Table 4: Sodium phosphate buffer preparation - 0.2M, pH 7.4

	mol. wt.	g/25 mls dist. H_2O	M
Na_2HPO_4	141.96	0.7098	0.2
NaH_2PO_4	120.0	0.6	0.2

Table 5: Procedure for immunocytochemical staining of NPY in rat brain tissue.

Stage	Procedure
1	50 µm sections were cut and free-floated in 0.1 M sodium phospate buffer, pH 7.4
2	Sections were washed 3 times for 5 mins each in 0.1 M sodium phospate buffer, pH 7.4
3	Tissue was incubated in methanol containing 1% H_2O_2 for 30 mins.
4	Samples were washed 3 times for 5 mins each in 0.1 M sodium phospate buffer, pH 7.4
5	Tissue was incubated in 0.1M sodium phosphate buffer containing 1% Triton X100 and 4% goat serum (to block background IgG i.e. non-specific binding) for 1 hour.
6	Samples were washed 3 times for 5 mins each in 0.1 M sodium phospate buffer, pH 7.4
7	Samples were incubated with the appropriate antibody overnight at 4°C
8	Samples were washed 3 times for 5 mins each in 0.1 M sodium phospate buffer, pH 7.4
9	Samples were incubated in biotin anti-rabbit solution for 45 mins (100ml : 25ml 1% goat serum in 0.5 % Triton X100 in 0.1 M sodium phospate buffer).
10	Samples were washed 3 times for 5 mins each in 0.1 M sodium phospate buffer, pH 7.4
11	Solutions A (avidin) and B(biotin) from a Vectastain kit* were mixed together 30 mins prior to step 12
12	Samples were incubated for 45 mins in avidin-biotin peroxidase (100µl each of A and B from Vectastain kit : 25µl 1% goat serum in 0.5 % Triton X100 in 0.1 M sodium phospate buffer).
13	Samples were washed 3 times for 5 mins each in 0.1 M sodium phospate buffer, pH 7.4
14	Samples were incubated with DAB* for >20 mins to produce a black stain.
15	Samples were washed in distilled water for 5 mins.
16	Samples were mounted on gelatinised slides.
17	Slides were immersed in 70% alcohol for 5 mins
18	Slides were immersed in 100% alcohol for 5 mins
19	Slides were immersed in 100% alcohol for 5 mins
20	Slides were immersed in Histoclear for 5 mins.
21	Slides were immersed in Histoclear for 5 mins.
22	Slides were mounted in DPX medium, coverslipped and and left to air dry.

* Vector Laboratories Ltd., Peterborough, UK.

RESULTS

Figures 9-14 illustrate the SCN and the IGL from a control (non-sham, non-lesioned) animal. The SCN and IGL are stained with cresyl violet cell body stain to reveal the overall shape of these structures, and stained for NPY-immunoreactivity within these structures.

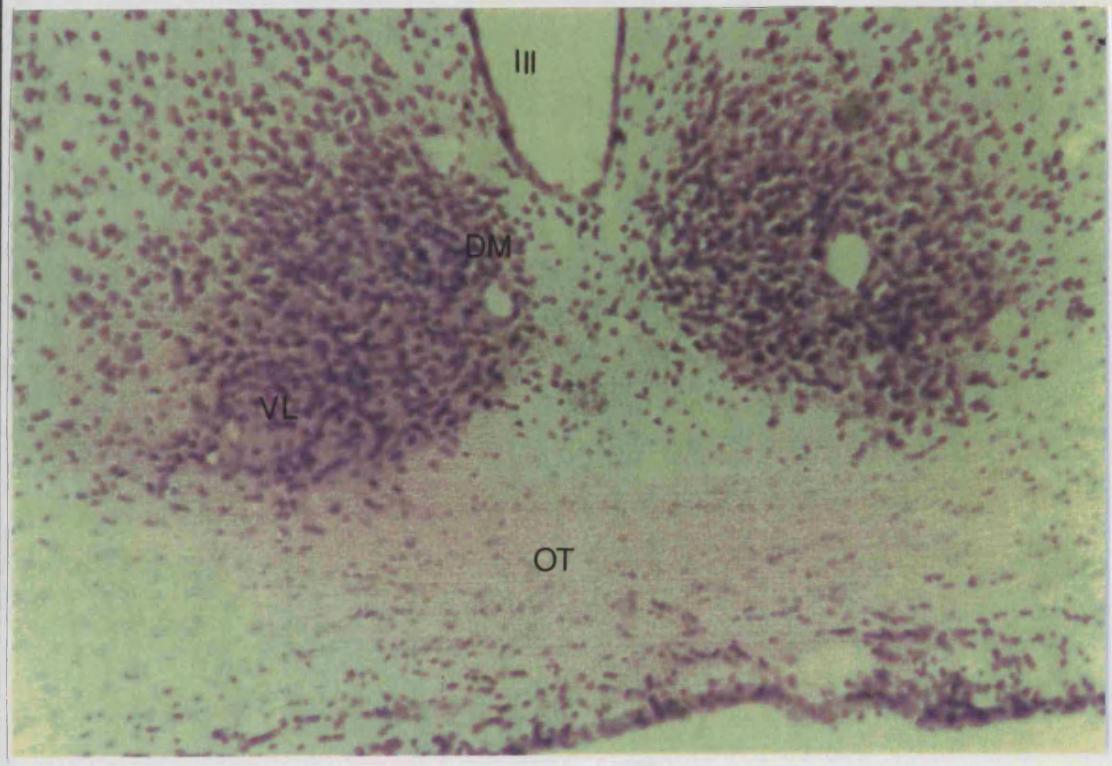


Figure 9. Cresyl violet cell body stain revealing the SCN. OT=optic tract; VL= ventrolateral SCN; DM=dorsomedial SCN; III=third ventricle. 10/0.3magnification.

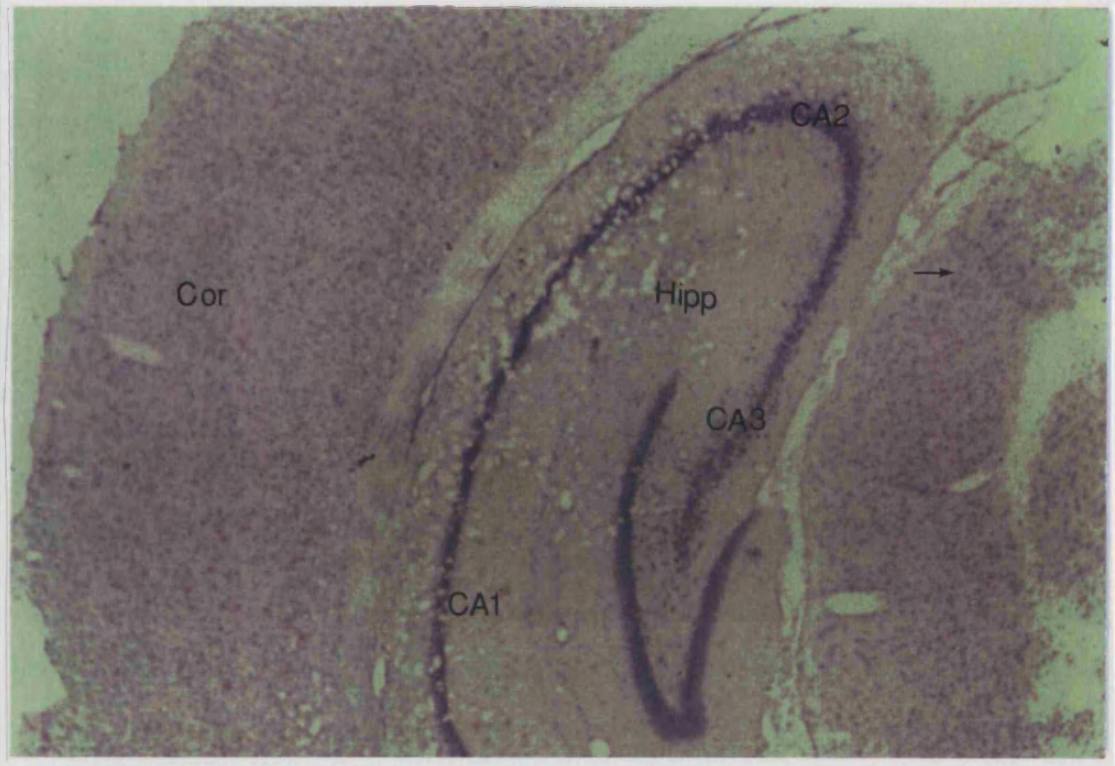


Figure 10. Cresyl violet stain revealing the IGL (indicated by the arrow) of the thalamus. Cor=cortex; Hipp=hippocampus including layers CA1; CA2; CA3. 2.5/0.08magnification

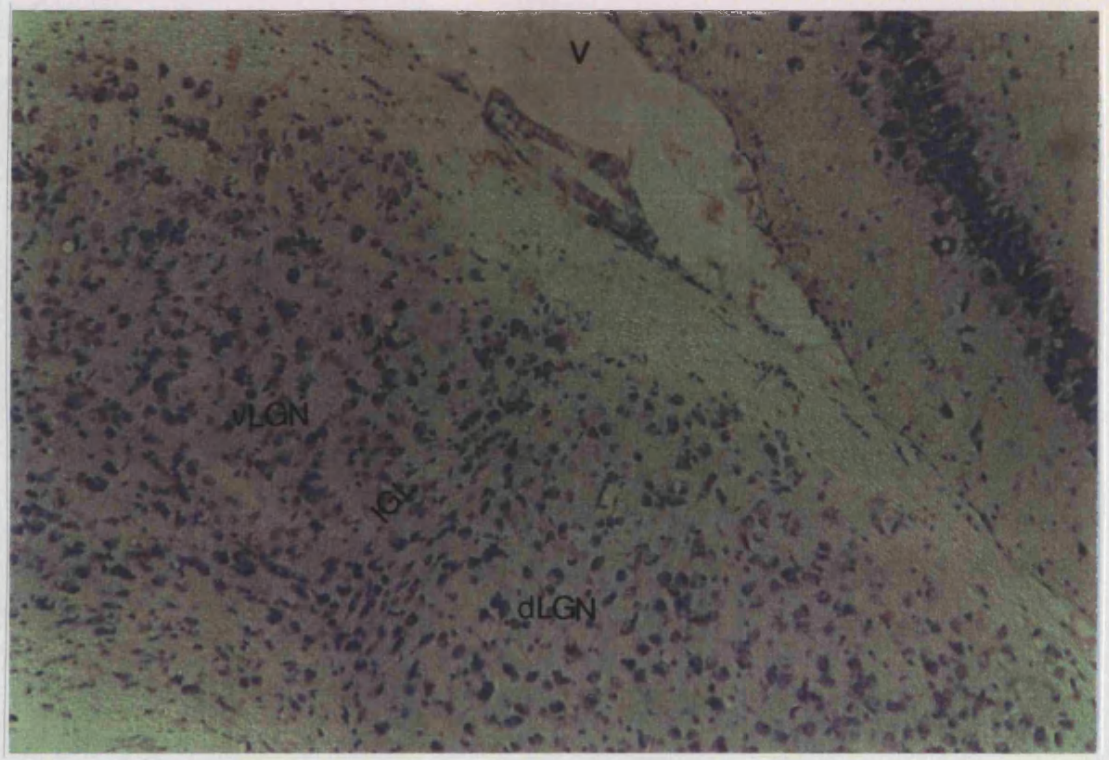


Figure 11. Close-up of a cresyl violet stained IGL. The cell bodies having a different orientation within the thalamus are cells of the IGL. dLGN=dorsal lateral geniculate nuclei; vLGN=ventral lateral geniculate nuclei; OT=optic tract; V=lateral ventricle. 10/0.3magnification.

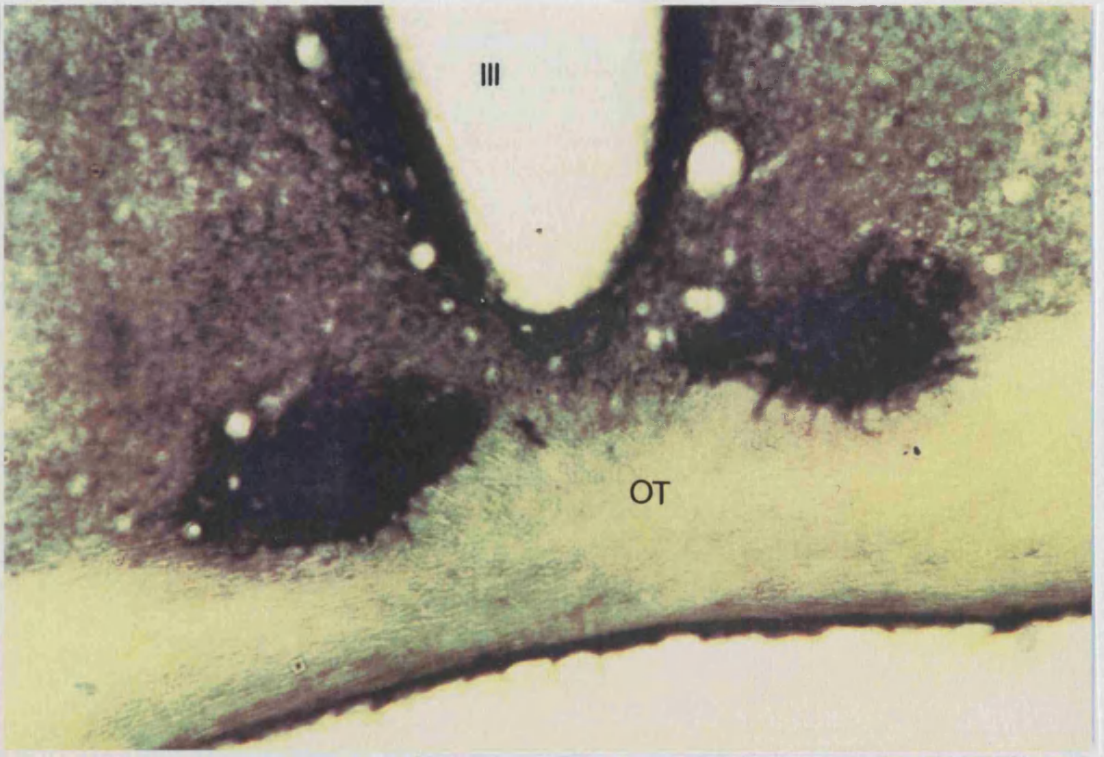


Figure 12. NPY-immunoperoxidase staining of the SCN. The NPY is present characteristically in the ventrolateral subdivision of the SCN. OT=optic tract; III=third ventricle. 24/0.55magnification.

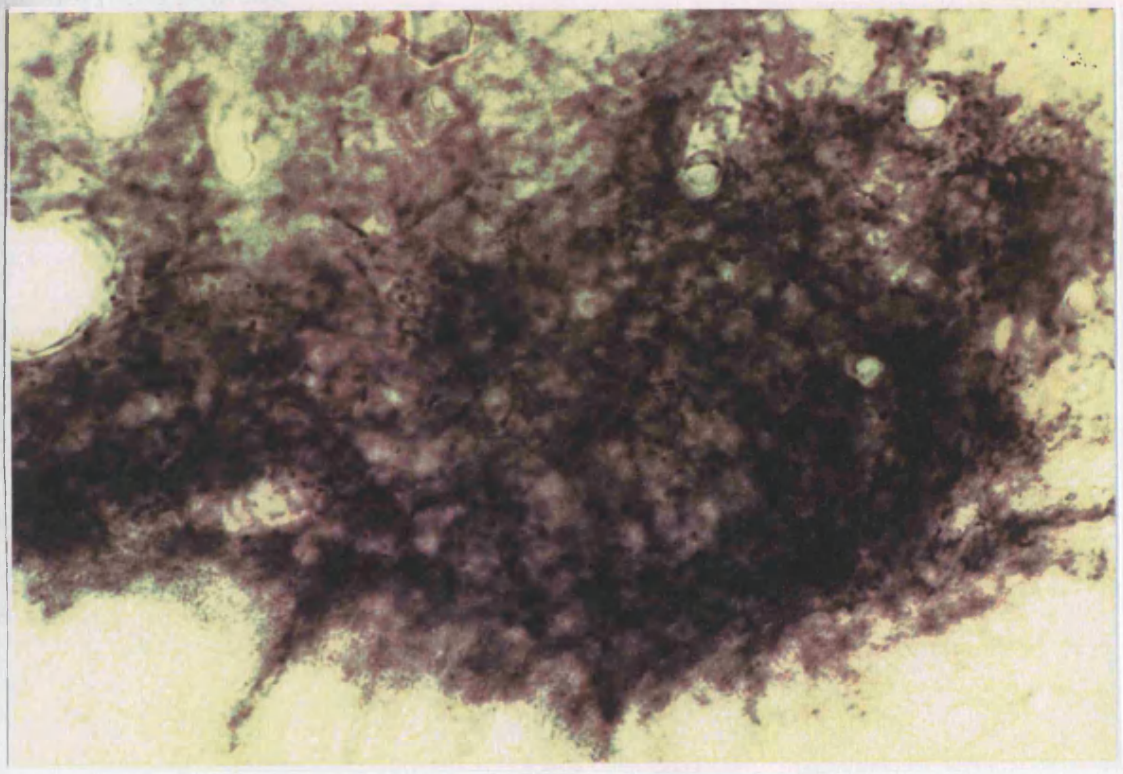


Figure 13. Immunoperoxidase stain of a single SCN, revealing dense staining for NPY-containing neurons. 2.5/0.08magnification.

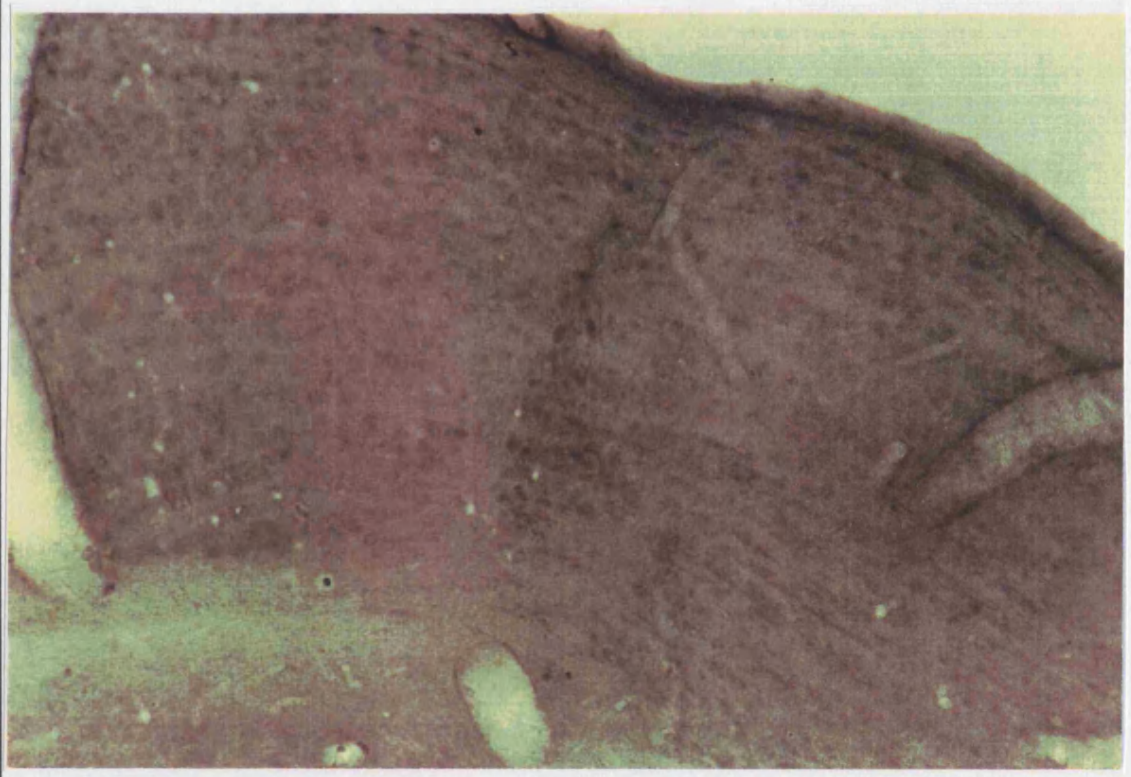


Figure 14. Immunoperoxidase localisation of NPY-containing neurons in the IGL of the thalamus. 10/0.03magnification.

Sham-operated animals.

Five animals were injected with phosphate-buffered saline only into the IGL area of the thalamus. Figure 15 shows a sample picture of a sham-operated animal. It can be seen that gliosis occurred adjacent to the needle tract, through the hippocampus.

Quisqualate lesions.

Thirty-five quisqualate lesions were performed. However, the area of gliosis caused by the quisqualate injection (Figure 16), was not much greater than that caused by the sham-lesion procedure. Furthermore, quisqualate injections were ineffective in destroying the IGL, since immunoreactive staining of the SCN in 17 animals revealed NPY still to be present in the same amounts as control animals.

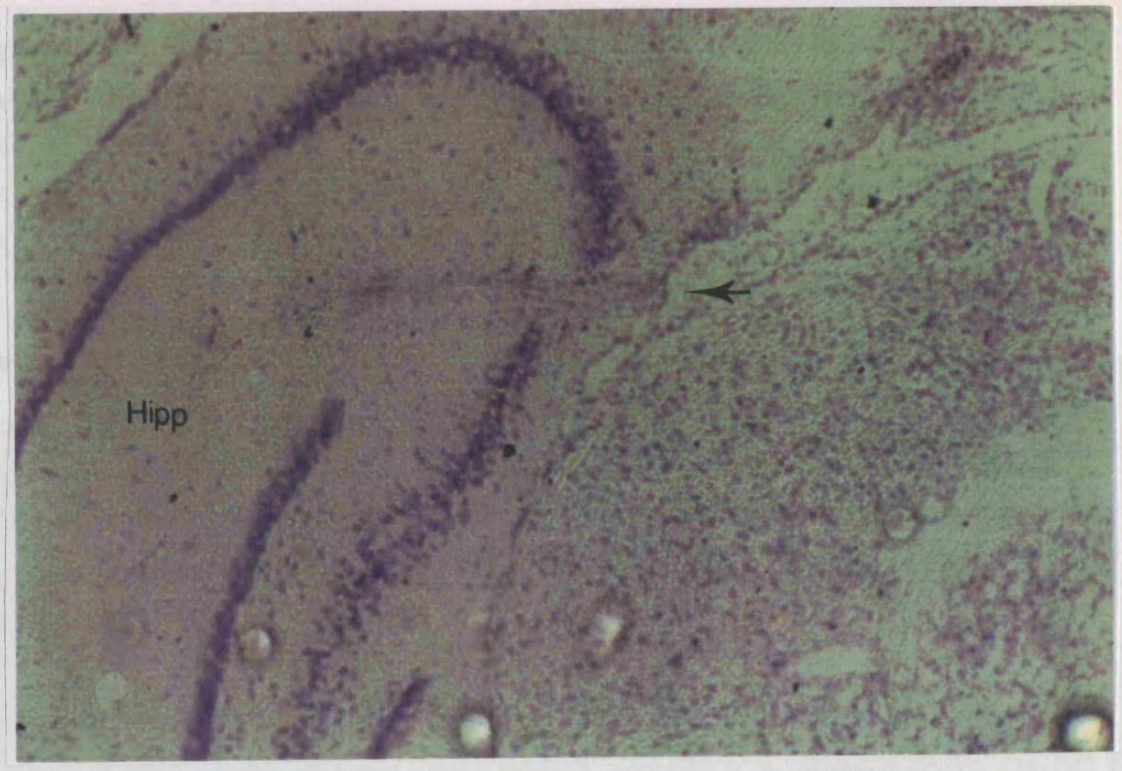


Figure 15. Cresyl violet stain from a sham-operated animal. The needle tract is visible as a line of gliosis passing through the right-side hippocampus. Hipp=Hippocampus. 2.5/0.08 magnification.

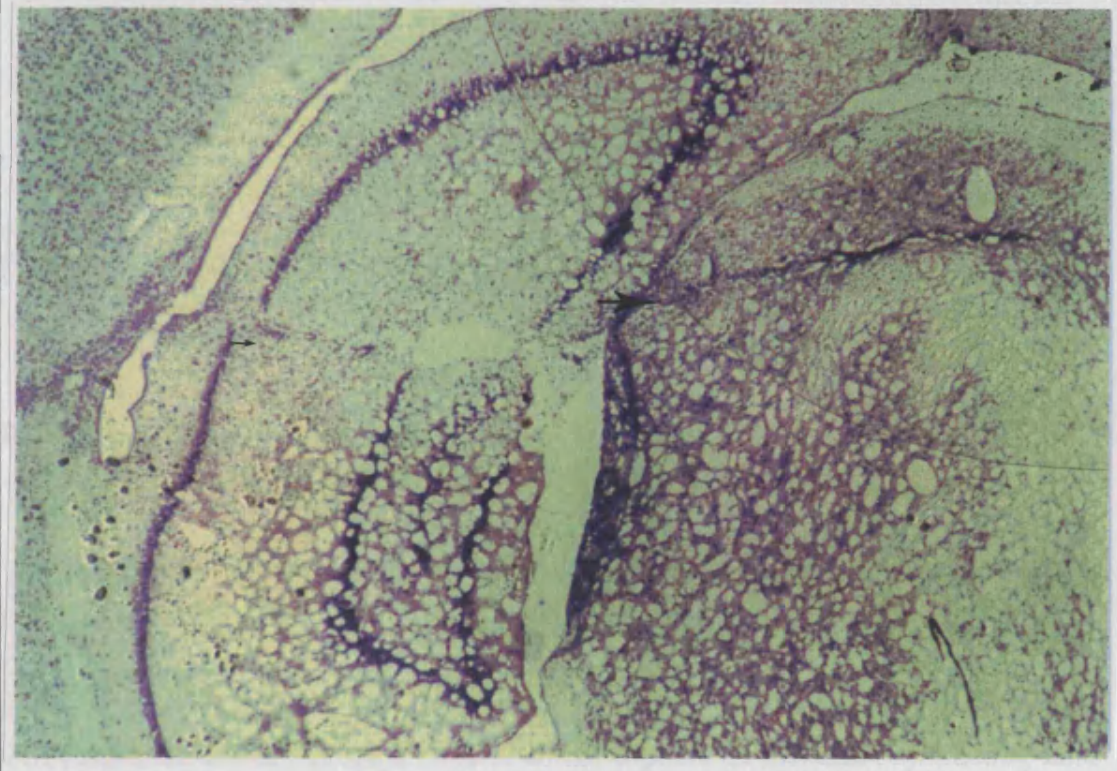


Figure 16. Cresyl violet stained section of the right-side thalamus of a quisqualate-lesioned animal. Note that the gliosis does not extend significantly beyond the needle tract. The tissue was poorly preserved as can be seen by the holed-texture of the tissue. 2.5/0.08magnification.

Kainate lesions.

Thirteen lesions with kainate ranging from 1, 2, 5 and 10mM, did not destroy the IGL. Figure 17 illustrates tissue from a 5 mM kainate lesion. Gliosis does not extend further than that seen with quisqualate injection, and this again was insufficient to block the functioning of the IGL as indicated by the presence of NPY-staining in the SCN in 5 of the samples.

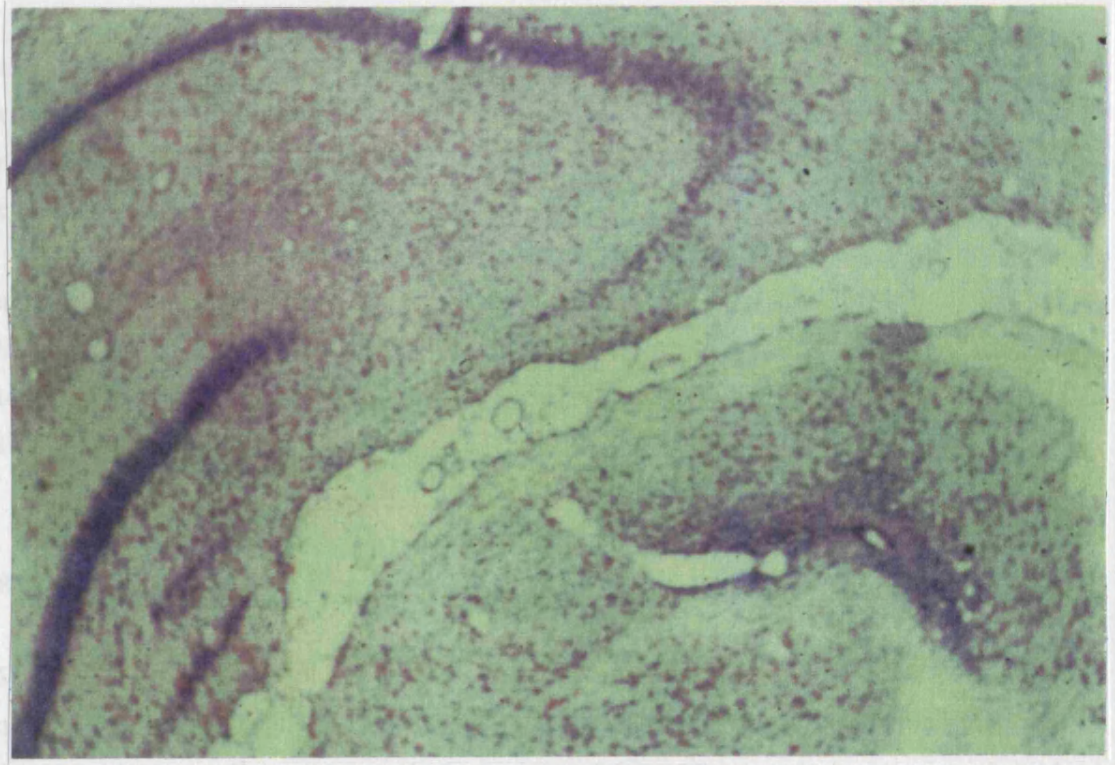


Figure 17. Cresyl violet stained section of the right-side thalamus of a kainate, IGL-lesioned animal. 4/0.12magnification.

Excitotoxic lesioning with quisqualate and kainate produced an area of gliosis in the thalamus which was unexpectedly small, and all SCN tissue stained positive for NPY, indicating that the IGL had not been effectively destroyed. SCN-NPY staining was therefore discontinued at this stage because of the expense of the antibody-staining kits. During the remainder of the study, the effectiveness of the excitotoxins in lesioning the IGL was assessed by viewing cresyl violet-stained sections.

Ibotenate lesions.

Ibotenate lesions of the IGL were carried out on 5 animals using 10 mg/ml ibotenic acid. The extent of gliosis was similar to that seen with quisqualate and kainate. The area of destruction did not spread sufficiently far from the needle tract to ensure functional removal of the IGL. Tissue preparation for cresyl violet staining was poor and as a result photographs obtained are of poor quality and are not included as illustrations.

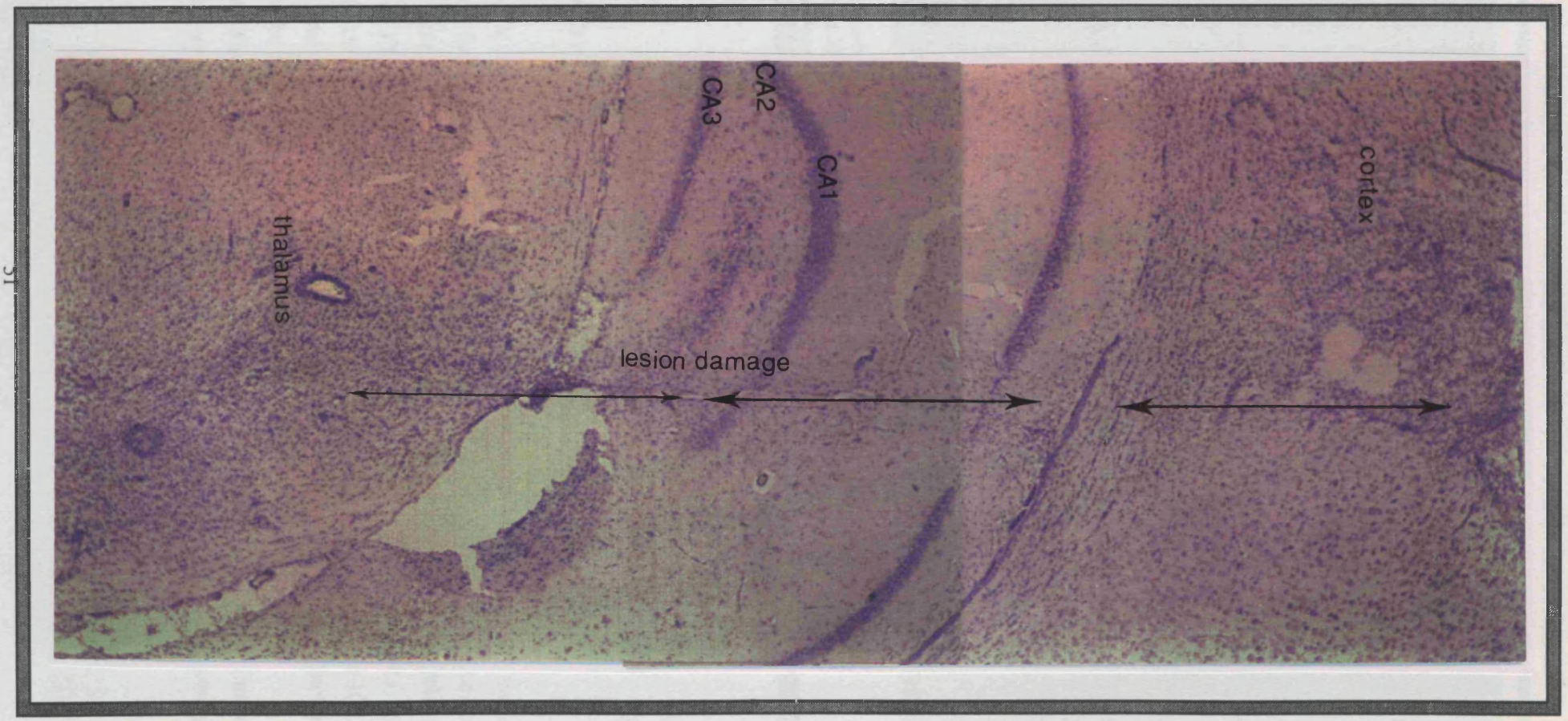
NMDA STUDY

Neither quisqualate, kainate nor ibotenate was effective at lesioning the IGL and all produced a minimal spread of gliosis in this area. This result raised the question of whether there was a problem with the lesioning technique. For example, did the procedure result in the correct delivery of excitotoxin to the designated area? A positive control was required to answer this question, and it was decided to lesion the IGL with NMDA in order to determine whether the results of other workers could be replicated (Johnson et al., 1988; 1989; Smale & Morin, 1990). Unilateral injections of NMDA (0.2M; Johnson et al., 1988) into the IGL were carried out using the same procedure and coordinates as for IGL lesioning.

RESULTS

NMDA lesions were performed on 9 animals. In each animal this excitotoxin caused large amounts of destruction in the cortex, hippocampus and thalamus as represented by the extensive gliosis in these tissues (Figures 18 and 19). All animals lesioned with NMDA exhibited severe convulsions which were not abated by two separate doses of 1 mg/Kg i.m. valium. These severe convulsions and the large area of destruction caused by NMDA ruled out further use of this excitotoxin.

Figure 18. Cresyl violet stain of the right side cortex, hippocampus and thalamus of an NMDA-lesioned animal Note the additional damage to the cortex and hippocampus (CA1, CA2 & CA3 layers). 2.5/0.08magnification.



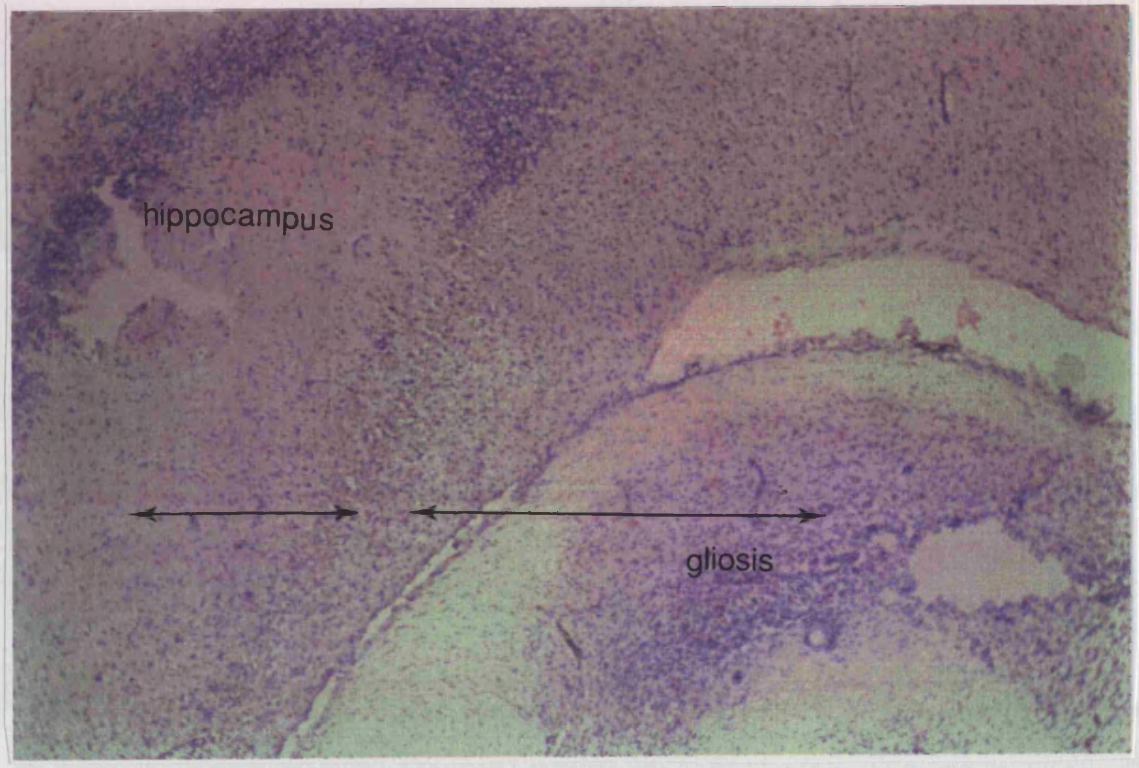


Figure 19. Cresyl violet stain of the right-side thalamus of an NMDA-lesioned animal (different to that in Figure 20). There is extensive gliosis in the thalamus at the area of the IGL, but also in the hippocampus with the CA3 region missing. 4/0.12magnification.

HIPPOCAMPUS STUDY

Having proved the lesioning technique to be effective (NMDA produced severe tissue damage), the reason for the low amounts of cell death caused by quisqualate, kainate and ibotenate was still unknown. In an attempt to assess whether there were diffusion problems with these EAAs in this brain area, kainate was injected into a different brain area, the hippocampus. The concentration of kainate chosen was mid-range of that used for IGL-lesioning (5 mM), and the hippocampus was chosen as the test area because this structure is already known to be susceptible to kainate toxicity (Watkins, 1984).

The following injection coordinates, calculated from bregma using the atlas of Paxinos & Watson (1986) were used, otherwise the procedure was the same as that for IGL-lesioning.

Table 6: Stereotaxic coordinates used for lesions of the hippocampus.

	AP (anterio-posterior) distance (mm)	ML (distance from midline) (mm)	DV distance from cortical surface (mm)
hippocampus	4.3	2.0	2.5

RESULTS

The hippocampus of 7 animals was injected with kainate (5 mM; 0.5µl) and each revealed a large area of tissue destruction, with gliosis extending down through the cortex and into the hippocampus. Within the hippocampus, destruction of the CA1 and CA3 layers was evident (Figure 20).

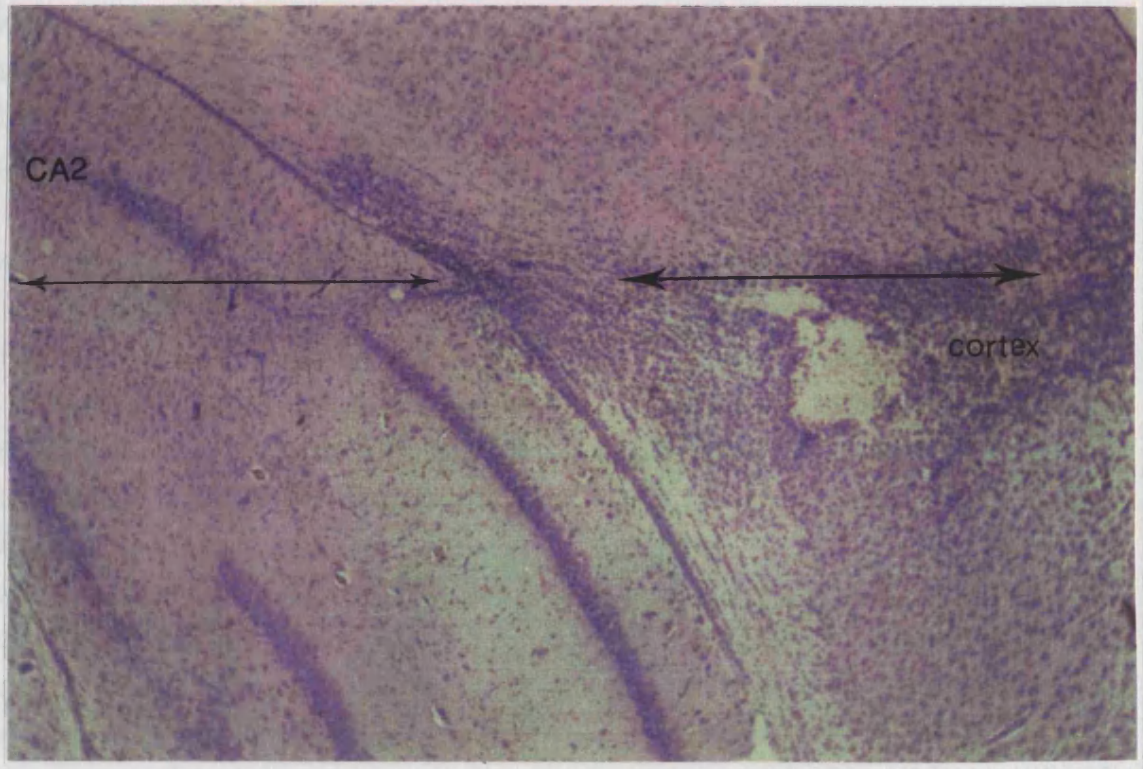


Figure 20. Cresyl violet stained section of the left side hippocampus of a kainate-lesioned animal. There is extensive gliosis in the cortex, extending down to the CA2 layer of the hippocampus. 4/0.12 magnification.

DISCUSSION

The present study has compared four EAA neurotoxins (quisqualate, kainate, ibotenate and NMDA) in terms of their effectiveness and suitability for lesioning the thalamic IGL. NMDA was found to cause extensive destruction of the IGL and surrounding areas to a similar extent to that reported by other researchers (Johnson et al., 1988; 1989; Smale & Morin, 1990). However, despite the report by Woodward and Coull, (1988) that kainate injection into the dLGN resulted in extensive damage, with gliosis spreading into adjacent thalamic tissue and into the overlying hippocampus, kainate failed to produce significant IGL lesions in the current study (even when used at higher concentrations than in the Woodward and Coull study). The same lesion insufficiency was found with quisqualate and ibotenate injection into the area of the IGL.

One practical aspect of the procedure which can be ruled out as being responsible for the minimal area of gliosis is tissue atrophy. It is known that the extent of cell loss following a lesion is best determined for most structures after an animal survival time of 3 to 4 days (Jarrard, 1991). At this time-point gliosis is maximal and degeneration of affected axonal systems is apparent. With longer periods of survival, the lesioned area undergoes considerable atrophy. Brain lesions in the present experiments were assessed within 5 days following surgery, so the reason for the small size of damage is not likely to be due to tissue atrophy.

There are two other aspects of the present methodology which may possibly account for reduced areas of cell destruction with the excitotoxins. Intramuscular administration of valium (diazepam) to all animals immediately following EAA injection was necessary to prevent convulsions and could not be omitted under Home Office regulations. However, evidence exists to suggest that the administration of this anticonvulsant may have reduced lesion size. McDonald and co-workers (1992) reported that the anticonvulsants diazepam and phenytoin were effective against quisqualate-induced toxicity. Similarly, anticonvulsant compounds have also been demonstrated to reduce kainate-mediated brain injury in rodents (Zaczek & Coyle, 1982). Therefore, administration of the anticonvulsant, valium, may have reduced the size of gliosis seen with quisqualate and kainate. However, animals which received kainate injections into the hippocampus were also treated routinely with valium, and the lesion size seen in this structure was considerable. NMDA mediated toxicity has been shown not to be affected by the administration of diazepam (McDonald & Johnston, 1990), and the present studies with NMDA, in which large areas of destruction were seen, support this result. Finally, there are no reports in the literature with which to compare the effects of anticonvulsants on ibotenate toxicity, but the present study suggests that ibotenate toxicity is also reduced by the administration of diazepam.

An alternative explanation for the reduced lesion size seen with quisqualate and kainate compared to NMDA in the thalamus, may be that there are comparatively few non-NMDA receptors in this area. This point is illustrated by the kainate results from the present study. When injected in the same concentrations, kainate produced extensive damage in the hippocampus but not in the thalamus. This is because injection of kainate into the hippocampus in rats *in vivo* preferentially destroys hippocampal CA3 neurons, which are potently excited by kainate and are particularly rich in high affinity kainate binding sites (Watkins, 1984). In addition kainate is the most potent EAA in the basal forebrain, whereas quisqualate was virtually unable to produce a lesion in this structure (Winn et al., 1991). Thus, EAA receptor density is known to vary across the structures of the brain. NMDA receptor density is richest in the hippocampus (Olverman et al., 1984). Binding with [^{3}H] AMPA has shown quisqualate receptor distribution to be highest in the cerebral cortex, and lowest in the cerebellum (Honoré et al., 1982). The differences in receptor density have been outlined in the nucleus basalis magnocellularis by Dunnett et al., (1987). Using the same four neurotoxins, this group found different profiles of damage within this structure, and suggested that this was due to either differences in their diffusion properties or in their action at different receptor types. Hence, if there are fewer non-NMDA receptors in the thalamic area of the IGL, this would account for the small lesion size seen with quisqualate and kainate; however, this does not explain why an equally small lesion size was seen with ibotenate, which is thought to act via NMDA receptors (Winn, 1991; McDonald et al., 1992).

CONCLUSION

The aim of this set of experiments was to achieve successful lesioning of the IGL, whilst leaving adjacent structures undamaged. The attempt to do this using the selective neurotoxin, quisqualic acid, was not successful since the amount of degeneration produced was not sufficiently large to destroy the IGL area. In addition, lesioning the area with the neurotoxins kainate and ibotenate was equally unsuccessful. The failure of these excitotoxins to elicit significant tissue lesions may be due to a low concentration of these receptors in this tissue, although the adequate lesioning of this area by other researchers contradicts this (Woodward & Coull, 1988). Neither can the reduced lesions size be ascribed to a technical failure in excitotoxin delivery, since the IGL was extensively lesioned with NMDA using the same technique and apparatus.

With the reason for reduced gliosis for the more selective excitotoxins unknown, and NMDA lesioning representing no improvement on existing studies (Johnson et al., 1988; 1989; Smale & Morin, 1990), the study was terminated.

CHAPTER IV

Effects of three antidepressant drugs on the circadian rhythm of locomotor activity in the rat

INTRODUCTION

Circadian rhythm disorders have been implicated in the pathogenesis of clinical depression (Wehr & Wirz-Justice, 1982; Goodwin et al., 1982), and antidepressant drugs are effective in alleviating depression. However, no definitive link has yet been made between these two areas. In fact, animal studies have so far shown inconsistencies in the effects of antidepressants on circadian rhythms. Most experiments in which antidepressant compounds have been shown to have an effect on the circadian system have shown that antidepressants delay (lengthen) rhythms, an action which fits the phase advance hypothesis of depression proposed over a decade ago by Wirz-Justice & Campbell, (1982). However, as well as the many reports of lengthening (Duncan et al., 1986; 1988), there are examples of shortening of the free-running locomotor activity period (Possidente et al., 1992). Furthermore, some studies have reported additional effects of antidepressant drugs such as dissociation (Wirz-Justice & Campbell, 1982) or splitting (Kripke & Wybourney, 1980) of circadian locomotor activity rhythms. Moreover, there are accounts reporting no effect whatsoever when a variety of antidepressant compounds have been tested on several species ranging from plants to primates (Wehr & Wirz-Justice, 1982; Hallonquist et al., 1986). Recently, Wollnik (1992) concluded that, although some antidepressants affect certain parameters of circadian rhythmicity in the rat, these effects were not common to all classes of antidepressant. In this respect, circadian studies mirror biochemical studies in that no common mechanism of action has been found to link these compounds which have a common therapeutic outcome.

Despite the ability of many antidepressants to block neuronal reuptake of monoamines, this effect has never been systematically correlated with alterations in circadian rhythm parameters. The purpose of this study was to investigate the effect of three antidepressant drugs on the period of the free-running activity rhythm of the rat, with particular reference to the abilities of the compounds to inhibit neuronal uptake of serotonin (5-HT) or noradrenaline (NA). The three drugs chosen were paroxetine, imipramine and clorgyline.

Paroxetine, is a comparatively new antidepressant drug, which reached the market in 1991 and belongs to the class of serotonin selective reuptake inhibitors (SSRI's). These compounds are known to bind to the 5-HT transporter site on the presynaptic membrane and selectively block 5-HT reuptake. The circadian effects of paroxetine have not yet been investigated, although another SSRI, fluoxetine, has been shown to shorten the period of wheel-running activity rhythms in mice (Possidente et al., 1992). Imipramine is from the tricyclic antidepressant class of drugs, which are still the most widely prescribed drugs for depressive illness (Paykel & Hale, 1986). Imipramine was chosen for analysis since it is known to block monoamine reuptake into presynaptic nerve terminals, with relative selectivity for NA (Dewar et al., 1993).

Animal studies have shown inconsistencies in the effects of imipramine on circadian rhythms with decreases in rat locomotor activity period (Takahashi et al., 1987) and increases in hamster activity period (Wirz-Justice & Campbell, 1982) being reported. It was hoped that the present studies would go some way to confirming the effects of this drug on the rat circadian system.

Clorgyline is a monoamine oxidase inhibitor (MAOI). MAO type-A is located on the outer surface of mitochondria and is responsible for the breakdown of 5-HT, NA and dopamine. Inhibition of this enzyme leads to increased concentrations of these monoamines in releasable stores within the nerve terminal, allegedly giving rise to clorgyline's antidepressant effect (Lipper et al., 1979; Rudorfer & Potter, 1989). Hence, clorgyline was chosen because it is an antidepressant compound which is not known to interact with monoamine uptake systems. The majority of studies employing clorgyline have been performed using the hamster. Continuous administration of the drug via osmotic mini-pumps has been shown to lengthen the period of hamster wheel running activity (Wirz-Justice et al., 1980; Wehr & Wirz-Justice, 1982; Duncan et al., 1986; 1988); to increase the activity-rest ratio and to alter the PRC to light (Duncan et al., 1988); to alter the period and phase of the circadian rhythm of wheel-running (Ozaki et al., 1993; Tamarkin et al., 1983), and to slow the circadian pacemaker (Wirz-Justice & Campbell, 1982). In fact, chronic clorgyline treatment is suggested to affect the circadian system in a manner similar to that of IGL ablation, for example, in decreasing the magnitude of light-induced phase advances (Duncan et al., 1988; Harrington & Rusak., 1991). As with the effects of imipramine, a species difference exists between the rat and the hamster for the effects of clorgyline on the circadian system. Clorgyline failed to alter the period of food intake in the Wistar rat (Rietveld et al., 1986). This species difference may be due to the fact that the hamster brain contains more than 95% MAO type-A, whereas the rat brain contains only 60-80% MAO type-A (Edwards & Malsbury, 1978). This situation reiterates the point that although much general knowledge has been accumulated concerning the mammalian circadian system, it is important to take into account the differences between species.

AIM

The aim of these studies was firstly to examine changes in rat circadian locomotor activity rhythms during chronic antidepressant administration, and secondly to correlate this with measured levels of brain monoamine uptake activity. This attempt to correlate circadian effects with quantifiable changes in neurochemistry is a novel one.

EXPERIMENT 1

SUBJECTS

Thirty male Wistar rats (body weight 300±5 g at the beginning of the experiment), supplied by Charles River (Margate, Kent) were used. Prior to the experiment, animals were group housed (6 per group) under a 12:12 hour light/dark cycle (lights on 07.00h; lights off 19.00h). Illumination was supplied by overhead fluorescent tubing, and the light intensity was approximately 100 lux at the level of the rat cages. Rats were fed standard rat chow (Special Diet Services, Witham, Essex) and water *ad lib*. Ambient temperature was regulated at 23°C.

APPARATUS

Details of the Dataquest III system used to measure circadian locomotor activity rhythms can be found in Chapter II. Briefly, the apparatus comprises 30 individual wire-bottomed activity cages (56 x36 x18 cm) each containing a running wheel (345 mm diameter). An attached wheel-magnet and fixed reed proximity switch send an impulse to a receiver below the cage at each wheel revolution. Information from each receiver is fed into a consolidation matrix before being down-loaded every 10 mins to an IBM-compatible PC in the adjacent room. These experiments were conducted in a light sealed room with independent lighting control, and access to the room was through another darkened room. Drinking water and food were checked daily. Waste trays were changed twice weekly at random times of day. Activity was recorded continuously and actograms were inspected at regular intervals throughout the experiment.

EXPERIMENTAL PROCEDURE

Rats were housed singly in activity wheel cages and the lighting changed to constant dark conditions (DD) at the 07.00h dark/light transition point. After 4 weeks in DD, when actogram inspection revealed the free-running rhythm to be stable, animals were randomly allocated to one of four treatment groups (paroxetine, clorgyline, imipramine or 0.9% w/v saline as vehicle; n=7 for each) and implanted with Alzet mini-osmotic pumps containing the relevant antidepressant solution. Implantation was calculated to take place between CT1 and CT3 for each individual animal, since this is within the 'dead zone' of the phase response curve to light and disturbance at this point is predicted to have no effect on rhythm (Daan & Pittendrigh, 1976b). The order of pump implantation was randomly assigned and operations were performed over four successive days. This was done to allow the subsequent biochemical assays to be performed in manageable group-sizes. Following implantation, animals were returned to a clean wheel cage and locomotor activity rhythms were monitored for the 14-day duration of pump activity. A separate group of control animals (n=2) were

subjected to anaesthesia and reversal, plus a cage change, but no pump implantation. The volume of food and water intake was measured at random times on Monday, Wednesday & Friday throughout the experiment under dim red illumination. At the end of each experiment animals were weighed, then sacrificed and brain monoamine uptake levels were determined.

Mini-Osmotic Pumps

The Alzet pump model 2ML2 (Alza Corporation, Palo Alto, CA, USA) weighs 5g, measures 1.3 cm diameter by 4.8 cm long and has a solution volume of 2 ml. It delivers a constant output of solution over 14 days at a constant rate of 5 µl per hour *in vitro.*

Since the volume delivery rate per hour is fixed in the manufacturing process for each model, the dose of drug exuded is adjusted by varying the concentration of the drug solution used to fill the reservoir. This was calculated according to the formulae:

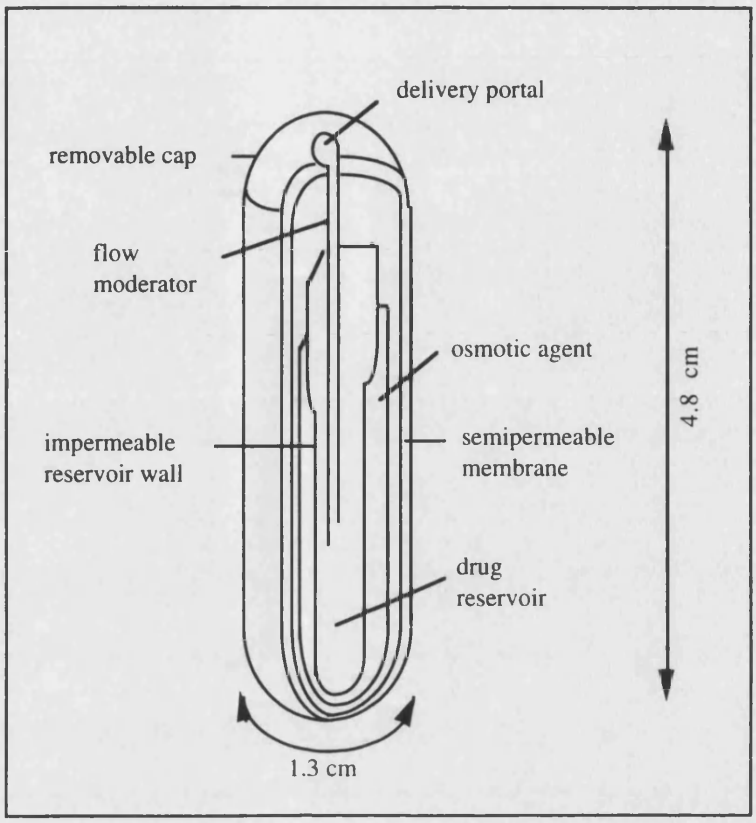
$$\frac{\text{required drug dose (mg / Kg / day)}}{1000\ \text{g}} \times \text{animal weight (g)} = \text{X mg / day}$$

$$5.0\ \mu\text{l per hour} = 120\ \mu\text{l per day}$$

$$\therefore \text{ stock solution} = \{\text{X mg / day in } 120\ \mu\text{l}\} \times 14 \text{ days}$$
$$= \{\text{X} / 120 \times 14\}\ \text{g / ml}$$

Before filling, the two separate pump pieces were weighed together. The top piece consists of the removable cap, delivery portal and flow moderator; the bottom piece is the body of the pump consisting of the reservoir space and surrounding osmotic agent, separated from the outer semipermeable membrane by an impermeable reservoir wall (Figure 21). Pumps were filled with a 5 ml syringe attached to a 20µm filter and the blunt-tipped 25 gauge filling tube, all supplied by Alza. Ensuring that this filling system was free from bubbles, the filling tube was inserted through the opening, and down to the bottom of the reservoir. The solution was injected slowly until it appeared at the outlet. The tube was then removed and the flow modulator was inserted. Each pump was weighed after filling and the difference in weight before and after gave the net weight of the solution loaded. A weight change of 2 ± 0.1 grams indicated that the pump had been filled correctly with a 2 ml volume. This filling procedure took place under sterile conditions in a flow hood using aseptic techniques, immediately prior to implantation.

Figure 21. Cross section of an Alzet osmotic pump illustrating the mechanism of action.



Drug Dosage

The following doses of antidepressant drug were used:

Imipramine - 10 mg/Kg/day (Greco et al., 1990).

Clorgyline - 1 mg/Kg/day (Aulakh et al., 1983).

Paroxetine - 0.5 mg/Kg/day (Kleinlogel & Burki, 1987). This concentration was lower than that desired, but was the determined by the solubility of paroxetine for administration in the small volume of the osmotic pumps.

Implantation Surgery

Animals were anaesthetized in the wheel room with a combination of Sublimaze® (fentanyl 0.6 mg/Kg) and Domitor® (medetomidine 0.03 mg/Kg), and taken to the operating theatre in a light-tight container with their eyes covered with a damp cloth. Pump implantation was performed in the operating theatre under dim red light. After shaving and cleansing the surgical site with the antiseptic solution Hibitane®, a small incision (1.5 cm) was made in the intrascapular region. A deep subcutaneous pocket was made in the flank of the animal for the pump, which was inserted with the delivery portal away from the wound site and the wound sealed with wound clips (Royal®-35; 0.46 mm diameter wire auto-clips). Animals were then returned to the wheel room in the same light-tight container before reversal of the anaesthetic with

Nubain® (nalbuphine) and antisedan (atipamezole), each at a dose of 0.02 mg/Kg. When they regained consciousness, animals were placed immediately into a clean wheel cage. This procedure was performed on each of the four separate days of pump implantation, with 7 or 8 animals being implanted on each day. Actual implantation of the pump took only one minute per animal, and each batch of animals was anaesthetised, implanted, reversed and returned to the wheel within 40 minutes.

Anaesthetic drugs have been shown to disrupt light input to the mammalian circadian system by preventing phase advances of the locomotor activity rhythm (Colwell et al., 1993). Although these researchers did not test the fentanyl/medetomidine combination used in this experiment, the question of anaesthetic influences was addressed by the inclusion of the anaesthetic and reversal-only group. Since this group contained n=2 animals, results from this group are not shown graphically in the results section, but are given in the text for reference.

BIOCHEMICAL ANALYSIS: UPTAKE OF [^{3}H]-5HT INTO CRUDE SYNAPTOSOMES EX VIVO

Theoretical basis of uptake assay

The ability of the antidepressant drugs to inhibit the neuronal reuptake of the monoamines NA and 5-HT *in vivo* was measured using an *ex vivo* assay of brain tissue from drug-treated animals. Tissue removed from each animal was exposed to either tritiated 5-HT or tritiated NA for a specified time under optimal conditions for the uptake process to occur, and the amount of radioactivity which entered the tissue was counted. The presence in the tissue of the antidepressant compounds paroxetine and imipramine following *in vivo* dosing was expected to inhibit the pre-synaptic reuptake of the radio-labelled monoamines, resulting in a lower radioactive count for these tissue samples compared to those from animals which had been saline-treated. Tissue from clorgyline treated animals was expected to display the same degree of radioactive uptake as saline control animals, since clorgyline is not known to affect the uptake mechanism.

Paroxetine and imipramine are competitive inhibitors of the uptake site (Marcusson et al., 1989) and compete with the monoamine substrate for this site, so that their inhibitory effects can be overcome at sufficiently high substrate concentrations. Competitive inhibition was characterised kinetically using a double reciprocal plot of substrate concentration against uptake rate (Lineweaver-Burke plot) (Stryer, 1981). The intercept of the y axis gave the Vmax (the maximum velocity of uptake) and the slope (Km; the rate constant for uptake), was directly related to the potency of the compound at inhibiting uptake. Theoretically, the Vmax should not be changed by a

competitive inhibitor, whereas the slope of the plot should vary with the inhibitor used and with substrate concentration.

Tissue preparation

A crude preparation of synaptosomes was produced from cortical tissue as follows: Rats were stunned by a blow to the back of the head and quickly guillotined. The brains were removed and the cortices dissected out, rinsed with 1ml ice-cold saline and stored on an ice-chilled weighing boat until needed (all tissue was used within one hour of removal). The cerebral cortices were weighed and homogenised individually in 10 volumes of ice-cold 0.32 M sucrose using a Potter's homogeniser (8 strokes) and centrifuged (5 min, 1000g, 4°C). The supernatants, containing the preparation of crude synaptosomes, were decanted and stored on ice until ready for use in the uptake assay.

UPTAKE ASSAY

The uptake of [^{3}H]-5-HT and [^{3}H]-NA into synaptosomes was assayed according to the method of Thomas and Nelson (1987). Briefly:

- Krebs buffer (pH 7.4) of the following composition was prepared and oxygenated by bubbling with 95%O_2:5%CO_2 at 37°C for one hour prior to use: (in mM); NaCl, 118; $MgSO_4.7H_2O$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 1.3; NaH_2PO_4, 1.2; Di-Na EDTA, 1.2; l-ascorbate, 0.06; glucose, 10; pargyline, 0.01; KCl, 4.8

- Triplicate assay tubes were loaded with 50µ l aliquots of the supernatant and pre-incubated for 10 min at 30°C with 350µl of oxygenated Krebs buffer (pH 7.4) and with 50 µl of one of three concentrations of either 5-HT (0, 15 and 100 nM) or NA (5, 45 and 195 nM) or with 'non-specific' compound. Uptake due to passive movements of the labelled monoamine into the synaptosomes (non-specific uptake) was assessed in the presence of desipramine (10 µM) for NA uptake and citalopram (1 µM) for 5-HT uptake, each dissolved in oxygenated Krebs buffer.

- After pre-incubation, 5 nM [^{3}H]-NA/5-HT was added in a 50µl volume of buffer to yield total 5-HT and NA concentrations of 5, 20 & 105 nM and 10, 50 & 200 nM respectively. Incubation was for 10 min at 30°C and the total incubation volume was 500µl (see Figures 22a and 22b for assay configuration and details).

- Incubation was stopped by rapid filtration through glass fibre filters (Whatman GF/B) with four 1 ml rinses with ice-cold buffer, using a 48-well Brandel Cell Harvester apparatus. Filters were counted for radioactivity by liquid scintillation

spectrometry (Packard Tri-Carb 2500), using 4 ml of Ultima Gold MV liquid scintillant per vial.

Figure 22a Assay configuration.

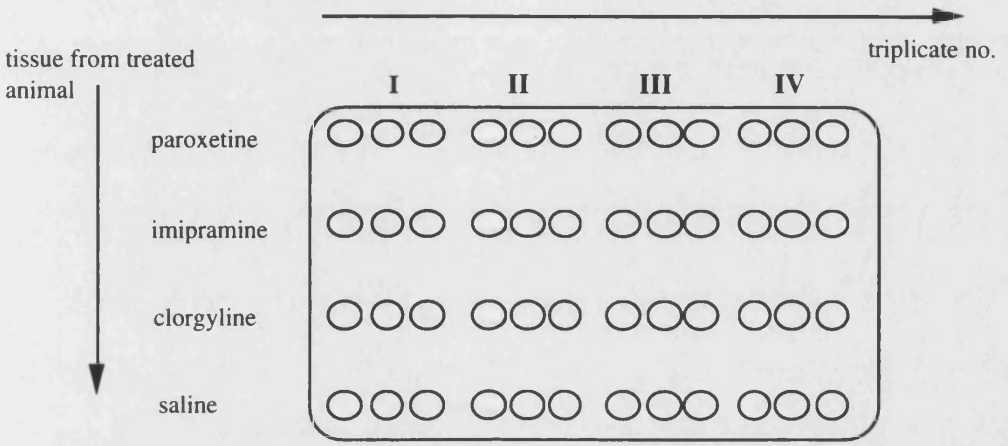


Figure 22b. Assay details.

(triplicate no.)	Volume (ml) I	II	III	IV	Incubation
non-tritiated monoamine		50 (A)	50 (B)		
Buffer	400	350	350	350	
non-specific uptake (desipramine or citalopram)				50	10 mins; 30 C
Synaptosomes	50	50	50	50	
Radioligand	50	50	50	50	10 mins; 30 C
Final volume	500	500	500	500	

Where A and B are different concentrations of monoamine.

Figure 22a: Assay configuration used for biochemical analysis. This illustrates how the 48-well tray was loaded ready for filtration by the 48-cell harvester. A separate tray was required for each monoamine.
Figure 22b illustrates the contents of each triplicate.

Table 7: Sources of drugs and chemicals used.

Drug/Chemical	Source
Medetomidine hydrochloride (Domitor)	SmithKline Beecham Animal Health Ltd., Surrey, UK.
Fentanyl citrate (Sublimaze)	Janssen Pharmaceuticals Ltd., Oxford, UK.
Nalbuphine hydrochloride (Nubain)	DuPont Pharmaceuticals Ltd., Hertfordshire, UK.
Atipamezole hydrochloride (Antisedan)	Norden Laboratories, Hertfordshire, UK.
Diazepam (Valium)	Roche Products Ltd., Hertfordshire, UK.
Imipramine hydrochloride	Sigma Chemicals, Poole, Dorset, UK
Clorgyline hydrochloride	Sigma Chemicals, Poole, Dorset, UK
Paroxetine hydrochloride	SmithKline Beecham Pharmaceuticals
citalopram hydrochloride.	Sigma Chemicals, Poole, Dorset, UK
desipramine hydrochloride	Sigma Chemicals, Poole, Dorset, UK
[^{3}H]-5-HT specific activity 24 µCi/mmol	Sigma Chemicals, Poole, Dorset, UK
[^{3}H]-NA specific activity 80 µCi/mmol	Sigma Chemicals, Poole, Dorset, UK

DATA ANALYSIS

BIOCHEMICAL ANALYSIS

The radioactivity entering the tissue due to non-specific uptake was subtracted from the counts (dpm) at each substrate concentration (dpm - non-specific count). The specific activity of the radioligands was then used to determine the dpm per microgram of tissue, and the former two values were used to calculate the *rate* of monoamine uptake (picomoles/hour/mg tissue). The inverse of this rate was plotted against the inverse subtrate concentration to yield the classical Lineweaver-Burke plot. Assays were carried out on four separate days because of the large number of tissue samples involved. Within each of the four assays, the slope of the saline treated animals for that assay was subtracted from each of the treatment slopes to give the relative amount of uptake inhibition as follows:

$$\frac{\text{treatment slope - saline slope}}{\text{treatment slope}} \times 100 = \text{uptake inhibition relative to saline}$$

Measuring the slope of the plot, rather than uptake at a single subtrate concentration, reduces error as it gives an estimate based on inhibition at all monoamine concentrations tested.

CIRCADIAN RHYTHM ANALYSIS

The effects of the antidepressant drugs on the following parameters of the animals' free-running locomotor activity rhythm were measured as described previously in Chapter II.

1. The period of the free-running rhythm (tau; τ); was measured for the 14 days prior to and the 14 days during treatment.
2. Phase changes of locomotor activity elicited by the antidepressant drugs were assessed by measuring the onset of activity.
3. The duration of daily activity (α) was calculated before and during treatment.
4. The average amount of daily activity per cycle per 10 minute bin (A), was calculated before and during treatment.

All analysis was performed blind with respect to animals and treatment group, using double plotted actograms and the TAU program (Minimmiter, Oregon, USA).

STATISTICS

Biochemical results from each assay were analysed by two-way ANOVA with drug treatment and assay day as variables, followed by *post hoc* Dunnett's test to identify significant effects. The slope values from the Lineweaver Burke plots were used for the analysis.

The effect of drug treatment on circadian period and phase was assessed by one-way ANOVA of these parameters between the treatment groups. Changes in α and A for each drug were assessed by Students t-test before and during treatment.

RESULTS

BIOCHEMISTRY

Figure 23 shows an example of the data obtained from one of the four *ex vivo* uptake assays. The Tables in Figure 23 contain data obtained from ^{3}[H]5-HT tissue counts in paroxetine-treated (top table), and saline-treated (bottom table), rats. Corresponding

plots of the rate of ^{3}[H]5-HT uptake into synaptosomes against substrate concentration, and the double reciprocal of this, are given below each table. To the right of each table are values for the correlation coefficient, slope factor and Y-intercept for the Lineweaver-Burke plot. Both the paroxetine and saline plots cross the y-axis at approximately the same point (0.51 and 0.4 units, respectively), but vary in slope factor (25.98 and 12.41, respectively), indicating competitive inhibition of the uptake process in the presence of the antidepressant compound, paroxetine.

Figure 23. Raw data and derived Lineweaver-Burke plots from radioactive uptake experiments.

paroxetine

[3H]5-HT Uptake data record sheet.

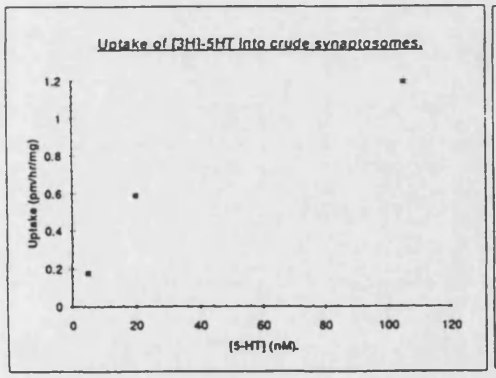
Animal No.: 14 Species: Rat Non-Spec. defined with: Citalopram (1uM)

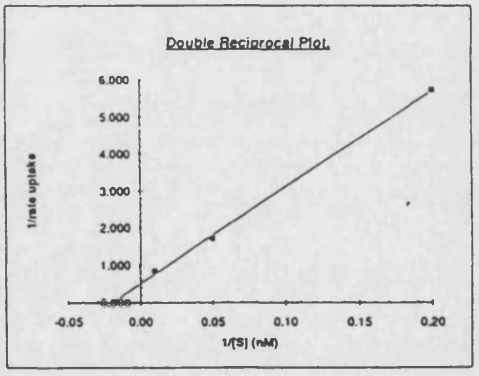
Date: 14/12/92 Tissue: Whole Cortex

Treatment group:

[5-HT] nM	dpm	dpm-ns	spec act	nmol/ml	uptake pm	pm/hr/mg	1/v	1/ conc
5	12741.00	7654.50	23.70	52614.00	0.145	0.175	5.728	0.200
20	11514.40	6427.90	5.93	13153.50	0.489	0.586	1.705	0.050
105	7577.63	2491.13	1.13	2505.43	0.994	1.193	0.838	0.010
non spec	5086.50							

Correl. Coef.; 0.9993
Slope Factor; 25.98
Y-Intercept; 0.51
Estimated Km; 51.0
Estimated Bmax; 1.96





saline

[3H]5-HT Uptake data record sheet.

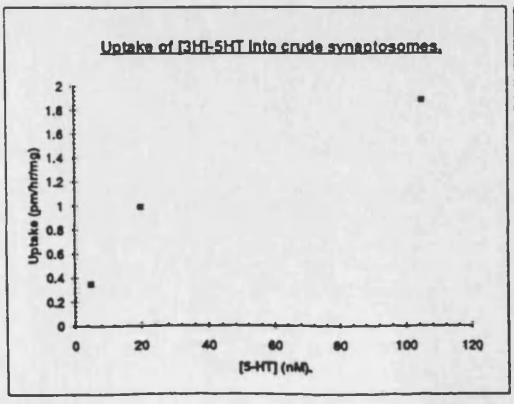
Animal No.: 30 Species: Rat Non-Spec. defined with: Citalopram (1uM)

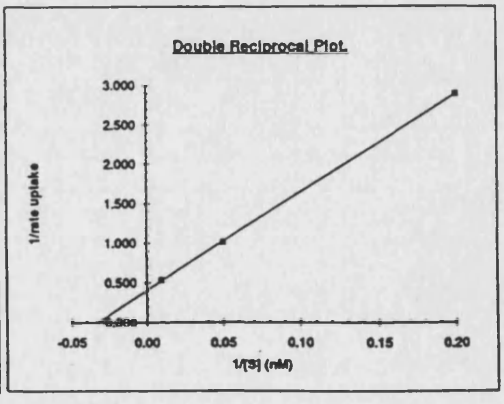
Date: 11/12/92 Tissue: Whole Cortex

Treatment group:

[5-HT] nM	dpm	dpm-ns	spec act	dpm/pot	uptake pm	pm/hr/mg	1/v	1/ conc
5	20770.05	15179.40	23.70	52614.00	0.289	0.346	2.888	0.200
20	16412.30	10821.65	5.93	13153.50	0.823	0.987	1.013	0.050
105	9515.75	3925.10	1.13	2505.43	1.567	1.880	0.532	0.010
non spec	5590.65							

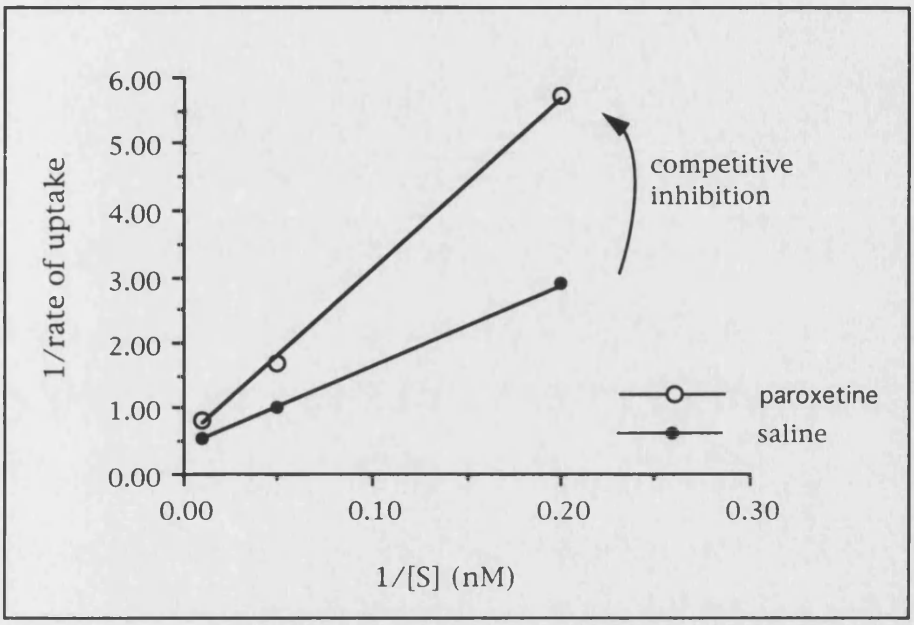
Correl. Coef.; 1.0000
Slope Factor; 12.41
Y-Intercept; 0.40
Estimated Km; 30.7
Estimated Bmax; 2.47





The two Lineweaver-Burke plots from the previous Figure are drawn on the same axis in Figure 24 to demonstrate the fact that the steepness of the plot increases in the presence of a competitive inhibitor (Stryer, 1981).

Figure 24. Lineweaver-Burke plot of 3[5-HT] uptake in rat cortical synaptosomes in the presence of paroxetine and saline.



Inhibition of monoamine uptake

Figure 25 illustrates the trends in inhibition of uptake of radioactivity produced by the antidepressant drugs. As anticipated, paroxetine showed a greater inhibition of [^{3}H]-5-HT uptake than [^{3}H]-NA uptake (inhibition = 30.66±13.9% vs 2.26±1.46% respectively). Imipramine inhibited the uptake of [^{3}H]-NA more than [^{3}H]-5-HT (relative inhibition = 43.34±8.77 vs 16.1±10.2% respectively), whilst clorgyline displayed an equal degree of uptake inhibition of both [^{3}H]-monoamines (relative inhibition = 17.4±2.79 vs 12.33±6.28 % respectively). Despite the clear trends in uptake inhibition, none of the values reached statistical significance.

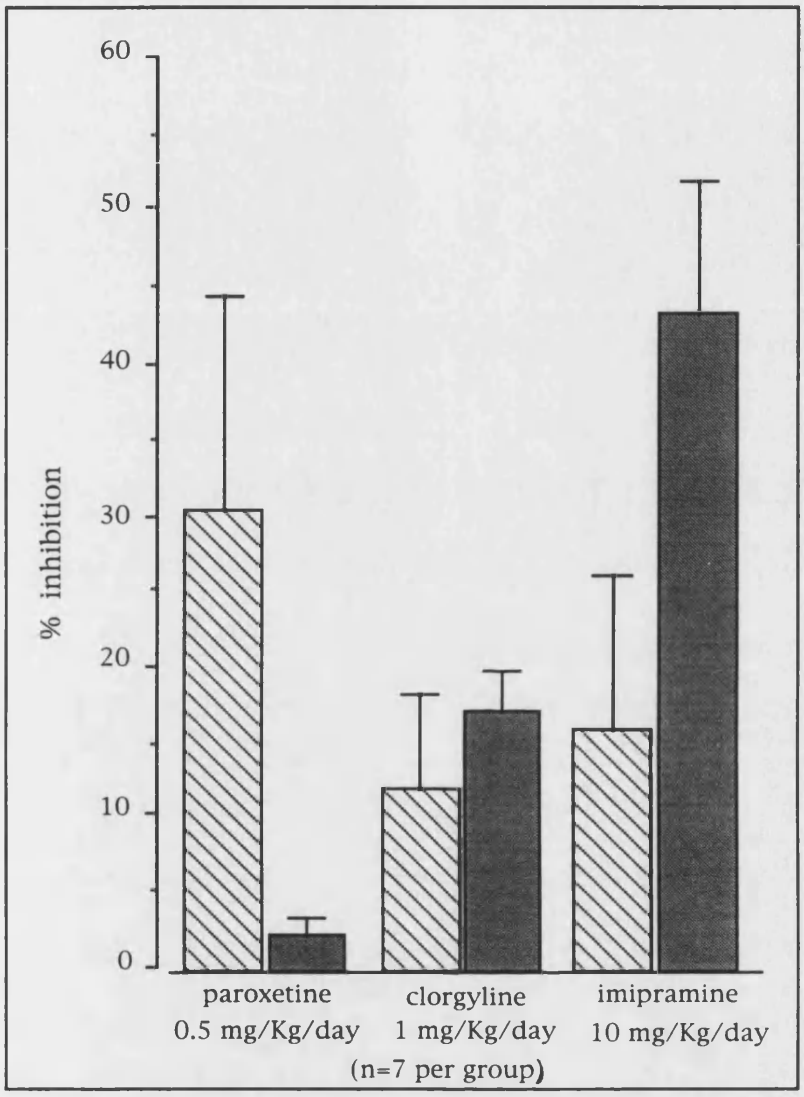


Figure 25. Inhibition of [3H] monoamine uptake into rat cortical synaptosomes from animals chronically pre-treated with antidepressants (osmotic pump administration). n=7 per group.

■ = [3H]-noradrenaline ▨ = [3H]-serotonin

Body weight.

Chronic clorgyline- and impramine-treated animals showed a significant weight loss over the two week period of drug administration compared to saline-treated animals (-26.097±1.83g and -1.53±5.08g for clorgyline and imipramine animals, respectively). Animals implanted with saline- and paroxetine-containing pumps gained only approximately 50% of the weight gained by the two non-implanted animals: 19.31±3.055g and 17.34±5.82g for paroxetine and saline animals and 34.79±0.26g for non-implanted animals (Figure 26).

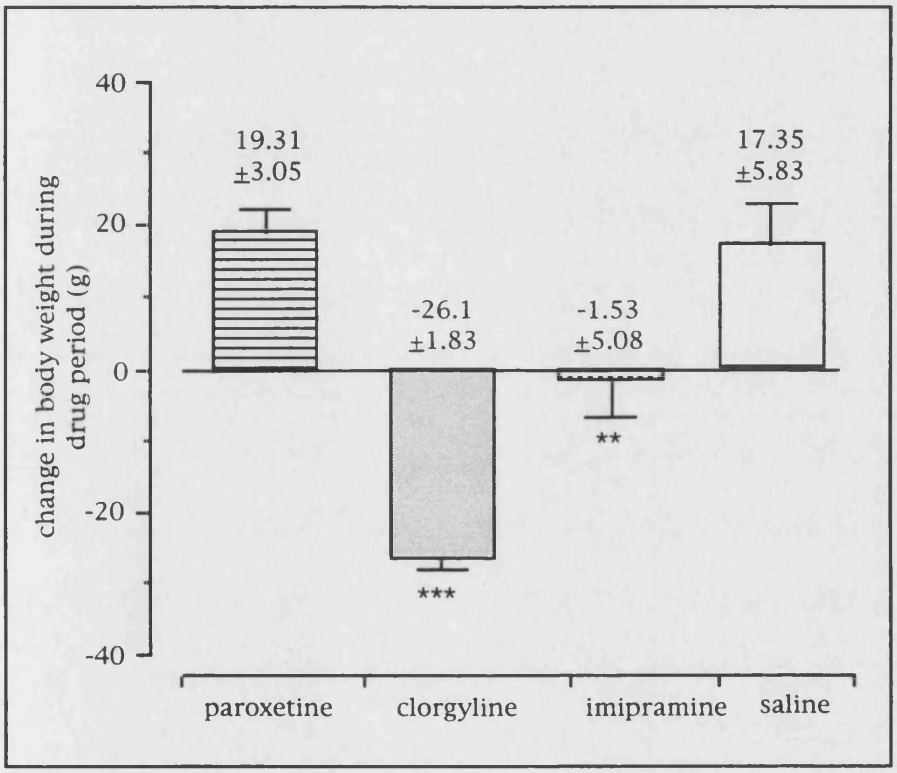


Figure 26. Influence of antidepressant treatment on animal weight during the two-week drug period. paroxetine=0.5 mg/kg/day; clorgyline=1 mg/Kg/day and imipramine= 10 mg/Kg/day. ** $p \leq 0.05$; *** $p \leq 0.001$ compared to saline

Food consumption.

A plot of food consumption measured over the experimental period is given in Figure 27. The overlying plots of food comsumption in paroxetine, clorgyline, imipramine and saline-treated animals show that antidepressant treatment had no significant effect on rat chow consumption. A slight increase in feeding was seen immediately following pump implantation, but this was not statistically significant when tested by two-way ANOVA.

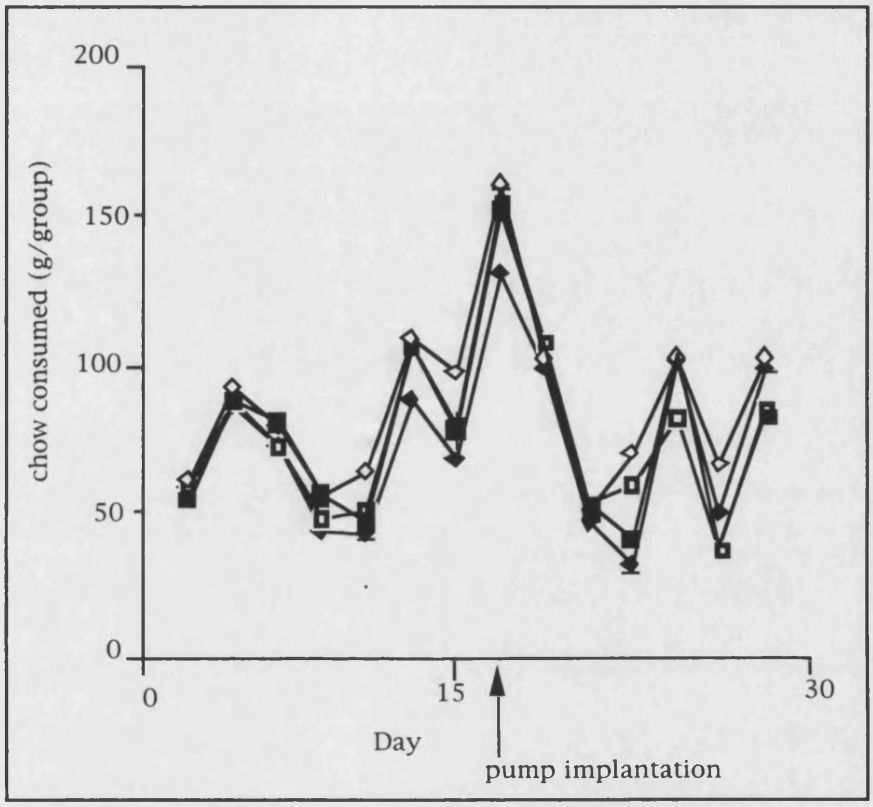


Figure 27. Food consumption over the experimental period. paroxetine 0.5 mg/Kg/day; clorgyline 1 mg/Kg/day and imipramine 10 mg/Kg/day. * $p \leq 0.05$.

Saline —◆— clorgyline —■— paroxetine —◇—
imipramine —▬—

CIRCADIAN RHYTHM RESULTS

Period.

None of the antidepressant drugs significantly altered the period of locomotor activity. Clorgyline tended to lengthen period (0.09 ± 0.06 hours), whilst imipramine apparently shortened the period (-0.03 ± 0.04 hours), but these changes were not statistically significant when compared with the period of saline treated animals

(Figure 28). Anaesthetic and reversal-only animals exhibited a 0.1±0.1 hour change in period. The mean and standard error of the mean (sem) are given above each bar.

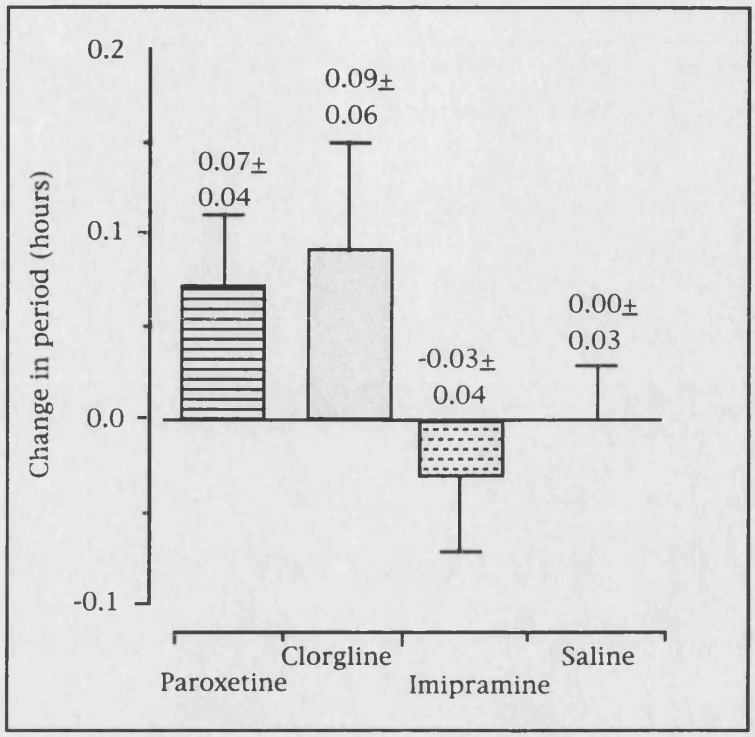


Figure 28. Effects of antidepressant drugs on the circadian period of locomotor activity in the rat following chronic administration via osmotic mini-pumps. paroxetine=0.5 mg/Kg/day; clorgyline=1 mg/Kg/day and imipramine=10 mg/Kg/day. (n=7 per goup).

Phase.

The steady-state phase of locomotor activity onset (i.e. that during the 14 days of drug treatment), was significantly advanced by 121.01±33.07 minutes in clorgyline-treated animals. Neither imipramine nor paroxetine treatment significantly affected the phase of locomotor activity, although an advance was seen in all groups (Figure 29). Anaesthetic and reversal animals also exhibited a non-significant phase advance of 47.64±15.88 minutes.

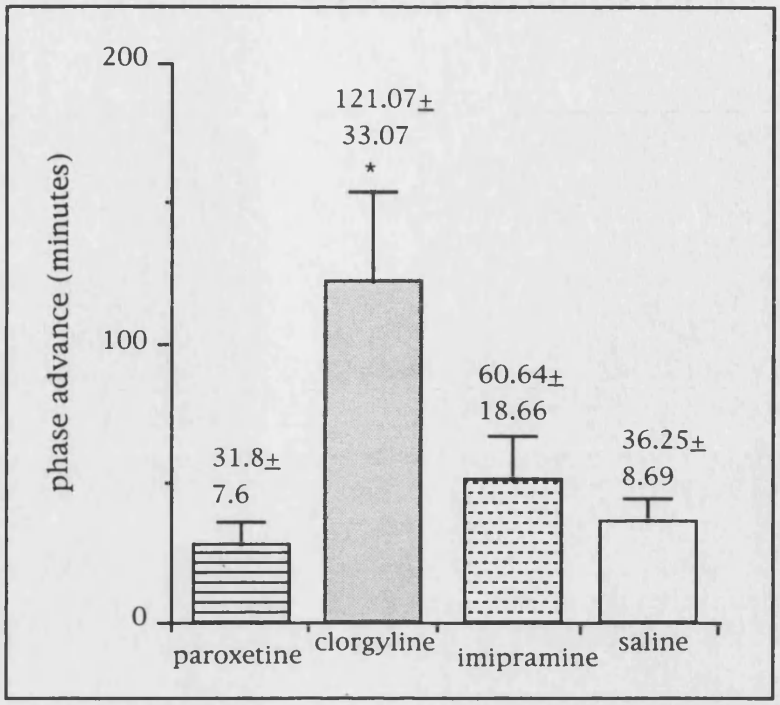


Figure 29. Phase shifts elicited by chronic antidepressant treatment. paroxetine=0.5 mg/Kg/day; clorgyline=1 mg/Kg/day and imipramine=10 mg/Kg/day. * = $p \leq 0.05$ and (n=7)

Figures 30a and 30b illustrate the actograms from three rats which were implanted with clorgyline-pumps on the day indicated by the arrow. A phase advance of the onset of locomotor activity is evident in these actograms. The lower actogram in Figure 30b is that of a saline pump-implanted animal, and it can be seen that the onset of locomotor activity did not alter in phase following pump implantation.

Figure 30a. Actograms from animals implanted with clorgyline-containing pumps. (The arrows to the right of each actogram indicate the day of pump implantion).

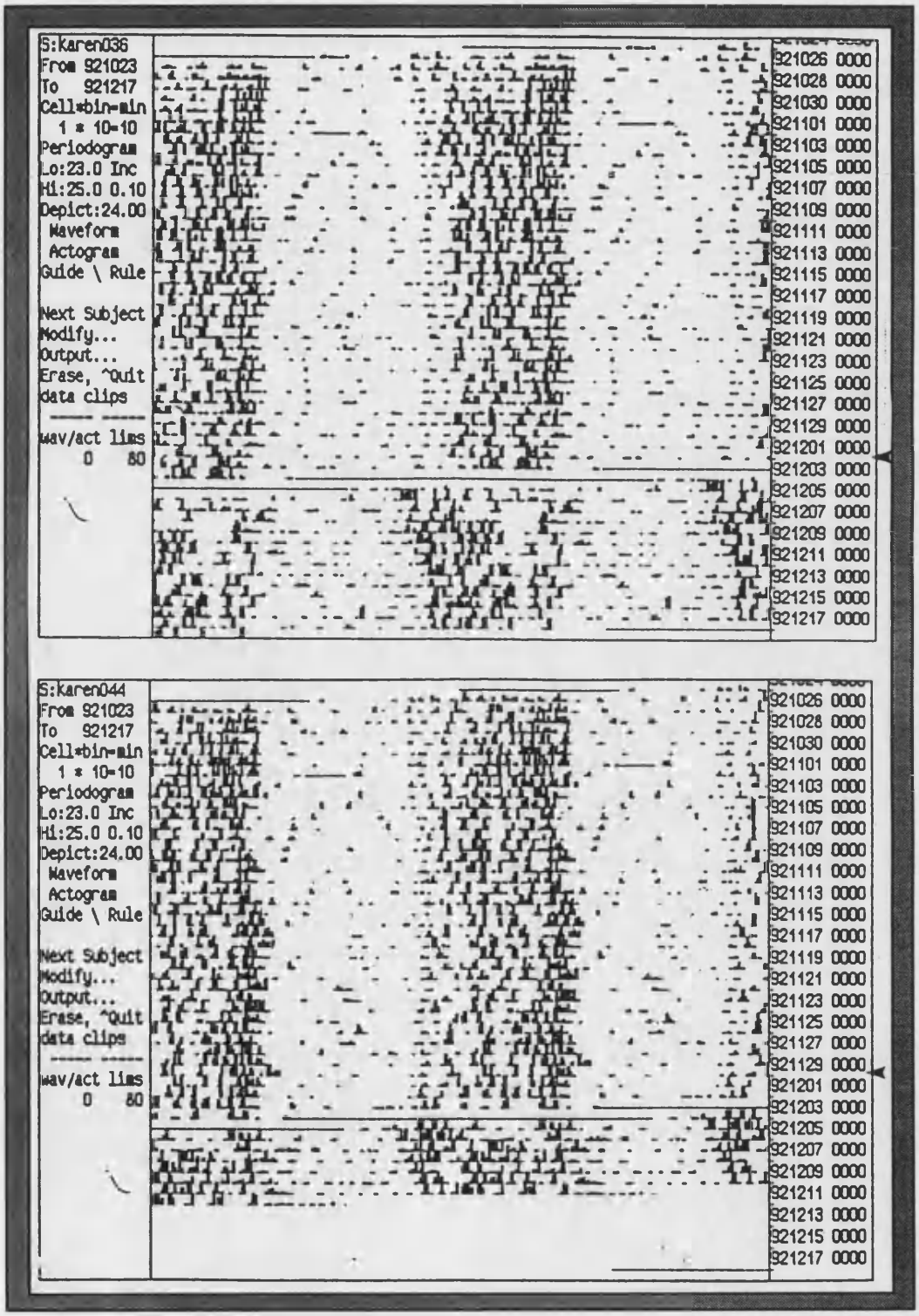
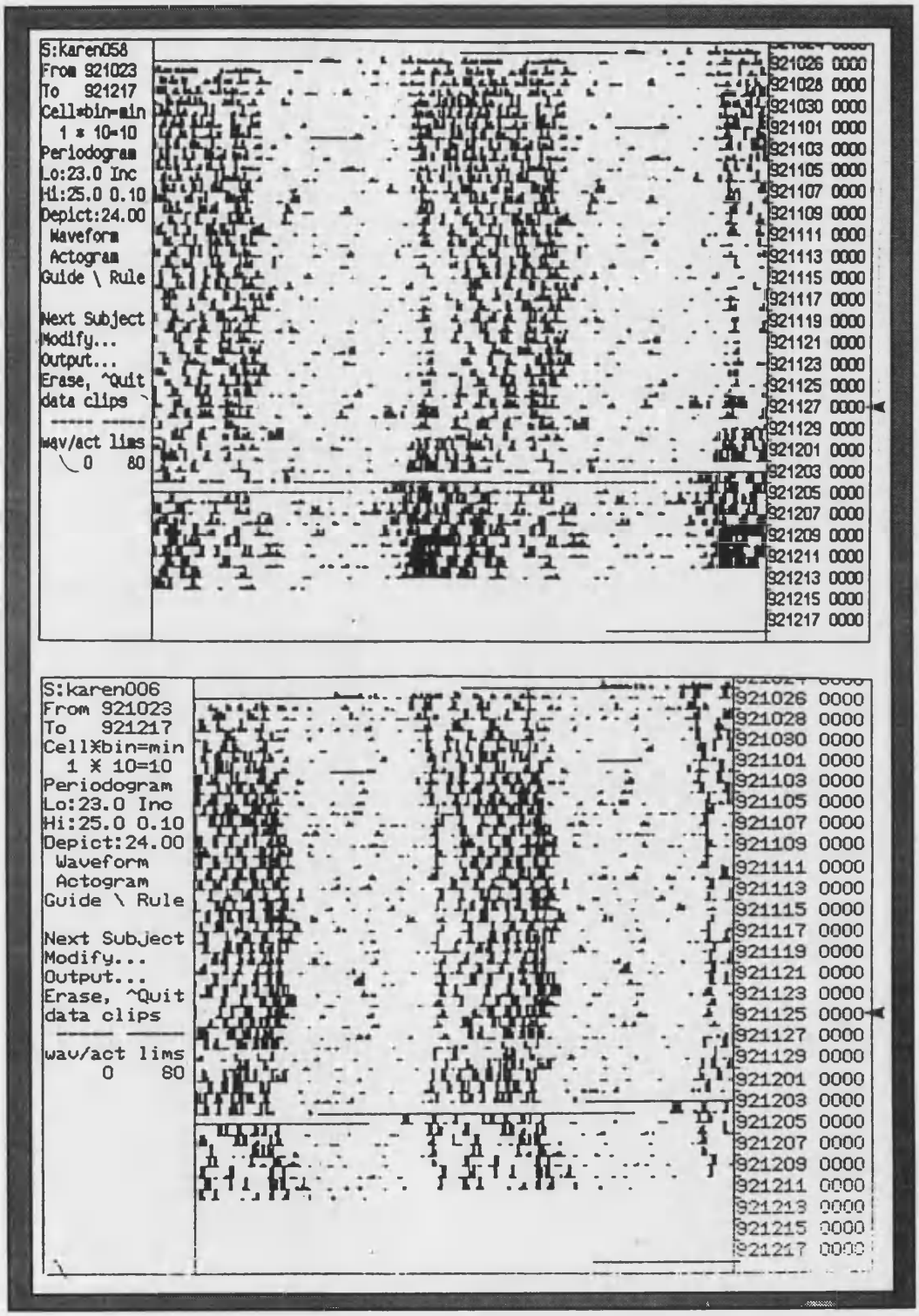


Figure 30b. Actograms from a clorgyline- (upper trace) and saline- (lower trace) pump implanted animal. (Arrows indicate the day of pump implantion).



Activity period (α)

The length of daily activity was significantly increased by an average of 91.07±32.05 minutes in clorgyline-treated animals, but was unaffected by other treatments. There was no significant change in alpha in anaesthetic and reversal animals (-26.48±5.3).

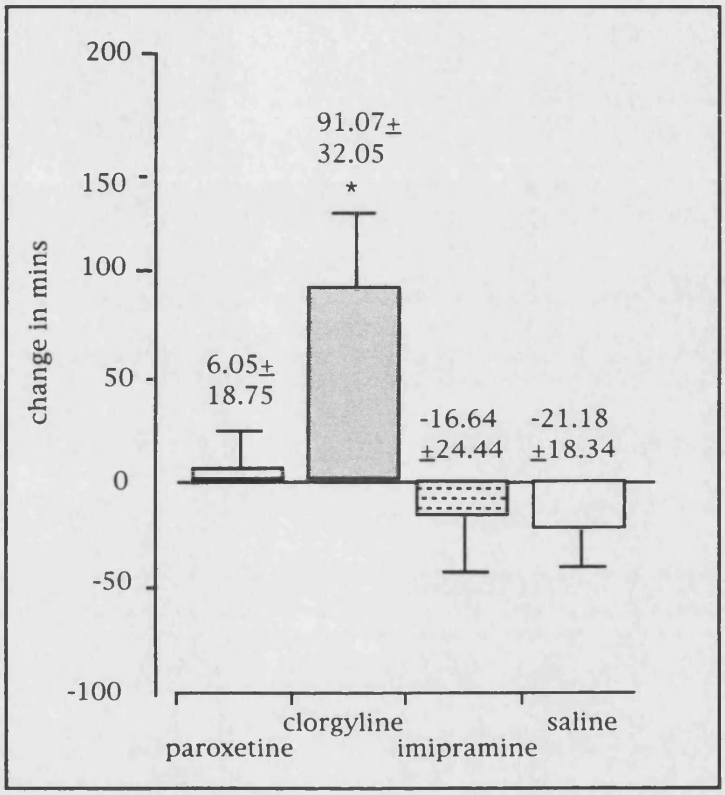


Figure 31. Changes in alpha with drug treatment (antidepressants administered via mini-pumps s.c.) parox=paroxetine 0.5 mg/Kg/day; clorg.=clorgyline 1 mg/Kg/day and imip=imipramine 10 mg/Kg/day. * $p \leq 0.05$: oneway ANOVA followed by Dunnett's test.

Amount of activity (A)

Paroxetine was found to significantly decrease the mean number of wheel revolutions per cycle per 10 min from 9.56±0.85 before treatment to 6.52±0.6 during treatment. Similarly imipramine and saline tended to decrease activity although these changes were not statistically significant. Clorgyline, however, tended to increase the average amount of activity but again this was not significant. These trends are shown in Figure 32. Anaesthetic and reversal animals showed a non-significant decrease in activity of 13.15±4.52 wheel revolutions per 10 minute bin.

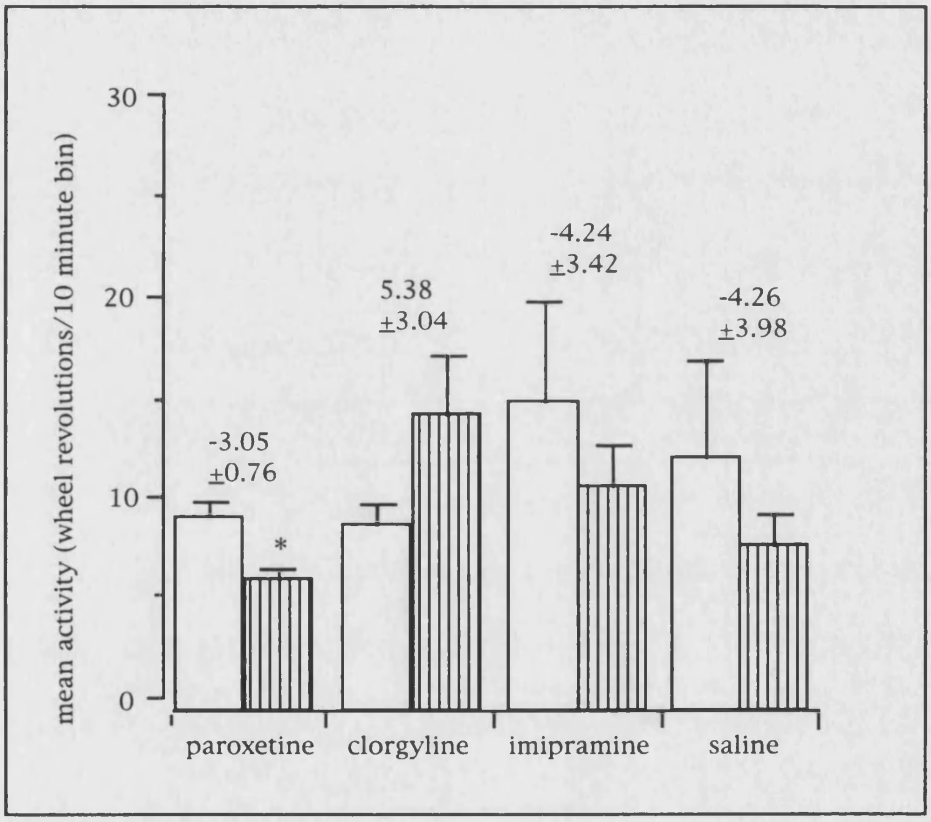


Figure 32. Effect of antidepressant drugs administered chronically via mini-pumps on amount of rat locomotor activity. ☐ average amount of activity per cycle per 10 min bin before treatment. ▥ average amount of activity per cycle per 10 min bin after treatment; * = p≤0.01 Paroxetine 0.5 mg/Kg/day; clorgyline 1 mg/Kg/day and = imipramine 10 mg/Kg/day (n=7 per goup).

Figures 33a and 33b illustrate actograms from paroxetine-treated animals showing the reduced wheel-running activity following pump implantation. For comparison, an actogram from a clorgyline pump-implanted animal, which increased wheel-running activity following pump implantation, is shown in Figure 33a. In addition, the

actogram from an imipramine-treated animal is shown for comparison in Figure 33b, since this animal did not alter its wheel-running activity following implantation.

Figure33a. Actograms from animals implanted with paroxetine- (upper trace) and clorgyline- (lower trace) containing pumps. (Arrows indicate day of pump implantation).

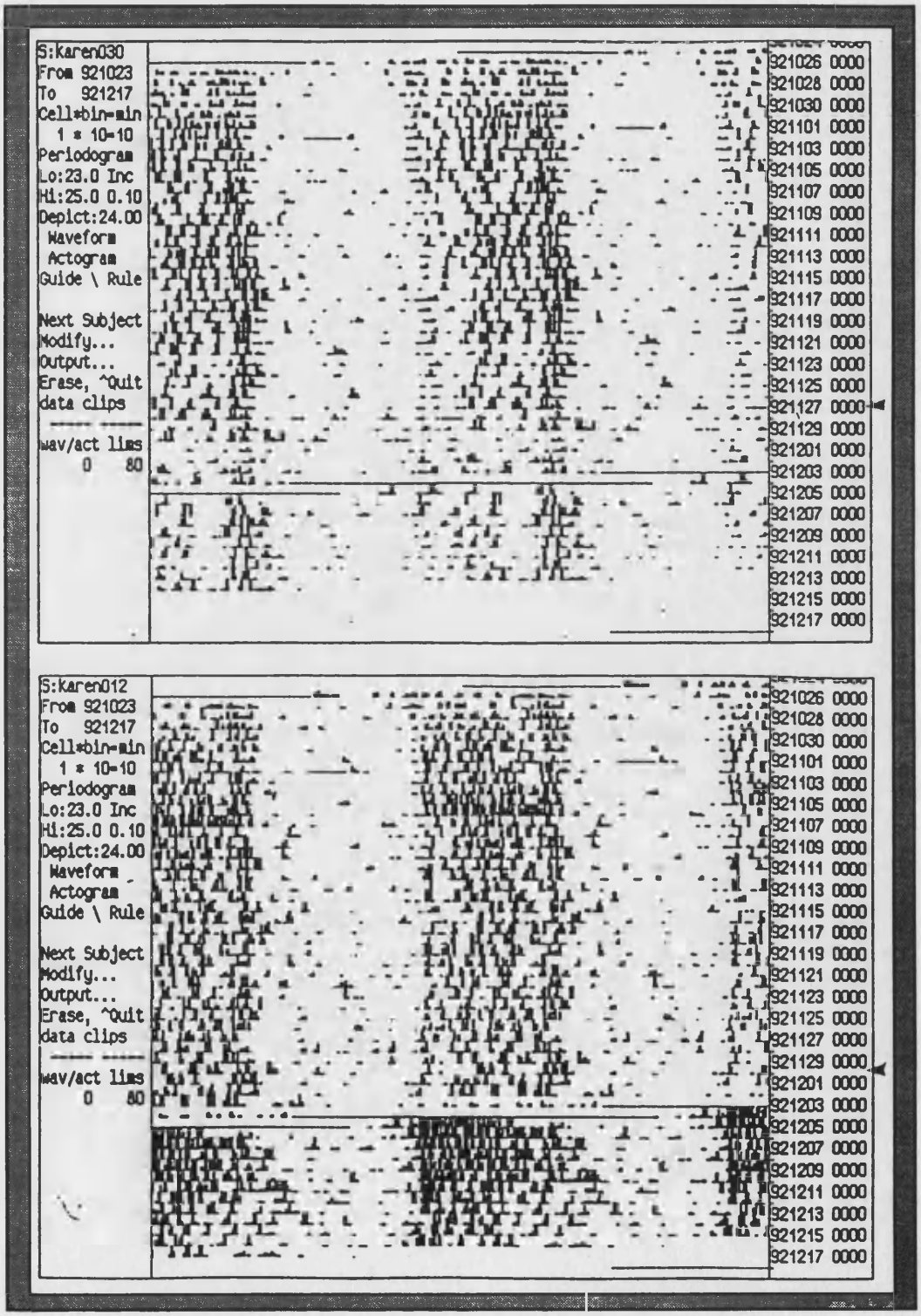
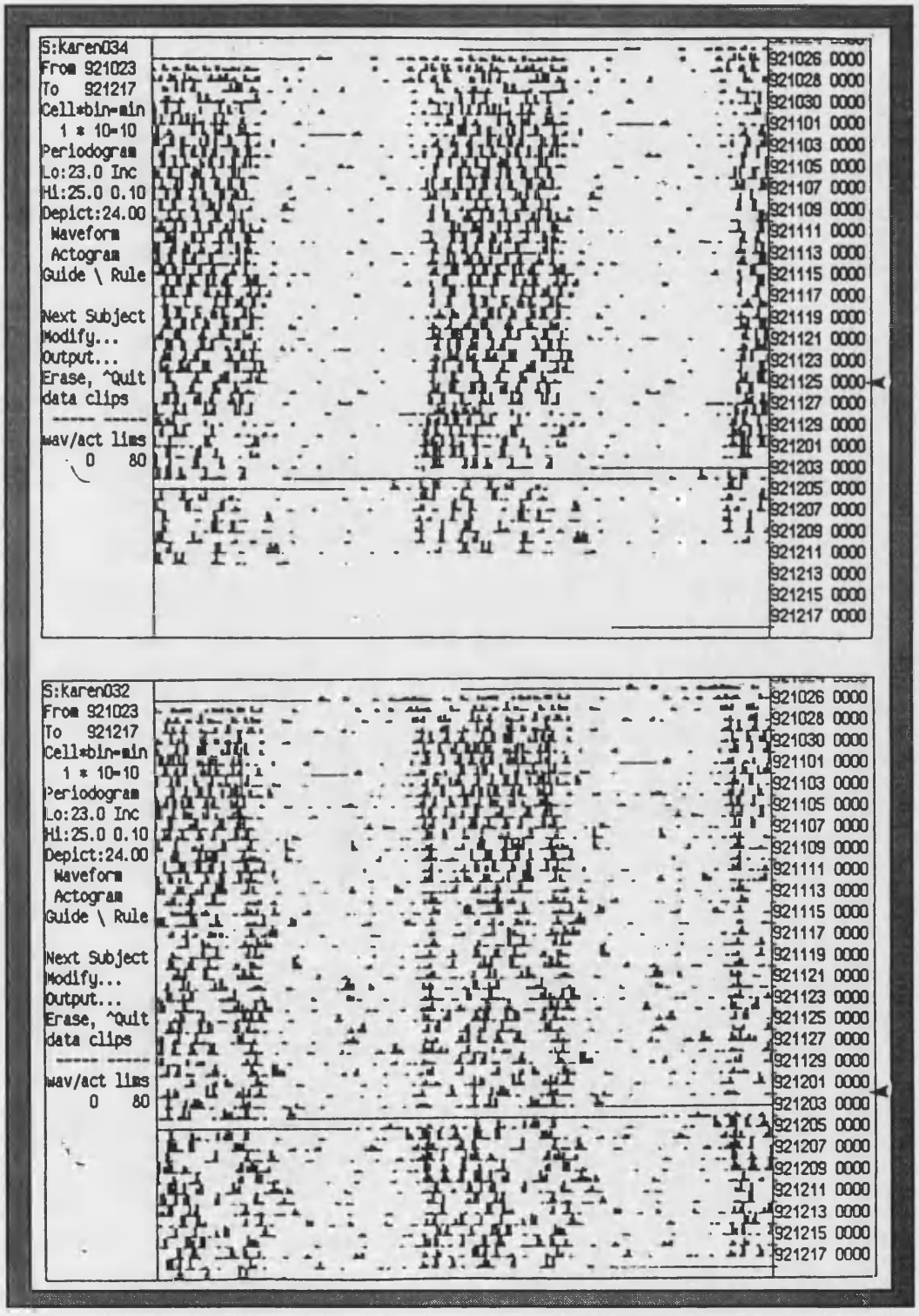


Figure33b. Actograms from animals implanted with paroxetine- (upper trace) and imipramine- (lower trace) containing pumps. (Arrows indicate day of pump implantation).



DISCUSSION

In many chronobiological studies use is made of osmotic mini-pumps as an accepted method of chronically administering drugs that avoids the excessive disturbance of the animal associated with repeated parenteral administration. However, many of the studies do not follow up behavioural measurements of the pumps' activity with biochemical assessment of satisfactory delivery of the pumps contents. The aim of this study was therefore to address this issue through the *ex vivo* measurement of monoamine uptake inhibition, a biochemical end-point of imipramine and paroxetine. Although this measurement was partially successful, in that trends in monoamine uptake inhibition were as anticipated for each antidepressant drug, (paroxetine was more selective for 5-HT than NA uptake inhibition, and imipramine was marginally more selective for NA than 5-HT), the level of uptake inhibition did not reach statistical significance. Whilst imipramine was administered in a concentration known to be biochemically effective at inhibiting uptake (Dewar et al., 1993), paroxetine had to be administered at a lower dose than desired (Kleinlogel & Burki, 1987) due to solubility problems. This, combined with the small n numbers introducing variability into the assay, may account certainly for the failure of paroxetine, and partly for the failure of imipramine, to inhibit monoamine uptake in the present study. The MAOI, clorgyline was not expected to inhibit monoamine uptake, since there is no evidence that this compound has a direct effect on the monoamine transport system. Hence, the minor uptake inhibition by this compound was surprising, and an appropriate theoretical explanation for this cannot be found. These results highlight the necessity to confirm the biological activity of drugs delivered from minipumps each time they are used.

From a physiological aspect, clorgyline- and imipramine-treated animals exhibited significant weight loss over and above that caused by the surgical implantation. Importantly, this cannot be attributed to a decrease in feeding, since all consumed the same quantities of rat chow during the experiment. This weight loss is in agreement with that found by other researchers using clorgyline in the hamster (Gordon & Duncan, 1994) and imipramine in the rat (Mogensen et al., 1994; Refinetti & Menaker, 1993). However, in each of these cases, the drugs also elicited a decrease in feeding which was not recorded in the present experiment.

Despite reports in the literature that imipramine (Refinetti & Menaker, 1993) and clorgyline (Duncan & Schull, 1994) depress general activity in rodents, paroxetine was the only compound to significantly reduce the average number of wheel revolutions per cycle per 10 minutes in the present experiment. It is likely that this is a sedative effect, because low doses of paroxetine, (<0.5 mg/Kg/day), have been shown to increase 'dozing' in rats (Kleinlogel & Burki, 1987). Since activity outside

the running-wheel was not monitored by telemetry or photocells, it is difficult to confirm or refute this explanation for decreased activity.

An observation to arise from this experiment is the fact that during the two week treatment period animals displayed rhythms with an approximate 24 hour periodicity, suggesting rhythm entrainment rather than the free-running state. Although the tau of the rat is said to be almost exclusively longer than 24 hours in DD (Honma et al., 1983; Stephan, 1983), it is nevertheless possible for animals to have a free-running period of 24 hours. However, if the animals were free-running, it might be expected that the time of onset of locomotor activity be distributed around the 24-hour clock for a group of 30 animals. Calculations from Experiment 1 revealed the group mean time of onset of activity to be 21.58$\pm$0.09 hours. This narrow range of onset time appears to support the suggestion that the animals were entrained to a stimulus occuring late in the evening. The source of this entrainment is difficult to explain, since the animals were housed in a light-sealed room, and corridor lighting went off automatically at 18.45h. Furthermore, the building was empty of staff and totally quiet at this time in the evening.

In terms of the circadian system, clorgyline treatment appeared to cause a significant phase advance and to increase in the length time for which the animals were active. However, when actograms were re-examined it can be seen that although pump implantation was initially timed to occur between CT 1-3 for each animal, a clorgyline-containing pump was in fact implanted into one animal at CT23. This animal responded to the perturbation at this time with the largest example of a phase advance of all animals (No. 36; Figure 30a). It therefore seems likely that time of implantation, and not the presence of antidepressant drug is responsible for the phase advance in this group. Similarly, the increase in alpha observed in clorgyline-treated animals can be explained by the advance in the onset of activity due to perturbation at this time-point, and again not due to the effects of the drug itself.
Finally, none of the antidepressant drugs had any statistically significant effect on the period of locomotor activity. This is in agreement with the work of Rietveld et al., (1986) who found no effect of clorgyline on the circadian period of food intake in the Wistar rat, and with Refinetti and Menaker (1993) who found no effect of imipramine on τ in the golden hamster.

In conclusion, this experiment has shown that the antidepressant compounds imipramine, clorgyline and paroxetine have no measurable effect on the rat circadian system. Unfortunately, despite the theoretical and practical advantages of osmotic minipumps as a route of administration which prompted their use in this experiment, it is apparent from the present results that these advantages were outweighed by the

effects of the surgical procedure on the circadian system and by the associated problems of drug solubility. For this reason, the experiment was repeated using drinking water as the route of drug administration. The three antidepressants chosen for Experiment 1 were stable in water, and the drinking water route of administration allows dosing to be maintained for longer than the two week limit of the 2ML2 osmotic pumps. This is a distinct advantage, because although the biochemical effects of antidepressant drugs are known to occur almost immediately, the clinical effects take approximately 2 weeks to manifest.

Finally, although there is evidence that rats are insensitive to red light (Honma et al., 1992), and consequently the dim red light used for food and water maintainance during in the DD wheel-room was predicted to have no effect on circadian rhythms, to remove any question of rhythm disturbance, the experiment was repeated in constant dim red light conditions.

EXPERIMENT 2

SUBJECTS

Thirty male Wistar rats (weighing 325±5 g at the beginning of the experiment; supplied by Charles River (Margate, Kent) were used. Prior to the experiment, animals were group housed as described in Experiment 1.

APPARATUS

Data capture was performed in the same way as for Experiment 2 using the Dataquest III circadian activity monitoring system.

EXPERIMENTAL PROCEDURE

Rats were transferred to activity wheels and maintained under constant dim red lighting conditions (<2 lux; Encapsulite Safelight λ=610nM). During the 4-week stabilisation period, water intake was monitored daily (Monday - Friday) in order to determine the average intake per animal, per 24 hours. When actogram inspection revealed free-running rhythms to be stable (day 0), animals were randomly assigned to a treatment group and administered either ordinary tap water (n=9) or solutions of the antidepressant drugs (n=7 per group), at concentrations calculated to give the following doses: imipramine (10 mg/Kg/day), clorgyline (1 mg/Kg/day) and paroxetine (10 mg/Kg/day). The volume of water drunk was measured daily during the treatment period, so that any necessary changes in drug concentration, (in order to maintain a constant daily drug intake), could be made. Rat chow consumption was monitored on Monday, Wednesday and Friday throughout the experiment.

On day 21, the ability of the antidepressants to alter the response of the activity rhythm to a pulse of light was measured. At CT21 each animal was taken from the wheel room into the adjacent room and exposed to a white light pulse of approximately 300 lux for 60 minutes. Animals were then returned to the constant dim red light conditions of the wheel room for a further 14 days activity monitoring. Drug treatment was continued throughout this time through to the end of the experiment. At the end of the experiment, animals were weighed and sacrificed for biochemical analysis of *ex vivo* synaptosomal monoamine uptake as in Experiment 1.

CALCULATIONS

From water intake measurements prior to drug treatment, it was calculated that the average daily water intake per rat was 50 ml. Based on this value, and an average body weight of 430 g, stock solutions of drugs were calculated according to the formula:

$$\text{(drug) mg / Kg / day} \times \frac{\text{average body weight (g)}}{1000} \text{ in 50 mls}$$

The dosage administered is thus dependent on the amount of water drunk by the animal. Since addition of some antidepressant drugs to drinking water has been shown to cause a decrease in water consumption (Mitchell, 1989), the amount of water drunk was monitored closely. Water bottles were weighed every day, and two consecutive 10% changes in the average volume drunk were required before the drug concentration was altered.

DATA ANALYSIS

Biochemical analysis

The ability of the antidepressant drugs to inhibit the uptake of the tritiated monoamines NA and 5-HT, was assessed as in Experiment 1. Whereas only three substrate concentrations were used in Experiment 1 to obtain the Lineweaver-Burke plot for uptake inhibition, five different concentrations were used (in triplicate) in this experiment to improve the estimation of monoamine uptake. The final concentrations of [^{3}H]-5-HT were 5, 7.5, 10, 20 and 80 nM while the final concentrations of [^{3}H]-NA were 5, 10, 20, 50 and 80 nM. As in Experiment 1, four separate biochemical assays were performed.

.Circadian rhythm analysis

• τ, α and A were measured during the two weeks prior to drug treatment and during the three weeks of drug treatment before the light pulse administration.

• Steady-state phase-shift to the light pulse at CT21 was calculated by determining the best-fit lines through activity onset, and where possible offset, for days 0 to 7 (pre phase-shift) and for days 8 to 14 (post phase-shift). The time between the extrapolated post-phase shift activity onset/offset and the pre-phase shift activity onset/offset on the day of light exposure gave the amount of phase shift (Mrosovsky, 1988).

The time of light pulse delivery was calculated for each animal as follows:

CT21 = CT12 (activity onset) + 9 circadian hours i.e.

$$9 \times \frac{\tau}{24} = \text{real time (hours)}$$

real time (hours) + onset time = time of light pulse

STATISTICS

Biochemical results from each assay were analysed by two-way ANOVA with drug treatment and assay day as variables, followed by *post hoc* Dunnett's test to identify significant effects.

The effect of drug treatment on circadian period and phase was assessed by one-way ANOVA of these parameters between the treatment groups. Changes in α and A for each drug were assessed by Students t-test before and during treatment.

RESULTS

BIOCHEMICAL RESULTS

Figure 34 illustrates the Lineweaver-Burke plots obtained from the *ex vivo* uptake study for paroxetine, clorgyline, imipramine and control animals. The ability of paroxetine to significantly inhibit ^{3}[H]5-HT uptake is reflected in the steepness of this plot compared to that of imipramine and clorgyline, the slopes of which do not differ significantly from the water-only slope. The same is true for the ability of clorgyline to significantly affect ^{3}[H]NA uptake compared with the other compounds.

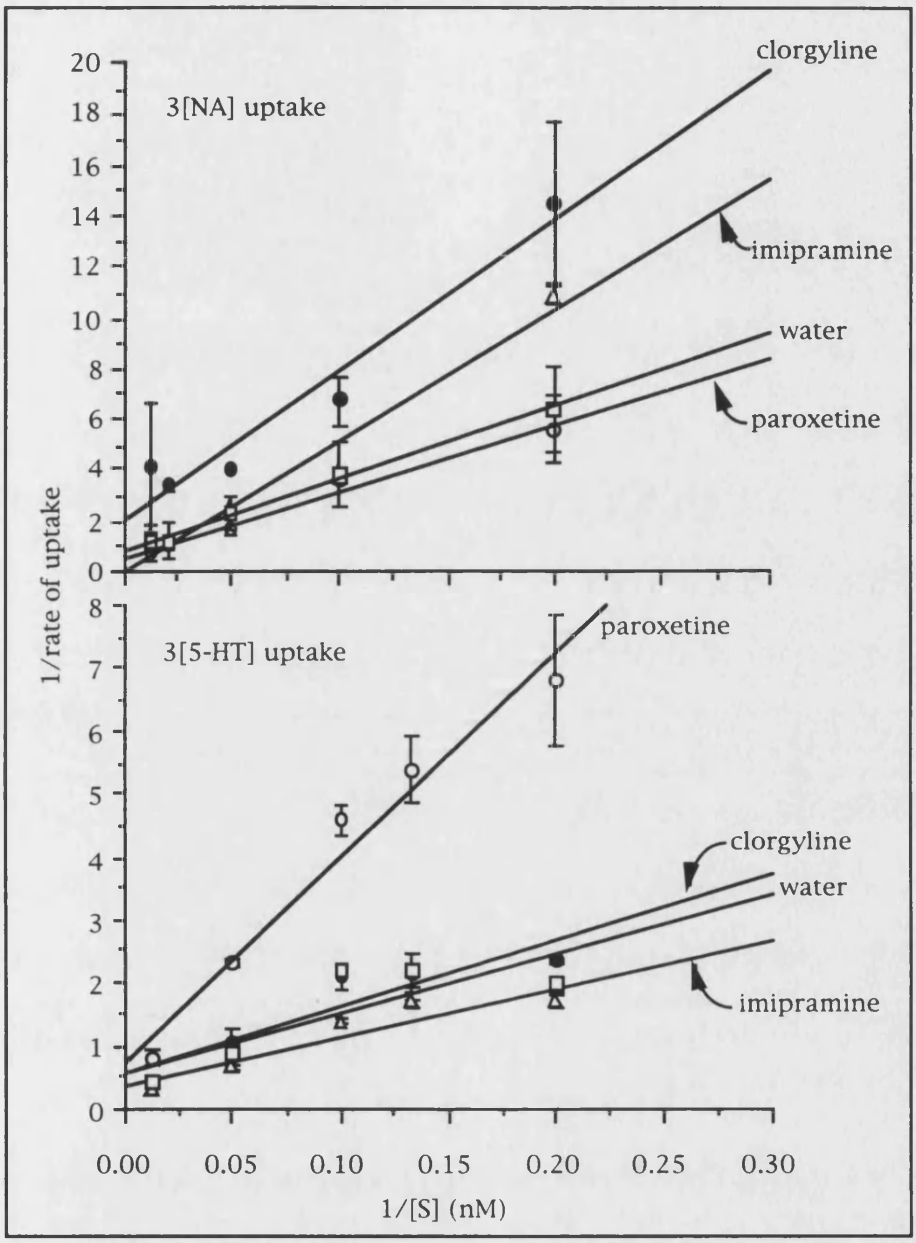


Figure 34. Lineweaver-Burke plot of 3[NA] and 3[5-HT] uptake in rat cortical synaptosomes in the presence of ○ paroxetine, △ imipramine, ● clorgyline and □ control (water).

Figure 35 shows the overall trends in uptake inhibition for both tritiated monoamines. Paroxetine significantly inhibited [^{3}H]-5-HT uptake (inhibition relative to control=68.49%) without affecting NA uptake. Imipramine had no significant effect on uptake of either monoamine. Clorgyline significantly inhibited the uptake of [^{3}H]-NA (inhibition relative to control=45.5%).

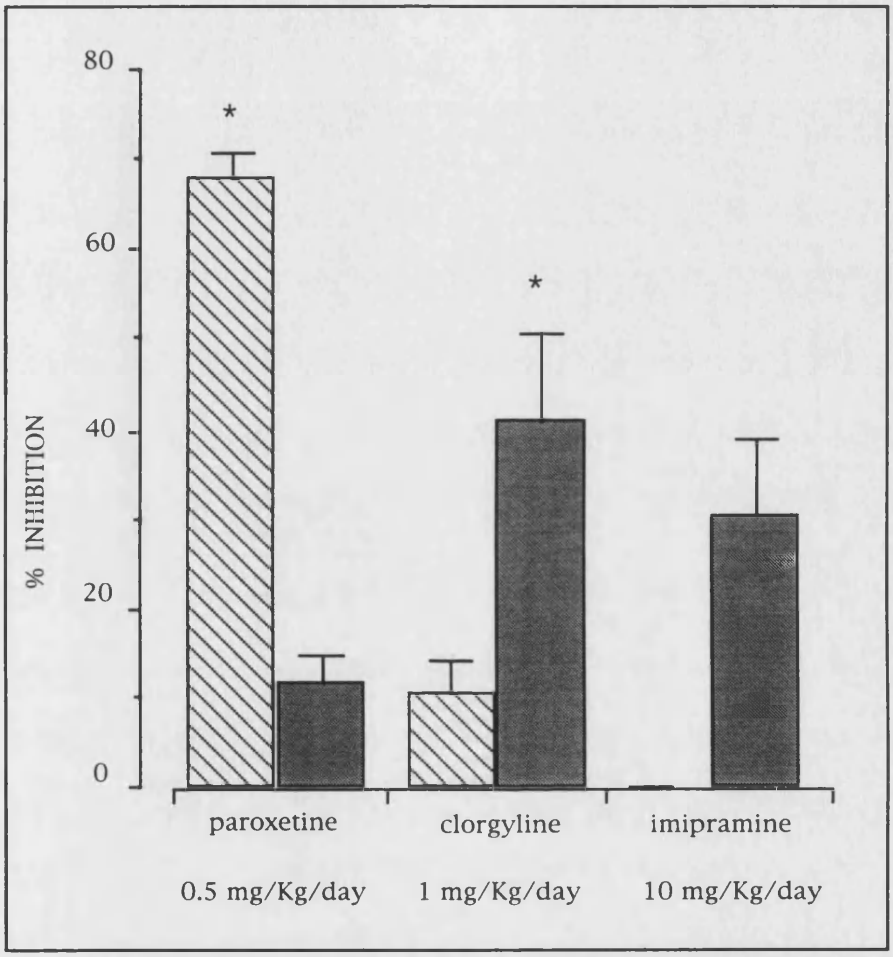


Figure 35. Inhibition of [3H]-monoamine uptake into rat cortical synaptosomes * $p \leq 0.05$; twoway ANOVA followed by Dunnett's test.
▨ = [3H]-noradrenaline (n=6); ▧ = [3H]-serotonin (n=4).

Body weight

All animals gained weight throughout the course of the five week drug treatment; Figure 36 illustrates the weight changes over this period. The smallest weight gain was seen in imipramine-and clorgyline-treated animals although the difference from that of other groups was not found to be statistically significant.

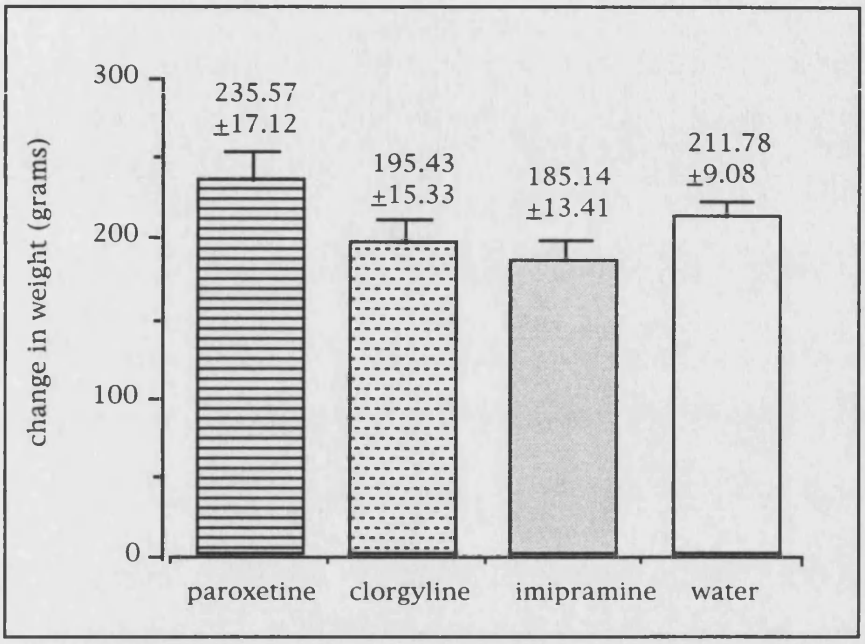
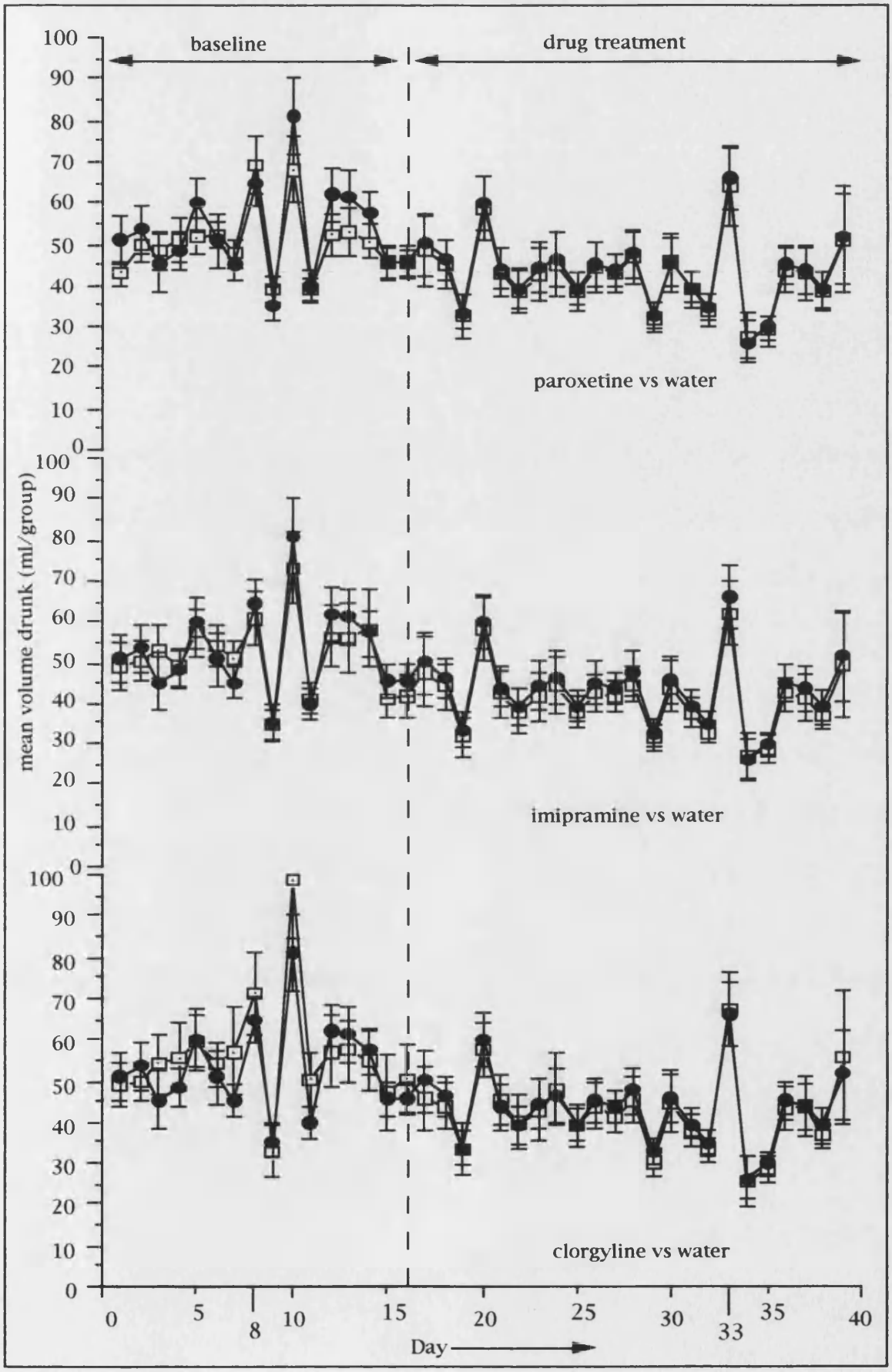


Figure 36. Change in weight over a 5 week drug period, of animals treated chronically with antidepressants in drinking water. Paroxetine 10 mg/Kg/day; clorgyline 1 mg/Kg/day and imipramine 10 mg/Kg/day (n=7 per group); water (n=9).

Changes in volume of solution drunk

Figure 37 illustrates water consumption throughout the experiment. Each treatment group is plotted separately against the values for the control (water-only) group to allow direct visual comparison with control. The baseline period refers to the days before drug was added to the drinking water. Although the amount of solution drunk per 24 hours appeared to decrease by approximately 10% during drug treatment, this decrease was seen in all groups including the water-only group. Therefore, addition of paroxetine, clorgyline and imipramine to water did not significantly affect intake. Days 8, 9 and 10 are days on which cages were changed. Day 33 is the day of the light pulse administration at CT21. In both cases there was an increase in the amount drunk which can be clearly seen.

Figure 37. Amount of water drunk by rats prior to (baseline), and during, drug treatment. Each measurement is made over an approximate 24 hour period. The mean and sem of each drug group □ (n=7), is plotted with that of the water group ● (n=9), for direct comparison.



CIRCADIAN RHYTHM RESULTS

Period

The free-running period of locomotor activity was shown to lengthen over the course of the experiment in all groups (Figure 38). This increase was greatest in paroxetine-treated animals (0.16±0.02 hours), although this value did not reach statistical significance compared to the water-only group (F=2.33; p=0.097).

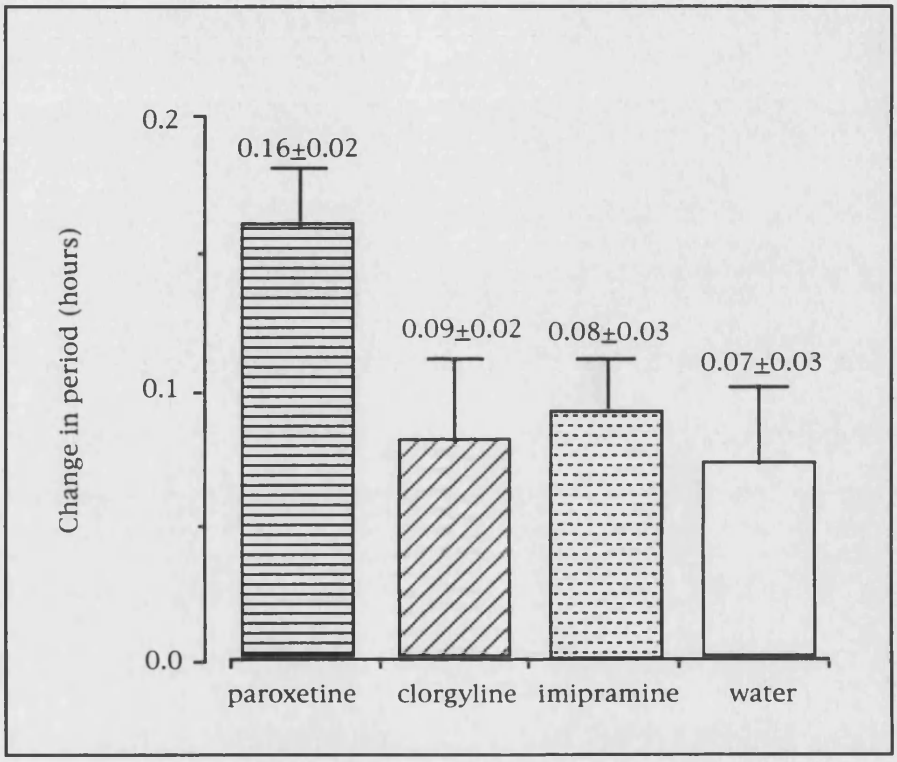


Figure 38. Effects of antidepressant drugs on rat circadian locomotor activity period (administration via drinking water). paroxetine = 10 mg/Kg/day; imipramine = 10 mg/Kg/day and clorgyline = 1 mg/Kg/day. n=9 for water (control) group and n=7 for antidepressant groups.

A representative actogram from each group is shown in Figures 39a and 39b. The increase in tau over time is visible in each actogram. The day of drug addition to water is indicated by an arrow.

Figure 39a. Actograms from animals treated chronically with paroxetine (upper trace) and clorgyline (lower trace).

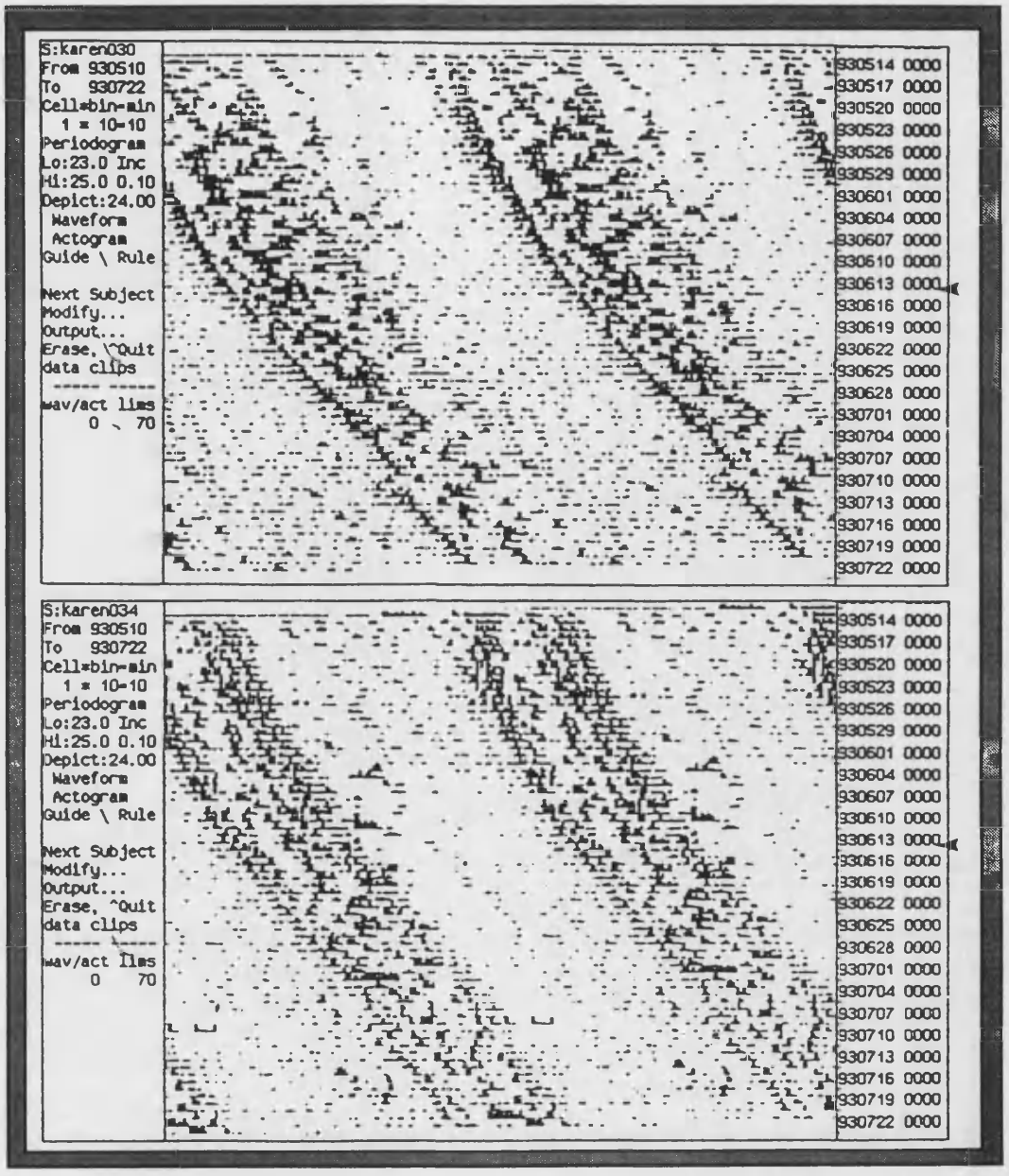
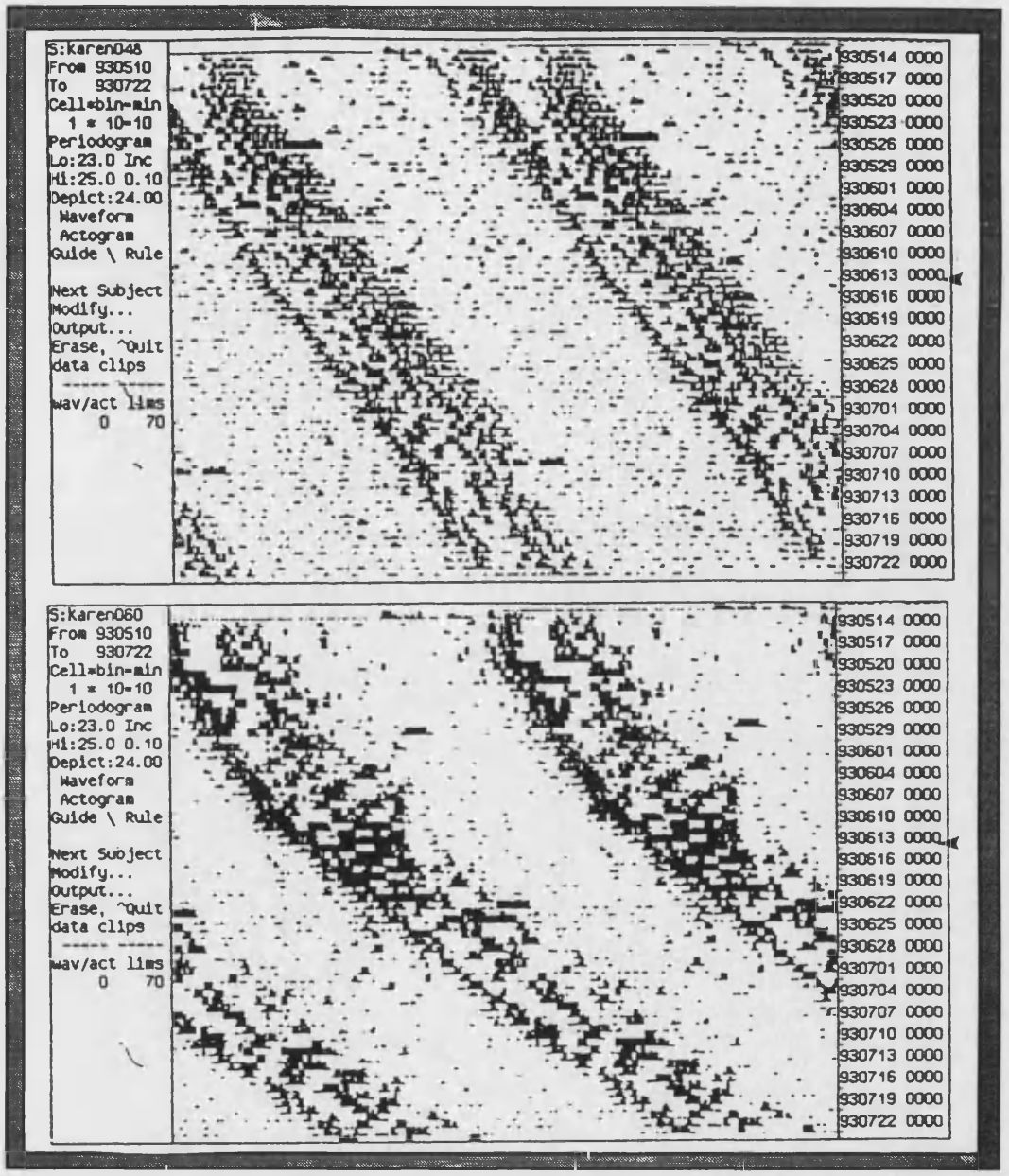


Figure 39b. Actogram from an animal treated chronically with imipramine (upper trace), plus an actogram from a control animal (lower trace).



Alpha (α)

Antidepressant drug treatment did not significantly affect the length of time for which the animals were active (α); furthermore, this value decreased in all animals during the course of the experiment (Figure 40).

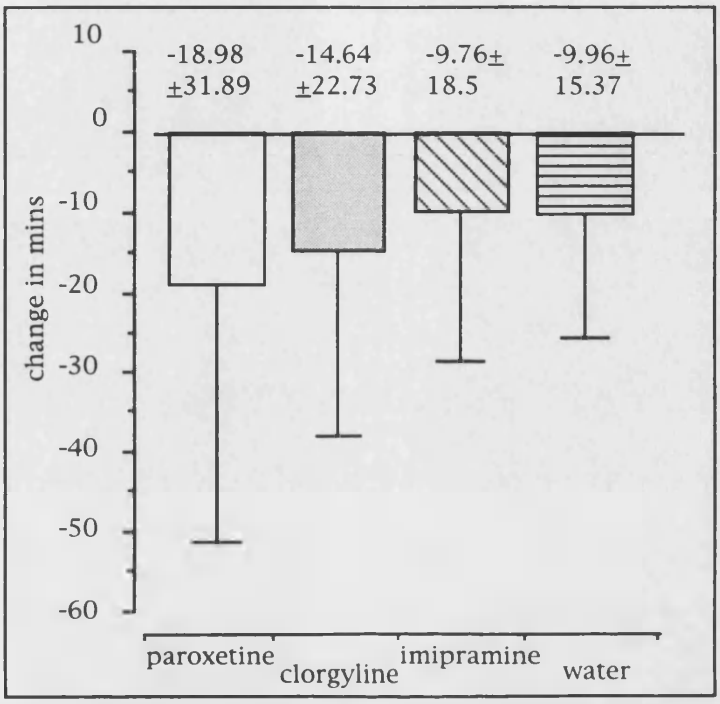
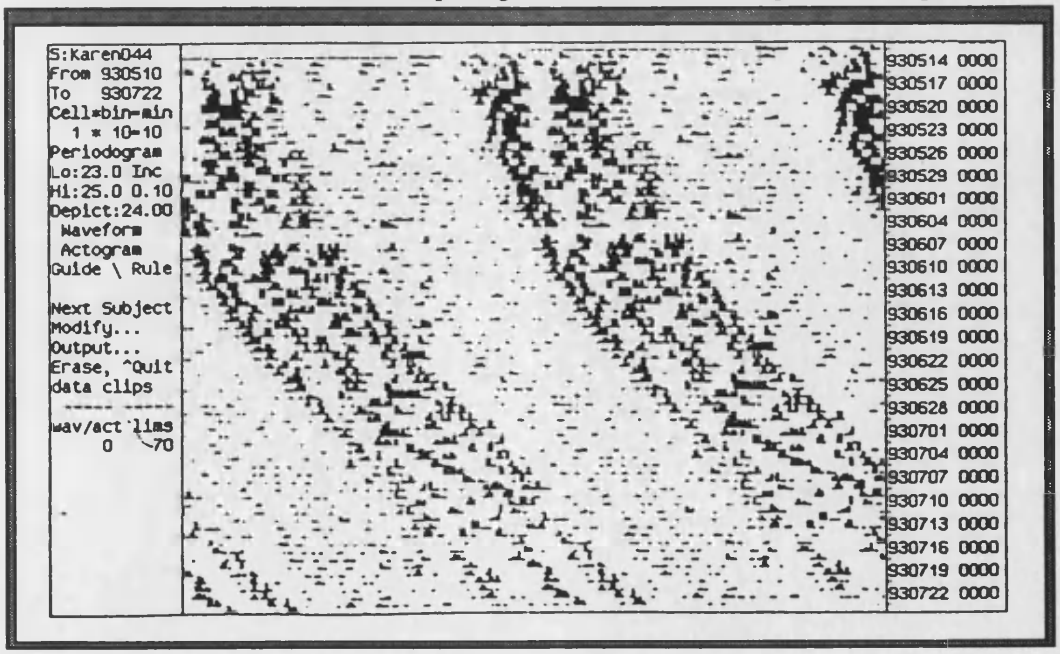


Figure 40. Changes in alpha with treatment (antidepressant administered via drinking water). Paroxetine=10 mg/Kg/day; clorgyline=1 mg/Kg/day and imipramine=10 mg/Kg/day.

Figure 41 is an actogram from a paroxetine-treated animal showing a minor reduction in α and A when comparing the before and during treatment periods.



Amount of activity (A)

Chronic antidepressant reatment did not significantly affect the average amount of activity per 10 minute bin per cycle (A) (Figure 42).

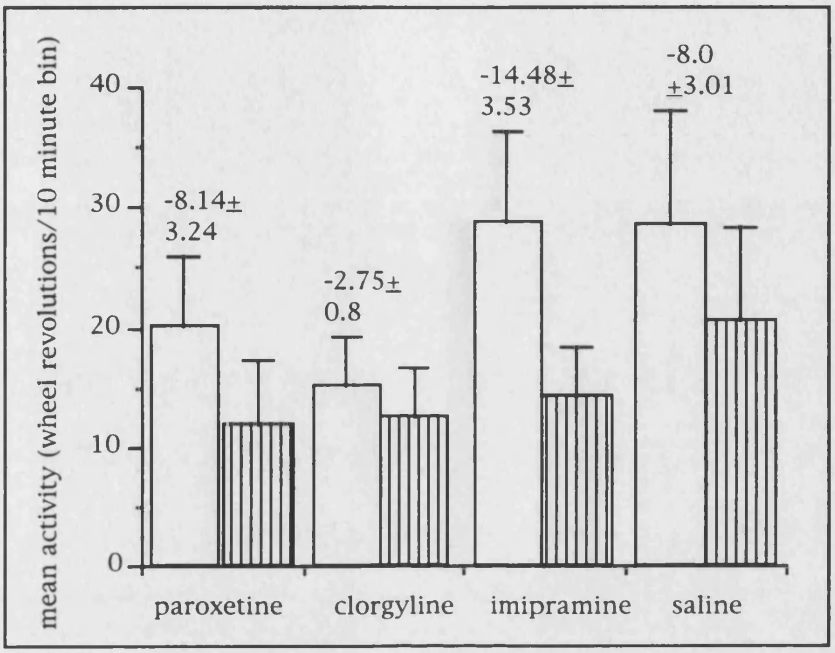


Figure 42. Effect of antidepressant drugs administered chronically via drinking water on amount of rat locomotor activity. ☐ average amount of activity per cycle per 10 min bin before treatment. ▥ average amount of activity per cycle per 10 min bin after treatment;

Phase shift at CT21

The effects of the light pulse administered at CT21 on onset and offset of free-running locomotor activity were assessed, and the mean phase shift (± sem) for each group is illustrated in Figure 43. The values of the shift in minutes for each group are:
Onset - paroxetine 59.99±26; imipramine 40.65 ± 18.57; clorgyline 54.37 ± 24.37 and water-only 12.67 ± 18.13.
Offset - paroxetine 29.6± 46.33; imipramine 71.15 ± 24.31; clorgyline 93.91 ± 36.41 and water-only 22.77 ± 22.77. None of the groups exhibited a statistically significant phase change to the light pulse when the results were tested with oneway ANOVA.

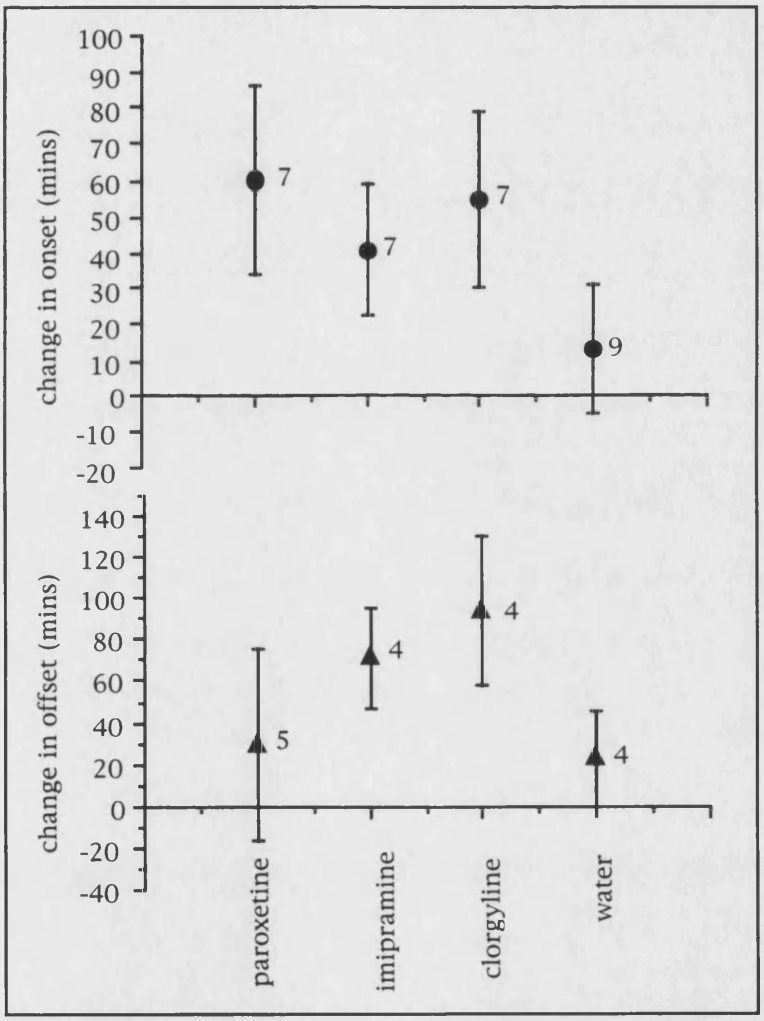


Figure 43. Distribution of the mean change (± sem) in the onset ● and offset ▲ of locomotor activity following a 1 hour light pulse at CT21. Paroxetine=10 mg/Kg/day; imipramine=10 mg/Kg/day; clorgyine =1 mg/Kg/day. The numbers by the side of each symbol represent the number of animals used for that analysis.

Comparison of Experiment 1 and Experiment 2 data: Free-running period

The histograms in Figure 44 illustrate the distribution of free-running periods from rats in Experiment 1 (upper plot) and Experiment 2 (lower plot), measured prior to treatment. It can be seen that the tau group mean of animals from Experiment 1 was close to 24 hours (23.99±0.01 hours), whereas the mean for animals in Experiment 2 was longer than 24 hours (24.38±0.03). This difference in the free-running period before drug administration was statistically significant.

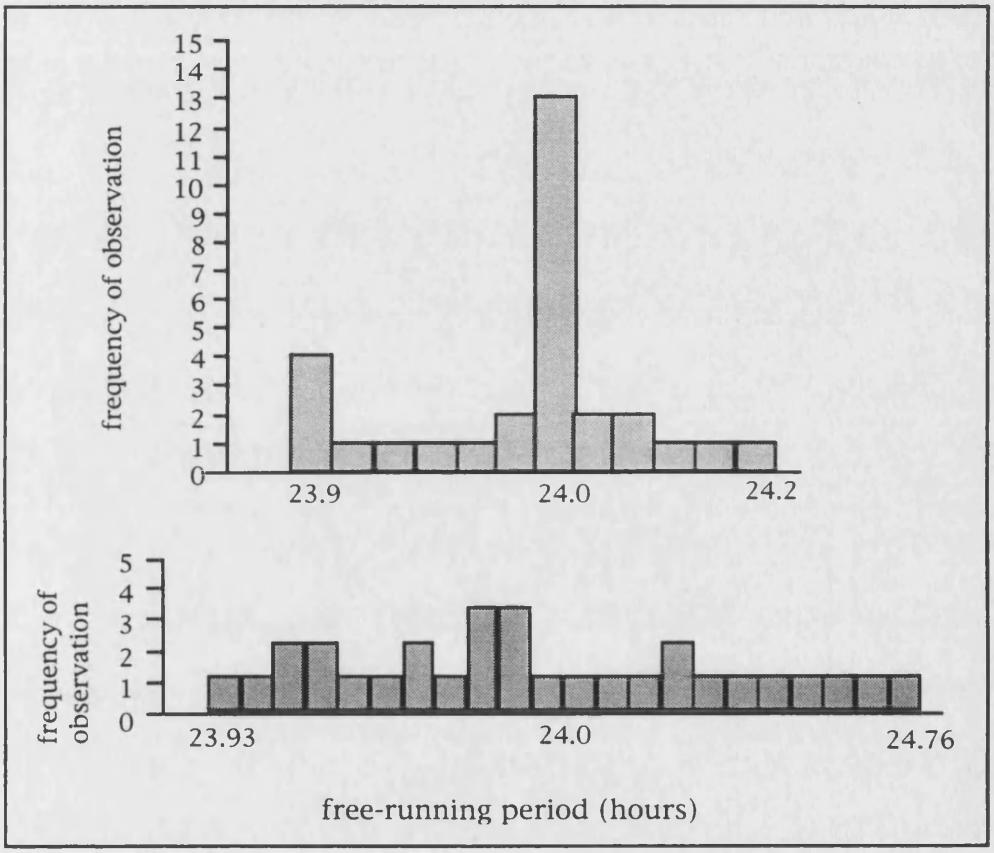


Figure 44. Histogram of the free-running period before treatment in animals from Experiment 1 (upper plot) (n=30; mean=23.99; s.e.m.=0.01) and Experiment 2 (lower plot) (n=30; mean=24.38; s.e.m.=0.03).

Comparison of Experiment 1 and Experiment 2 data: alpha and amount of acitivity

The duration of α in animals from Experiment 1 was shorter than that for animals in Experiment 2, although this did not reach statistical significance (10.79 hours ± 12.07 mins and 11.23 hours ± 8.74 mins, respectively). However, animals from Experiment 2 had a more intense band of activity (i.e. a larger A), and averaged significantly more wheel revolutions per cycle per 10 min bin than those from Experiment 1 (23.54±3.65 and 14.01±2.31 respectively; $p \leq 0.05$). This information is tabulated below.

Table 8: Comparison of activity data generated in Experiments 1 and 2.

	α onset to offset band	A (revs/10 min bin)
Experiment 1 *(constant dark)*	11.23 hours ± 8.74 mins	14.01 ± 2.31
Experiment 2 *(dim red light)*	10.79 hours ± 12.07 mins	23.54 ± 3.65
t-test (p value)	ns	≤0.05

DISCUSSION

The administration of the antidepressant drugs imipramine, clorgyline and paroxetine to drinking water did not affect the amount of water consumed, establishing this as a suitable route of administration for these compounds. This route allowed an increased dose of paroxetine to be administered compared to that in the previous experiment, and the biochemical data from the present study confirm the ability of 10 mg/Kg paroxetine administered orally, to selectively block ^{3}H-[5-HT] uptake *ex vivo*. Although imipramine showed a marked tendency to selectively inhibit ^{3}H-[NA] uptake, this effect did not prove to be statistically signficant, a factor which may be attributed to the small sample size of the assay. The significant inhibition of ^{3}H-[NA] uptake by clorgyline (supporting the trend seen in Experiment 1), was surprising given that this antidepressant is believed to exert its clinical effects via monoamine oxidase inhibition. To determine whether this was a residual effect of clorgyline in the tissue during the assay, clorgyline was subsequently tested for its ability to inhibit monoamine reuptake *in vitro*. Only at concentrations as high as 10^{-4}M did clorgyline

have any effect on uptake, thus ruling out any residual, *in vitro*, action of the drug during the assay.

The decreased body weight seen in Experiment 1 in animals implanted with clorgyline- and imipramine-containing pumps did not occur when these drugs were given orally. In fact, body weight did not vary significantly between any of the groups in the present experiment, and all animals gained weight over the drug period. This result highlights the variation of effect of the same drug when administered via different routes.

None of the antidepressant drugs had any significant effect on the circadian system. Neither the free-running period of locomotor activity, nor the length of activity time, nor the phase of activity onset was altered. The lack of effect of clorgyline on phase and alpha in particular, supports the idea that the changes seen in these parameters in clorgyline-treated animals in Experiment 1 were due to the physical pertubations of surgery, and not to the effects of the drug *per se*. None of the antidepressant drugs significantly affected the amount of wheel-running activity. This is in contrast to Experiment 1, where paroxetine caused a decrease in activity. This difference may reflect the higher dose of paroxetine facilitated by drinking water administration, substantiating the idea that low doses of paroxetine increase dozing in rats (Kleinlogel and Burki, 1987).

The effects of the light pulse administration at CT21 are difficult to explain, since none of the groups, including the water-only control group, elicited a significant phase shift of activity. An explanation which could account for this lack of effect may be that the PRC for this group of animals varied from that measured by Honma et al., (1985) for the same species (Figure 45), on which the timing of light pulse exposure was based. It can be speculated that if the PRC of the animals in the present experiment varied slightly from that in Figure 45, such that light pulse fell at the crossover point between phase advance and phase delay shifts, then the result would be no net phase shift. This hypothesis could explain the present results. In addition, when measuring the onset of locomotor activity, all antidepressant-treated animals tended to exhibit larger phase advances that control animals. Similarly, when measuring offset, both imipramine and clorgyline-treated animals tended to exhibit the largest phase advances (Figure 43). Although, these values were not statistically significant, (a factor which may be attributed to the small n number involved), it could be further speculated that antidepressant treatment altered the PRC to light of these animals. This tendency of the antidepressants to potentiate phase advances to a light pulse is in contrast to that reported in the hamster, where chronic clorgyline treatment reduced phase advances to light (Duncan et al., 1988; Harrington & Rusak.,

1991). However, this is only speculation, since no significant circadian effects were observed.

Figure 45 illustrates the PRCs obtained by Honma et al., (1985) for onset and offset of wheel-running activity in male Wistar rats to short light pulses (300 lux; 30 mins). Based on this PRC, the phase at which the light pulse was applied in the present experiment is indicated by the shaded bar.

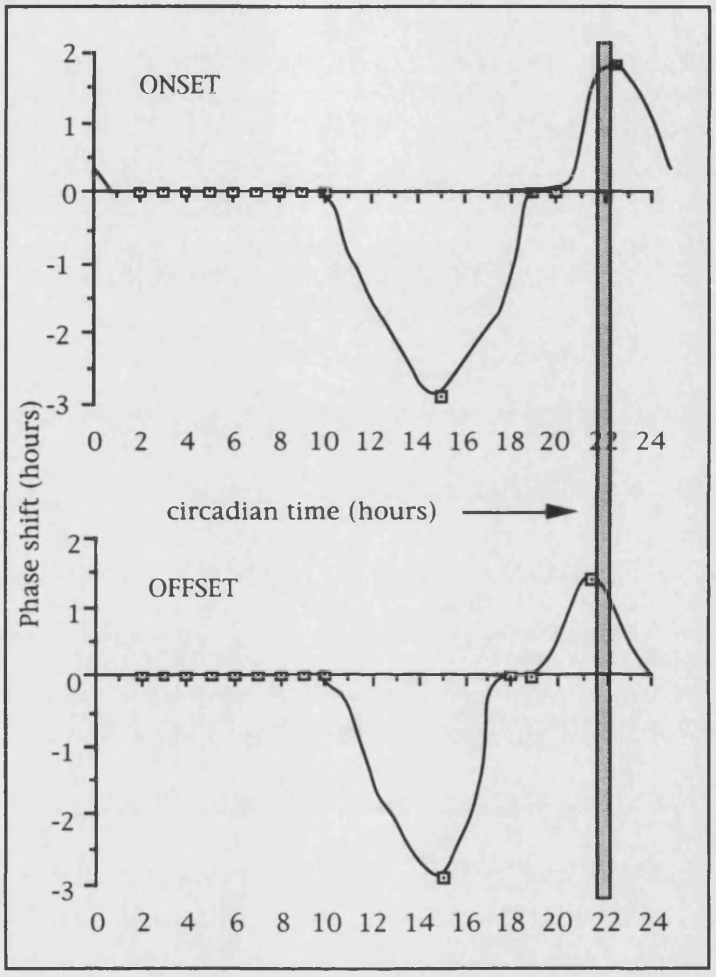


Figure 45. The PRC for locomotor activity onset and offset of the Wistar rat redrawn from Honma, et al., (1985) .

This experiment therefore confirms the circadian results obtained in Experiment 1 - that the antidepressant drugs had no significant effect on rat circadian locomotor activity rhythms.

Some general observations can be made between Experiments 1 and 2. When analysing the actograms for the first four weeks stabilisation period in the running-wheels, the two groups of animals had been treated and housed in the same manner. The only difference between the groups during this period was the lighting conditions employed. Experiment 1 animals were housed under constant darkness and Experiment 2 animals were housed under constant dim red light conditions. These different lighting conditions significantly affected a number of parameters of wheel-

running activity. Firstly, there was a significant difference in the free-running period during this time, with animals which were housed in constant dim red light conditions displaying a longer average period than those housed in constant darkness. Also, the time of onset of locomtor activity was markedly different between the two groups. The animals housed in constant dark conditions displayed a narrow range of onset times for the group (around 23.00h), to such an extent that entrainment to an external cue was debatable. The animals housed in contant dim red light conditions displayed a range of onset times for the group, which varied from 22.30h to 06.30h, ruling out any suggestion of entrainment of rhythms. Finally, animals housed in constant dim red light were significantly more active than those housed in constant dark, as determined by wheel revolutions per average cycle per 10 minutes. Although it has been proposed that rats are insensitive to red light (Honma et al., 1992), these results clearly indicate a strong influence of this type of lighting compared with constant darkness.

CONCLUSION

The aim of these studies was to explore the interaction between circadian rhythmicity and alterations in the levels of the monoamines noradrenaline and serotonin as mediated by antidepressant drugs. Because the antidepressants did not have any measurable effect on the circadian system, the results do not provide support for the hypothesis that the antidepressant action of these drugs is brought about by alterations in the circadian system. In addition, no correlation was found between the central inhibition of ^{3}H[5-HT] and ^{3}H[NA] uptake and effects on the circadian system.

CHAPTER V

Circadian locomotor activity rhythms in an animal model of depression: Chronic Mild Stress

INTRODUCTION

A large percentage of the animal studies which have attempted to link disturbances of circadian rhythms and depressive disorder have examined the effects of antidepressant drugs on circadian rhythms in normal animals. However, such studies could be considered to be based on a flawed concept because antidepressant drugs are renowned for showing few signs of their clinical activity in 'normal' subjects, a fact which may account for the inconclusive results obtained so far (Wollnik, 1992; Klemfuss & Kripke, 1994).

A more direct, and perhaps a more logical approach to this type of investigation, is to examine circadian rhythms in "depressed" animals, without the intervention of antidepressant drugs. This option of course poses the immediate anthropomorphic problem of what is a depressed animal? Indeed, much criticism is levelled at animal models of depression, which are, after all, based on a human disorder which has a large subjective component. Nevertheless, research in this field has so far yielded some potentially interesting results. In the learned helplessness animal model of depression, Stewart et al, (1990) have demonstrated that inescapable footshock lengthened rat circadian locomotor activity period. In addition, inescapable footshock has been shown to drastically reduce the amplitude of rat circadian body temperature rhythms (Kant et al., 1991). Whilst it is accepted that animal behavioural models of psychiatric disorders can never exactly simulate human psychopathology, they may go some way in helping to evaluate the central nervous system changes involved. Therefore, to complement the studies in Chapter IV which investigated the effects of antidepressant drugs on the rhythms of normal animals, this Chapter describes experiments aimed at elucidating circadian rhythm changes caused by an animal model of depression.

The starting point for such experiments is a convincing animal model of depression (Willner, 1984 & 1990). Some animal models serve merely as bio-assays for screening antidepressant drugs, and do not attempt to simulate the human symptoms of depression. These models include amphetamine-potentiation and reserpine-reversal, which are based on the ability of potential antidepressant compounds to affect chemically-induced behaviours (Moryl et al., 1993). Olfactory bulbectomy belongs to another group of depression models in which abnormal behaviour is created by brain damage (Jesberger & Richardson, 1985), which again does not equate with the factors thought to elicit human depression.

The diagnosis of major depression, according to the American Psychiatric Association (1987), requires the continual presence during a two week period of one core symptom and at least four subsidiary symptoms of the illness. The two recognised

core symptoms are depressed mood and loss of interest or pleasure (anhedonia). The seven subsidiary symptoms are fatigue/loss of energy, appetite disturbance/weight change, psychomotor retardation/agitation, insomnia/hypersomnia, feelings of worthlessness or excessive/inappropriate guilt, recurrent thoughts of death/suicide or a specific plan or attempt at suicide. Of the core symptoms, it is obviously not possible to determine mood in an animal model of depression, neither is it possible to determine the psychologically based subsidiary symptoms associated with human depression. However, it is possible to measure anhedonia, appetite disturbance/changes in weight and locomotor changes in animals. A valid animal model of depression would therefore be one which attempts to simulate these changes in behaviour. An example of such a model is that based on the effects of separation of an animal from its group, partner or parent. Separation models typically elicit symptoms such as changes in appetite and body weight, and decreases in social interaction and exploratory behaviour; however, the complexity and lengthy duration of these experiments (isolation may require one year in some paradigms) can prohibit the use of these models (Garzon & Del Rio, 1981).

The largest group of animal models of depression which aims to evoke symptomatic resemblance with the human condition is based on the generation of abnormal behaviours by stress (Willner, 1991). Stress is thought to be one of the major precipitating factors in depression (Breslau & Davis, 1986; Anisman & Zacharo, 1982; Kanner et al., 1981), and a positive correlation has been demonstrated between the onset of endogenous depression and the incidence of stressful life events in the preceding three months (Bidzinska, 1984). Learned helplessness is a much used animal depression model based on stress. In learned helplessness procedures, uncontrollable stress (such as inescapable footshocks) produces performance deficits in subsequent learning tasks, which are not seen in subjects exposed to the identical stressor but able to escape it (Willner, 1986). In addition, animals show a variety of other behavioural changes such as decreased locomotor activity, poor performance in appetite-related tasks and decreased body weight (Maier, 1984). Similarly, chronic unpredictable mild stress (CMS), is an animal model of depression based on stress which has been shown to elicit symptoms which parallel many of those seen in human depression. The CMS model has recently been reviewed and updated, and Willner and co-workers (1987), have produced a milder version of the original work of Katz (1981). The more recent version involves subjecting animals to a variety of stressors such as periods of food and water deprivation, white noise and strobe-lighting exposure instead of the more severe stressors originally used (such as electric shock, cold water and heat stress) (Katz, 1981; 1982).

CMS was chosen as the depression model for this study based on the following points:

• One of the most significant effects of stress observed in animal models of depression is the reduction in reward-related behaviour. Rats normally display very high preferences for a sweet solution when given a choice between sucrose and water (>80%), but exhibit decreases in this preference when exposed to CMS (Willner et al., 1987). This decrease in the efficacy of the reward is assumed to reflect an altered function within the brain systems which mediate reward behaviours and has been likened to the anhedonia associated with human depression (Fawcett et al., 1983; American Psychiatric Association, 1987; Willner et al., 1992).

• CMS has been proposed to satisfy three main criteria of an animal model of depression termed *face, predictive* and *construct* validity respectively (Willner, 1984; 1986; 1991). Briefly, these are defined as:

1. *Face*: symptomatic resemblance between the model and the clinical condition.

The face, or sympromatic validity of the CMS model has been confirmed by the decreased consumption of a palatable, sweet solution in response to stress (Muscat et al., 1988; Willner et al., 1987), the decrease in rewarding properties of natural (food) and drug (amphetamine and morphine) rewards in the conditioning place preference paradigm (CPP) (Papp et al., 1991), and the increased threshold for intracranial self stimulation (ICSS) (Moreau et al., 1992). All of these tests are proposed to be a measure of anhedonia. Furthermore, changes in locomotor activity (Papp et al., 1993) and body weight (Matthews et al., 1995) have been recorded in CMS-treated animals.

2. *Predictive*: ability of the model to respond selectively to antidepressant drugs.

The predictive validity of the CMS model has been verified with a wide range of antidepressant drugs such as the tricyclic compounds imipramine and desipramine (Papp et al., 1994; Moreau et al., 1992; Willner et al., 1987; Katz, 1982), the reversible MAO-A inhibitor moclobemide (Moreau et al., 1993a), the atypical antidepressants mianserin, iprindole, buproprion (Katz 1982) and maprotiline (Muscat et al., 1992b), and the SSRI, fluoxetine (Muscat et al., 1992b). Importantly, the effects of CMS were not reversed by the anxiolytic compound, chlordiazepoxide (Muscat et al., 1992a).

3. *Construct*: clinically appropriate theoretical rationale.

A combination of unpredictable stressful procedures may provide the best approximation to clinical depression, as none of the aetiological factors identified in human depression alone is sufficient to elicit this condition.

The CMS model can be criticised as being heavily focused on a single symptom of depression - anhedonia, and although this is a core symptom, it is not a prerequesite for a diagnosis of major depression (Willner, 1991). However, CMS has recently been demonstrated to evoke additional parallels to human depression in the form of

increased corticosterone levels and an impaired complement immune response (Ayensu et al., 1995), further validating CMS as a valid animal model.

To summarise, the following experiments were carried out in order to determine whether exposure to CMS produces alterations in rat circadian locomotor activity rhythms. A correlation between these two areas would support the hypotheses that biological rhythms are linked to depressive disorder. The experiments were conducted in three phases: 1) the CMS model was established within the laboratory 2) the reversibility of these effects by antidepressant compounds was tested and 3) the effects of CMS treatment on locomotor activity rhythms were determined.

METHODS

In this and all subsequent experiments in this section, the following methods and apparatus were used.

SUBJECTS

Male Hooded Lister rats supplied by Charles River, Margate, Kent, were individually housed in opaque plastic cages (45 cm long, 24 cm wide and 20 cm high) with standard rat chow (Special Diet Services; Witham, Essex) and water being freely available. Ambient temperature was 23°C and the lighting schedule was a 12:12 hour light/dark cycle (lights on 7.00a.m, 120 lux measured around cage level, lights off 19.00h). Housing, feeding and lighting parameters were then altered as indicated in the CMS procotol.

APPARATUS

1. Confinement boxes:

During periods of 'confinement', animals were placed in an opaque plastic box measuring 130 mm x 155 mm x 120 mm deep, with a minimum depth of 50 mm where the stainless steel feeding lid protrudes into the cage. This size of cage greatly restricts movement without actual physical restraint of the animal.

2. Stroboscopic lighting:

Stroboscopic lighting was provided by a portable stroboscopic light at a rate of 300 flashes per minute. The light was controlled using a domestic clock timer.

3. White noise generator:

White noise was provided by a conventional radio switched off-station. Again, the appliance was controlled by a domestic clock timer.

4. Locomotor activity apparatus

Locomotor activity was measured using a Benwick Electronics Activity Monitor (AM1051) system (Benwick Electronics, East Anglia, UK). The procedure involved placing animals in an individual clear plexiglass cage (45 cm long, 24 cm wide and 20 cm high) which was itself placed in the centre of a 2 x 3 beam matrix of infrared lights. Horizontal movement was detected when the beams were broken, and this impulse was recorded on an IBM-compatible PC using the Amlogger computer program (Benwick Electronics). Beam breaks were collected every 3 minutes for a 60 minute duration, to allow a detailed examination of the activity throughout the test period. Either the timecourse (number of beam breaks per 3 min block) or the total number of beam breaks for the 60 minute test period was plotted in the analyses. Locomotor activity testing was kept to a minimum of one test per week, which took place in the later stages of the CMS regime and no more than four separate tests were applied to any single group of animals. This is because the nature of the test was to examine locomotor activity in a novel environment and repeated testing of animals in

the locomotor apparatus may have lead to habituation to the test surroundings and a decreased baseline activity.

SUCROSE HABITUATION WEEK

All animals were first habituated to a 1% sucrose solution according to the timetable below (Table 9). Briefly, this procedure involved a 48 hour exposure to the sucrose solution, followed by sucrose preference testing on three successive days. A 20 hour period of food and water deprivation preceded each sucrose preference test. Fluid consumption was determined by weighing drinking bottles.

Table 9: Timetable for sucrose habituation week (week 0).

Day	Time	Procedure
Sunday (day 0)	17.00h	exposure to a 1% sucrose solution.
Monday (day 1)	17.00h	sucrose intake measurement at 24 hours
Tuesday (day 2)	17.00h	sucrose intake measurement at 48 hours; water deprivation (food *ad lib.*).
Wednesday (day 3)	13.00h-14.00h 14.00h-17.00h 17.00h	sucrose preference test food and water *ad lib.* water deprivation (food *ad lib.*).
Thursday (day 4)	13.00h-14.00h 14.00h-17.00h 17.00h	sucrose preference test food and water *ad lib.* water deprivation (food *ad lib.*).
Friday (day 5)	13.00h-14.00h 14.00h	sucrose preference test food and water *ad lib.*

A concentration of 1% sucrose was chosen based on reports that describe sucrose preference to fit a bell-shaped curve over the concentration range 0.01 to 5% (Pucilowski et al., 1993; Muscat et al., 1992; Willner et al., 1992; Phillips et al., 1991). At low concentrations, on the ascending limb of the concentration-intake curve, sucrose intake rises proportionately with concentration, and intake is directly related to reward value. However, at high concentrations on the descending limb of the concentration-intake curve, intake is no longer related in a simple way to reward value. The reason for this paradoxical decrease in intake at high sucrose concentrations is unclear, and has been proposed to be the result of post-ingestive satiety effects (Geary and Smith, 1985), although this hypothesis is under question (Muscat et al., 1991; Phillips et al., 1991). Therefore, 1% is a concentration at which changes in responsiveness to reward are reflected in corresponding changes in sucrose intake, since other concentrations may provide a misleading measure of reward value. Once the animals had been habituated to the sweet solution, they were tested for their preference for sucrose over water as described below:

SUCROSE PREFERENCE TESTING.

Testing occurred at 09.00h in the animal's home cage following a period of food and water deprivation from 17.00h the previous evening. Testing consisted of presenting a bottle of ordinary drinking water and one containing the 1% sucrose solution. The two bottles were placed to the left and right of the feeding compartment of each animal and sides were reversed at each test. The animals were allowed to drink freely from either of these bottles for a one hour period. The ratio of sucrose-to-water drunk was calculated to give a percentage preference for sucrose for each animal according to the formula:

$$\text{percentage sucrose preference} = \frac{\text{(amount of sucrose)}}{\text{(amount of water + amount of sucrose)}} \times 100$$

Day 5 sucrose preference test scores (third preference test in sucrose habituation week) were used as the *baseline value* with which to separate animals into two groups matched for sucrose intake. This was done by listing animals in ascending order of their percentage sucrose preference and alternating the group to which they were assigned. One group underwent the stress procedure outlined below whilst the other group served as a control group.

Stress procedure Stressed animals were housed singly and subjected to a series of stressors according to a weekly timetable adapted from that of Willner et al., (1987) and Moreau et al., (1993) (see Timetable in Appendix 1). The sum of individual stressors used was classified as being mildly stressful under the terms of the Animals (Scientific Procedures) Act, 1986, and all procedures in the present CMS schedule were approved by the U.K. Home Office. On a weekly basis, the stress regime consisted of confinement; stroboscopic lighting; white noise; food and water deprivation; water-only deprivation; grouped housing on a 'soggy' bed (sawdust bedding made damp by the addition of water); exposure to an empty water bottle; restricted feeding (rat chow pellets weighing 4-5 g in total scattered on the cage floor); sucrose preference testing; cage change; body weight measurement and daily maintenance of food and water.

The stressors were built into a weekly schedule such that no more than three stressors were allowed to be applied simultaneously and procedures such as reversed lighting were omitted to avoid confounding the subsequent circadian experiments. During the stress period rats were also tested in the locomotor activity apparatus previously described. At the end of the stress period changes in sucrose preference, body weight and locomotor activity continued to be monitored weekly until their return to control values; this was termed the 'extinction' period.

Control procedure: Control animals were singly housed and were deprived of food and water for 16 hours prior to the weekly sucrose preference test. Also, a weekly cage change, body weight measurement and daily food and water maintenance were performed (for Timetable see Appendix 2). Control animals were tested in the locomotor activity cages at the same time as stress group animals.

DATA ANALYSIS

All sucrose preference and body weight data were analyzed by ANOVA using a repeated measures design, with treatment as a between-subjects factor and successive tests as a within-subjects factor (Ludbrook, 1994). Only data during weeks of CMS application (excluding baseline and extinction values) were included in the analysis. Analyses were performed using SYSTAT for Windows (Systat Inc. Evanston, IL, U.S.A) which calculates the Greenhouse-Geisser and Huynh-Feldt epsilon adjustments for multisample asymmetry within repeated measures ANOVA and provides the corresponding corrected p values. This analysis was supplemented by t-tests of vertical pairwise contrasts at each weekly time point and the resultant p values were corrected to allow for multiple hypotheses using the Dunn-Sidàk adjustment:

$$p' = 1 - (1-p)^k$$

where p' is the adjusted p value and k is the number of comparisons which have been made. It is important to note that vertical contrast analysis is a minor addition to the repeated measures ANOVA testing and great biological importance should not be attached to the individual time points (week of CMS) which are highlighted as being significant. In the results sections, the F and p values are quoted for each statistical calculation.

Locomotor activity counts were transformed to $\log_{10}$ values because of the non-normal distribution of these data, and Student's t-test was performed on the logarithmically transformed values.

Fischer's Exact test was used for analysis of 2x2 Tables.

RESULTS

Since this animal model of depression is based on the decreased preference for a sweet solution, it is important to monitor any changes which occur not only in sucrose intake, but in water and total fluid intake to be able to correctly evaluate any changes in sucrose preference. Therefore, each results section details the changes in these four parameters before, during and (where relevant) after exposure to the CMS regime.

EXPERIMENT 1: ADAPTATION OF THE 'WILLNER ' MODEL (CMS1)

The aim of the following experiment was to determine if exposure to a regime of chronic mild stress (CMS) would cause a decreased preference for a sweet sucrose solution in male Hooded Lister rats.

SUBJECTS

Thirty male Hooded Lister rats (weighing 315±10 g at the beginning of the experiment; supplied by Charles River, Margate, Kent) were housed as described in the methods section.

EXPERIMENTAL PROCEDURE.

After division into two equally matched groups for sucrose preference, stress-group animals were subjected to CMS for six consecutive weeks with locomotor testing in weeks 4 and 6. At the end of this period animals were tested during extinction of the stress procedure for as long as it took behavioural changes to return to control levels; locomotor activity was measured in extinction week 1. Control animals were measured in the same way.

RESULTS.

Sucrose Preference Testing

Baseline:

Despite the tendency for pre-stressed animals to drink more sucrose and water than control animals in the final baseline test of the sucrose habituation week, Student's t-test revealed there to be no significant difference between the groups. The sucrose and water intake values were, in ml: 11.37±0.99 vs 10.44±0.81 and 5.61±0.87 vs 4.91±0.76 for the CMS and control groups, respectively. However, there was a significant difference between the two groups in the total amount of fluid intake in this baseline preference test (16.98±0.48 ml CMS vs 15.35±0.51 ml control; t=2.33, df 28; p=0.027). This difference in total fluid consumed did not translate into a significant difference in sucrose preference (66.66±5.27 % CMS and 68.03±4.94 % control; t=-0.19, df 28; p=0.85).

CMS-treatment:

The stress procedure had no significant effect on sucrose intake (treatment $F_{(1,28)}$=0.037, NS), although intake did vary significantly within subjects during the course of the experiment (week $F_{(5,140)}$=4.71; p≤0.002), fluctuations in sucrose intake

can be seen in Figure 46a. There was a significant interaction of week and stress treatment ($F_{(5,140)}$=6.81; $p \leq 0.001$), indicating that the fluctuations in sucrose intake were not the same in both groups.

There was no significant effect of the stress procedure on the amount of water consumed in the one hour test sessions (treatment $F_{(1,28)}$=2.2, NS), although there was significant variation in water intake within subjects during the course of the experiment (week $F_{(5,140)}$=14.0; $p \leq 0.001$) (Figure 46b). There was no interaction between week and CMS ($F_{(5,140)}$=2.38, NS), suggesting that the fluctuations in water intake were similar in magnitude and direction in both groups during the CMS procedure.

Total fluid intake was not significantly different between the two groups during the CMS period (treatment $F_{(1,28)}$=0.048, NS), although total intake fluctuated throughout the experimental period within subjects (week $F_{(5,140)}$=10.3; $p \leq 0.001$). There was a significant week and CMS interaction (week x treatment $F_{(5,140)}$=16.25; $p \leq 0.001$) (Figure 46c).

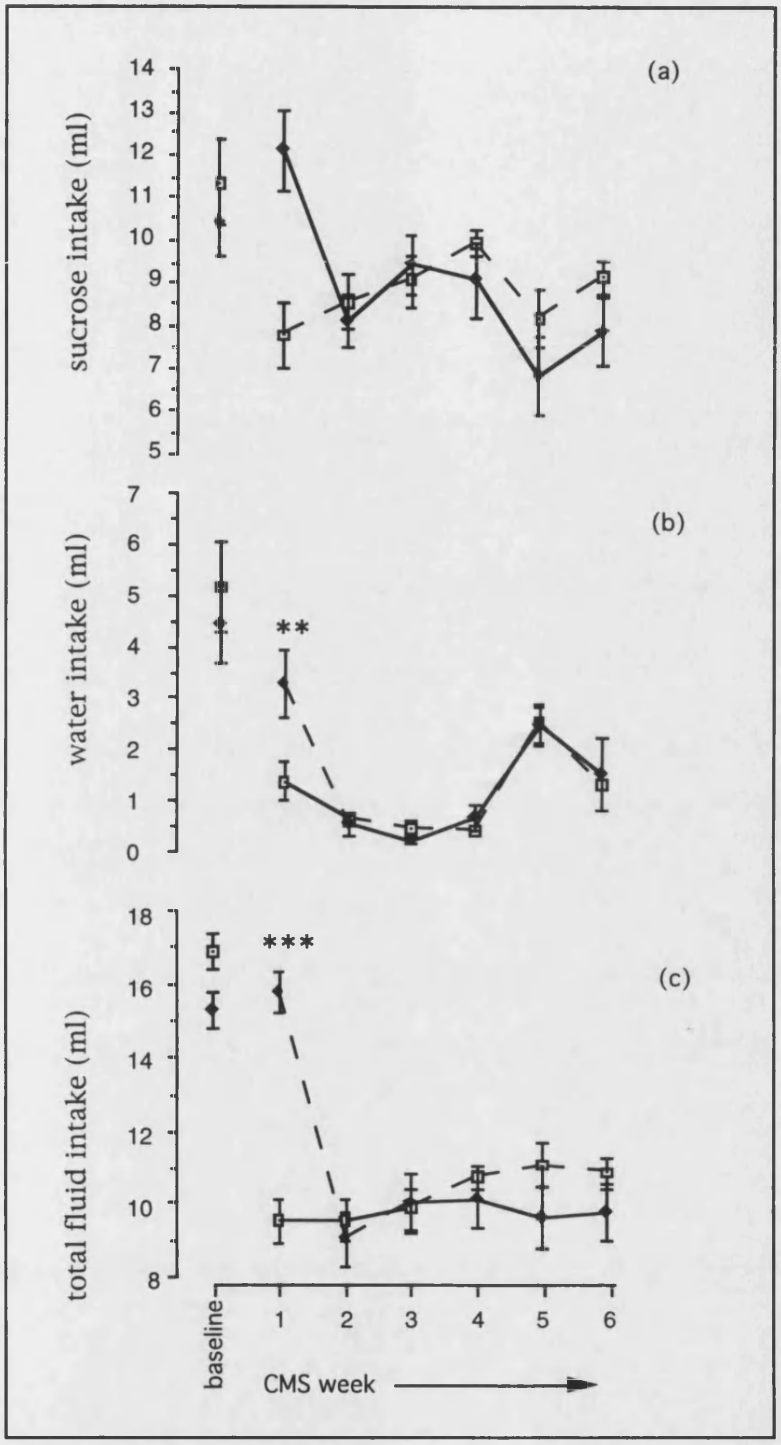


Figure 46a Sucrose intake, Figure 46b Water intake and Figure 46c Total fluid intake in CMS1 animals during weekly sucrose preference testing. Values are mean ± sem. Stress group — □ - control group n=15 per group. ** p≤0.01; *** p≤0.001 vs. control. ◆ Baseline values are taken from week 0, 3rd habituation test.

Overall, CMS-treatment did not significantly affect sucrose preference (treatment $F_{(1,28)}=0.87$, NS). However, the preference ratio varied significantly within subjects during the experimental procedure (week $F_{(5,140)}=11.92$; $p\leq 0.001$). This variation occurred in a similar manner in both groups as seen by the lack of a significant interaction (week x treatment $F_{(5,140)}=0.53$, NS). Figure 47 displays the sucrose preference test values for animals in the CMS 1 experiment.

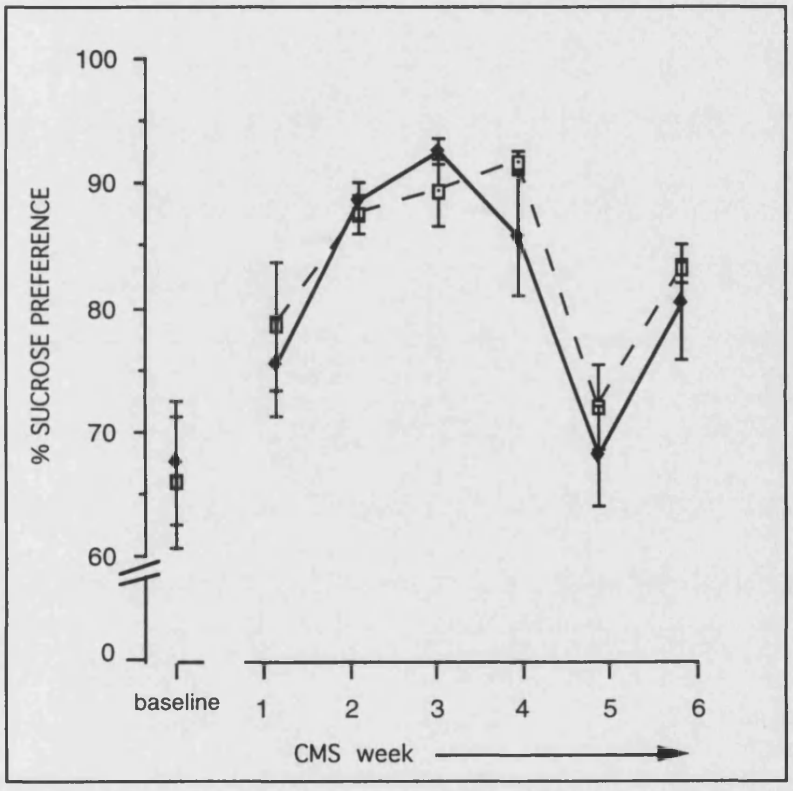


Figure 47. Sucrose preference testing at weekly intervals in CMS 1 animals and control group during 6 weeks exposure to CMS. Values are mean±sem. Stress group — ◻ - control group —◆— n=15 per group.

Locomotor Activity

Application of Student's t-test to log-transformed total activity counts for the 60 minute test period revealed stress and control animals to show equal activity in the locomotor activity apparatus in week 4 CMS, (CMS=3497±154 and control=3579±225 beam breaks). However, when tested in week 6 of the CMS protocol, CMS animals exhibited significantly lower activity during the 60 min test than control animals (1741.17±158.8 and 2387.53±169.57 beam breaks, respectively; t=-2.78; p=0.01: Figure 48). This deficit in locomotor activity disappeared within one week of stopping the stress procedure as indicated by testing in the first extinction week (2368.8±187.8 and 2049.67±220.1 beam breaks, respectively).

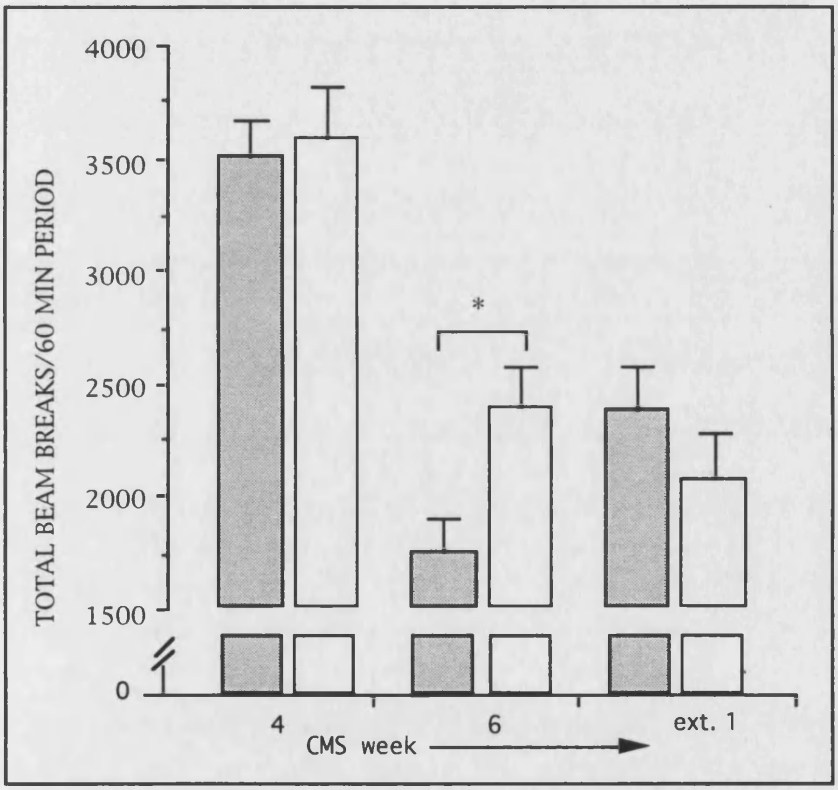


Figure 48. Locomotor activity testing in CMS1 animals and control group.
Values are mean±sem. Stress group ▩ control group □ n=15 per group.
* p≤0.05 vs control. ext. 1 = extension week 1.

Body weight

Repeated measures analysis revealed no effect of the CMS procedure on body weight (treatment $F_{(1,28)}$=0.934, NS), and all animals continued to gain weight throughout the course of the experiment at a rate of approximately 5.5% per week (week $F_{(5,140)}$=765.71, $p \leq 0.001$) (treatment x week $F_{(5,140)}$=7.41; $p \leq 0.004$). Animals from both groups ended the treatment period (week 6) at approximately 139% of their initial weight (Figure 49).

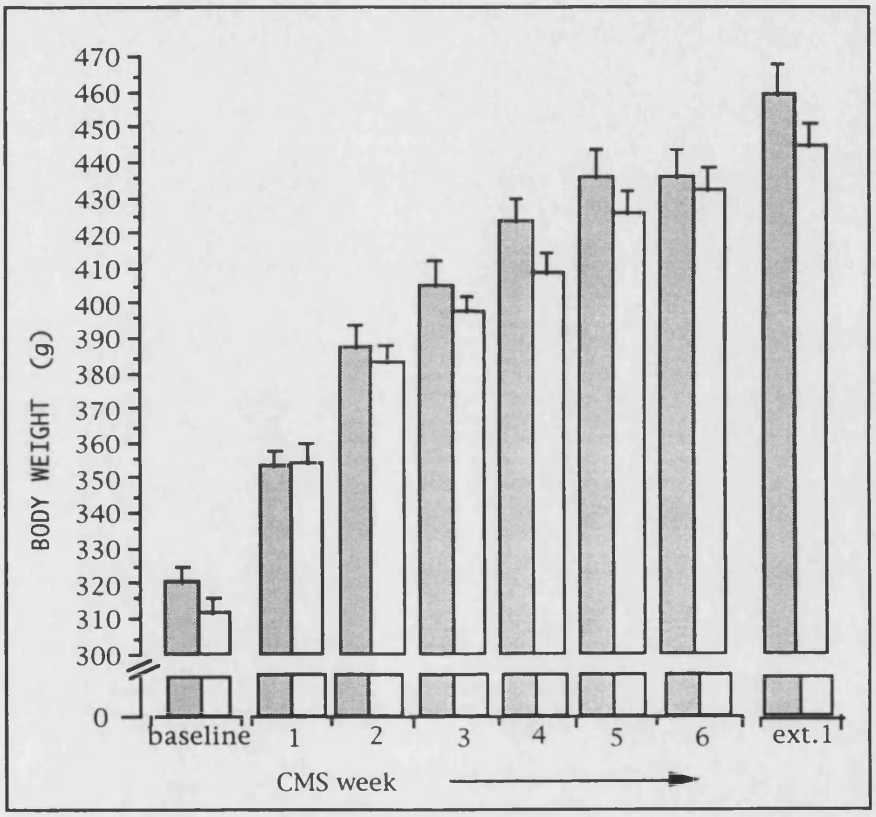


Figure 49. Changes in body weight during chronic mild stress treatment of CMS1 animals and control group. Values are mean±sem; stress group ■ control group □ n=15 per group. Baseline = experimental starting weight. ext 1.= extension week 1

DISCUSSION

The aim of the experiment was to demonstrate that animals subjected to CMS exhibited a decreased preference for a sucrose solution, indicative of a reduced responsivity to reward, and representative of an hedonic deficit similar to that observed in human depression. The results show that animal exposure to the protocol for six consecutive weeks did not only fail to cause the anticipated decrease in preference for a sweet solution, but also had no effect on body weight (weight loss is frequently observed in depressed patients (Hamilton, 1967). The only effect of the procedure was to cause a decrease in locomotor activity at the end of the six week period. CMS has been shown by other workers to elicit effects within a much shorter time period. Muscat et al., (1992), reported CMS to cause a substantial decrease in sucrose intake following nine days exposure to the regime. A shorter time-scale of exposure than 6 weeks is desirable because of the long duration of the circadian rhythm follow-up experiments.

Why then did exposure to CMS fail to generate a decreased preference for sucrose over water? The strain choice and age of animals used can be discounted as a reason for failure, since the majority of studies successfully demonstrating a decreased sucrose preference with CMS have also used male Hooded Lister rats weighing around 300 g (Willner et al., 1992) as in the present experiment. Instead, the reason that the protocol was ineffective may be non-specific, since the combination of stressors which yields a decreased sucrose preference is known to vary within laboratories (personal communications, Willner and Moreau). It was therefore decided to examine the individual areas of the CMS protocol to see if the stressor timetable could be re-organised to be more effective in terms of generating the decreased preference for sucrose. The present timetable differs from that used by Willner et al., (1987), in the following respects:

- two over-night periods of continuous lighting per week were not incorporated into the present timetable because this may complicate the interpretation of the circadian rhythm follow-up experiments.
- the weekly presence of a foreign object in the animal's cage overnight for 17 hours was deemed to be too weak a stressor and was omitted.
- cold-room exposure twice per week for 30 minutes was not carried out because this was impractical within the CMS-laboratory.
- bi-weekly periods of cage tilt on an increasing gradient was impractical.
- daily confinement to a small cage for two hour-long periods was not part of the Willner timetable, but was included in the present CMS protocol on the basis of the work of Moreau et al., (1992 & 1993a,b). Confinement is a form of restraint which

alone can be used to elicit depressive symptoms in animals (Desan et al., 1988; Plaznik et al., 1989).

In a methodological analysis of the CMS procedure, Muscat and Willner (1992) found no one element to be either necessary to cause a decrease in sucrose intake or sufficient to maintain the impairment for longer than 4 weeks (Muscat & Willner, 1992). Of the stressors applied, paired housing was shown to be the most potent, and its effectiveness was greatly enhanced by using multiple weekly exposures, although repeated exposure lead rapidly to habituation after 5 weeks of pairing. Furthermore, bi-weekly exposure to paired housing may be as effective as multiple exposure, and changing partners has been proposed to make paired housing more effective still (Muscat & Willner, 1992). Paired housing was used once per week as a stressor in the present schedule, with the same partners being paired each week.

Therefore, taking the importance of paired housing into consideration, it was decided to alter the CMS protocol to include two paired-housings per week (each of which placed the animal with a different partner). It was also decided to move the body weight measurement and sucrose preference test moved from mid-week to the end of the week to allow testing to cover a full week of CMS.

EXPERIMENT 2: REVISED TIMETABLE OF STRESS PROCEDURE (CMS2)

The aim of the following experiment was to determine if exposure to the revised CMS regime caused a decreased preference for a sweet sucrose solution in male Hooded Lister rats.

SUBJECTS

Thirty male Hooded Lister rats (weighing 295±5 g at the beginning of the experiment; supplied by Charles River, Margate, Kent) were housed as described for the CMS 1 experiment.

EXPERIMENTAL PROCEDURE

All animals underwent the same week 0 sucrose habituation period as those in Experiment 1 which involved 48 hour presentation of the 1% sucrose solution, followed by a series of three 1 hour tests of sucrose versus water on three successive days. In this particular experiment there was an unavoidable one week delay after the week 0 habituation before animals began the stress programme. During this week animals continued to be housed singly and were divided into two groups of 15 on the basis of a preference test score in this intervening week. In this experiment, stress group animals were subjected to a different regime of stressors than those in the CMS1 experiment. The differences were: two periods of food and water deprivation (19 and 16 hours); water-only deprivation lasting 15 hours; two periods of grouped housing with different partners, one of 16 hours on normal bedding and one of 20 hours on a 'soggy' bed (for Timetable see Appendix 3). The CMS regime was employed for five successive weeks followed by three weeks of extinction. The control group was deprived of food and water for 16 hours prior to sucrose preference testing, but otherwise had free access to food and water. Control animals were removed from their home cage for weighing once per week and were tested along with stress-group animals in the locomotor activity apparatus in weeks 2, 3, 5 and extinction week 1.

RESULTS.

Sucrose Preference Testing

Baseline:

Analysis of baseline values (final sucrose preference test of habituation week) by t-test, revealed there to be no significant difference between control and CMS animals in terms of sucrose, water and total fluid intakes. The values of these intakes were, in ml: sucrose 9.29±0.66 vs 7.59±0.76; water 1.35±0.24 vs 1.75±0.35 and total fluid

10.81±0.63 vs 9.33±0.70 for the CMS and control groups, respectively. Consequently, there was no significant difference between the two groups in the sucrose preference test prior to beginning CMS treatment (85.73±3.42 % CMS and 80.67±3.97 % control; t=0.966 , df 28; p=0.342).

CMS treatment:

There was no significant effect of the stress procedure on sucrose intake (treatment $F_{(1,28)}=2.5$, NS), although within subjects, intake did vary significantly during the course of the experiment (week $F_{(3,84)}=7.37$; $p\leq0.001$) (Figure 50a). The fluctuations in sucrose intake were different between groups such that there was a significant interaction of week and treatment ($F_{(3,84)}=8.26$; $p\leq0.001$).

CMS treatment significantly affected water intake (treatment $F_{(1,28)}=11.89$; $p\leq0.002$), since the stress group drank more water than control at each test (see Figure 50b). Water intake varied significantly within subjects during the experiment (week $F_{(3,84)}=4.29$; $p\leq0.015$); the most pronounced difference between stress and control occurred in week 2, when stress group animals consumed 1.98±0.18 ml compared to 0.83±0.11 ml for the control group (t-test $p\leq0.001$). There was no significant interaction between week and CMS ($F_{(3,84)}=0.9$, NS).

Total fluid intake was not significantly different between the two groups during the CMS period (treatment $F_{(1,28)}=0.34$, NS), although fluctuations did occur within subjects throughout the experimental period (week $F_{(3,84)}=3.35$; $p\leq0.03$), which were significantly different between the two groups (week x treatment $F_{(3,84)}=13.6$; $p\leq0.001$) (Figure 50c).

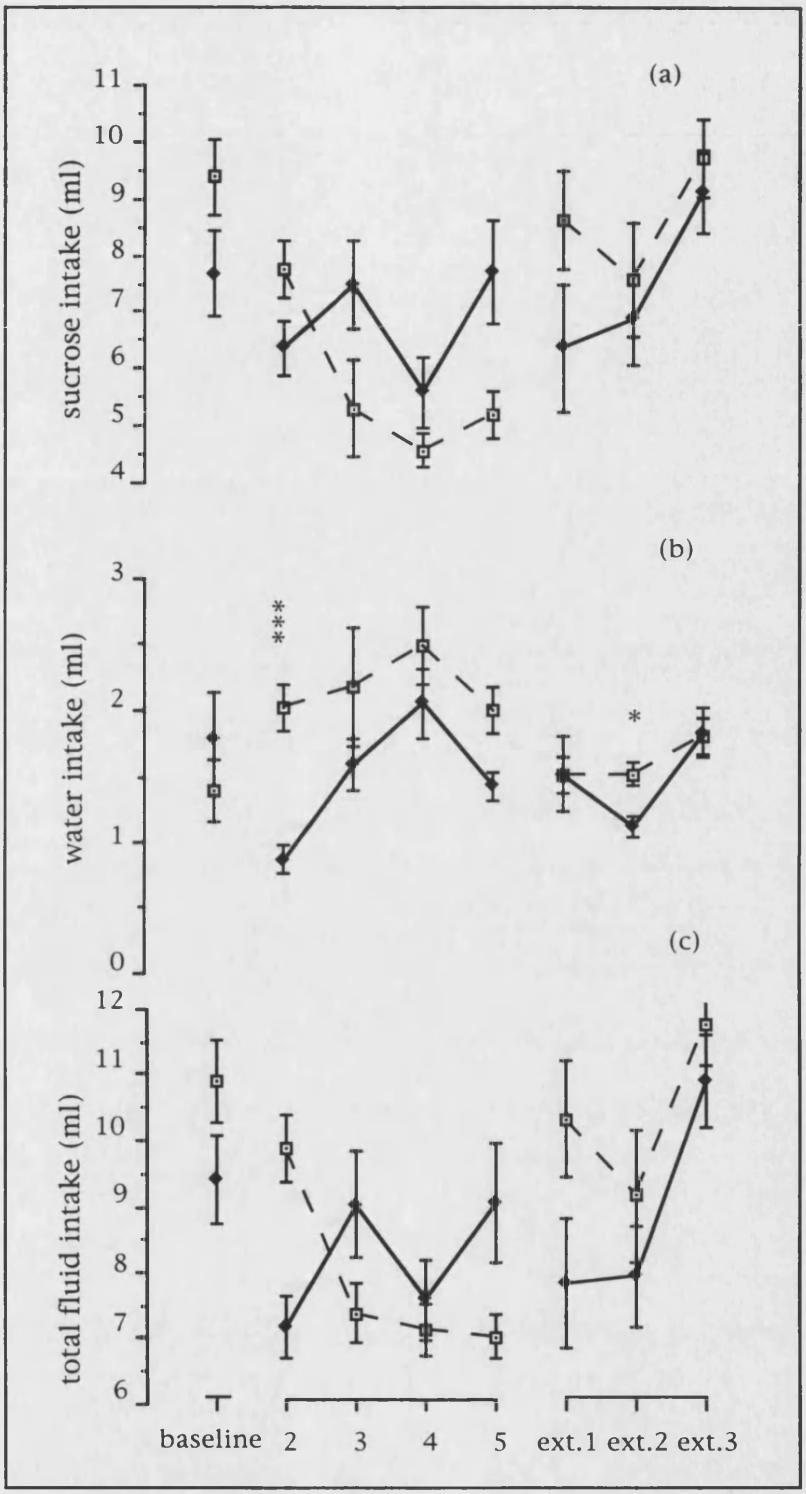


Figure 50a Sucrose intake, Figure 50b Water intake and Figure 50c Total fluid intake in CMS 2 animals during weekly sucrose preference testing. Values are mean ± sem. Stress group — ◻ - control group ◆ n=15 per group. Baseline values are taken from week 0, 3rd habituation test. *** $p \leq 0.001$ and * $p \leq 0.05$.

CMS-treatment significantly affected sucrose preference (treatment $F_{(1,28)}$=12.84; $p \leq 0.002$) and this varied significantly within subjects during the experimental procedure (week $F_{(3,84)}$=7.05; $p \leq 0.001$). Since changes in sucrose preference were in a similar in direction and magnitude during the course of the experiment in both groups, there was no significant week and stress interaction ($F_{3,84)}$=0.245, NS). Figure 51 displays the sucrose preference test values for the CMS 2 experiment.

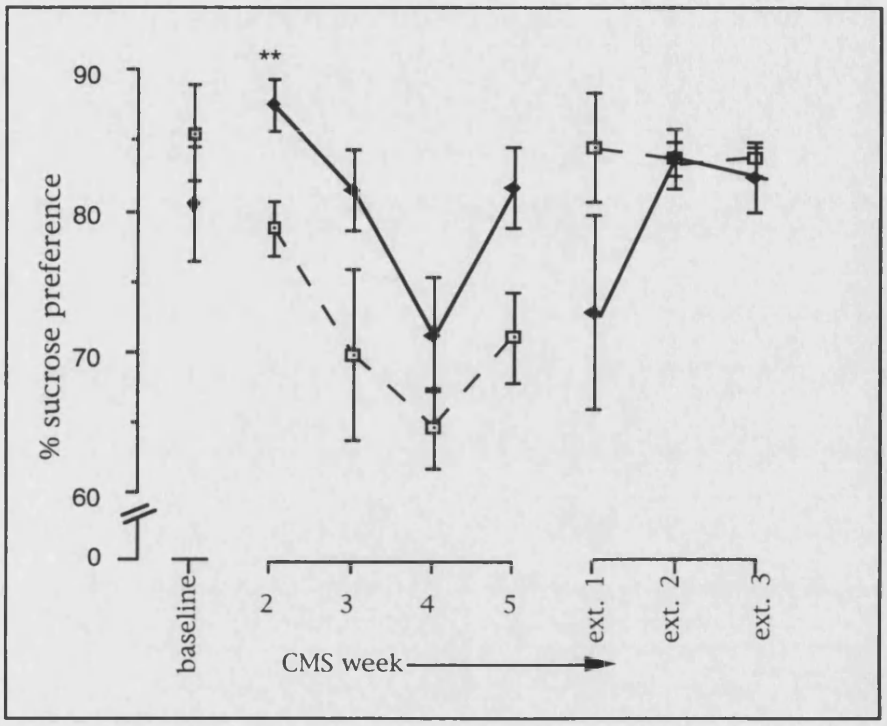


Figure 51. Plot of sucrose preference testing in CMS2 animals prior to and for three weeks after five succesive weeks of CMS treatment. CMS — ◻ - control —◆— n=15 per group values are mean±sem ** $p \leq 0.01$ vs control.

Extinction Period:

Sucrose, water, total fluid and sucrose preference values for each group of animals during the extinction period are also plotted in the previous two Figures. It can be seen that the stress procedure had no after-effect on sucrose intake (treatment $F_{(1,28)}$=1.185; NS). There was a significant within-subjects variation during the extinction period (week $F_{(2,56)}$=10.194; $p \leq 0.001$) which had ocurred during the CMS treatment. There was no significant week and treatment interaction during the extinction period (week $F_{(2,56)}$=1.57; NS).

This pattern was the same for water intake, with no significant difference between the two groups (treatment $F_{(1,28)}$=0.734; NS), a significant within subjects variation (week $F_{(2,56)}$=4.755; $p \leq 0.018$) and no significant interaction (week x treatment $F_{(2,56)}$=1.064; NS).

There was no after-effect of CMS treatment on total fluid intake (treatment $F_{(1,28)}=1.517$; NS); there was a significant within-subjects variation (week $F_{(2,56)}=19.206$; $p\leq0.001$) but no significant interaction (week x treatment $F_{(2,56)}=1.726$; NS).

Finally, during the extinction period, there was no significant difference between the CMS and control animals in terms of sucrose preference (treatment $F_{(1,28)}=0.768$; NS), no significant variation within subjects (week $F_{(2,56)}=0.772$; NS), and no significant interaction (week x treatment $F_{(2,56)}=2.904$; NS).

Locomotor Activity

Stress group animals were significantly less active than control animals when measured in week 2 of CMS exposure (CMS=2209$\pm$212.38 and control=2927.07$\pm$257.4 beam breaks respectively; t=-2.17, df 28; $p\leq0.05$). Although this trend continued when animals were tested in weeks 3 and 5 of CMS treatment, it was not statistically significant at these time points. These changes are illustrated in Figure 52. At the end of extinction week 1, there was no significant difference in locomotor activity between the 2 groups (CMS=2527$\pm$130.9 and control=2677.13$\pm$163.54 beam breaks, respectively).

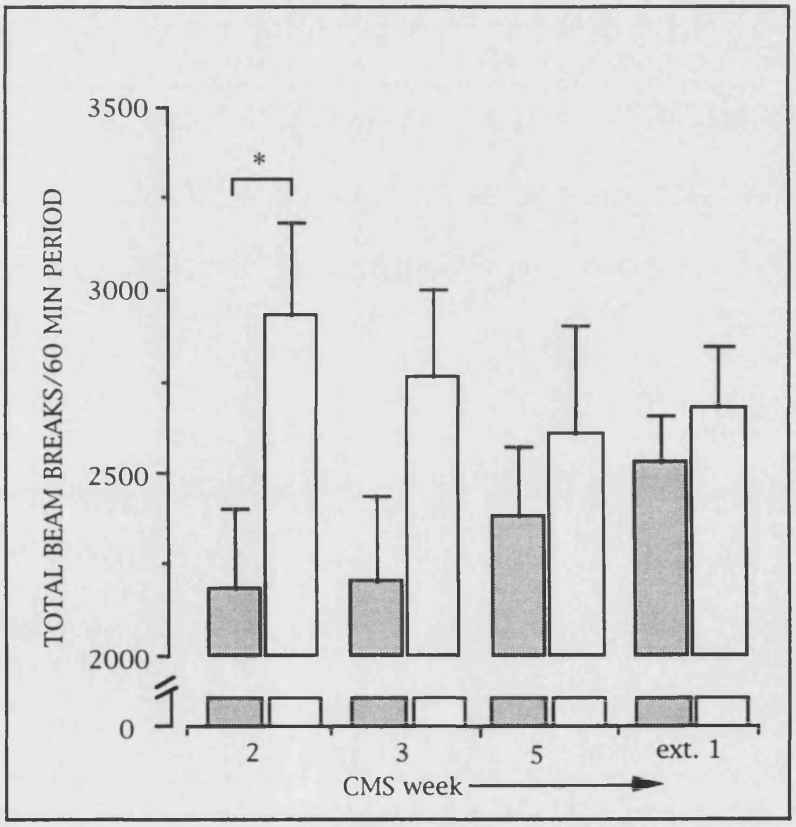


Figure 52. Locomotor activity testing in CMS 2 ☐ and control ☐ group animals; n=15 per group. Values are mean$\pm$sem *$p\leq0.05$ vs. control. ext. 1 = extension week 1.

Figure 53 plots the results obtained in week 2 CMS in the 60 minute locomotor activity test. The number of beam breaks per 3 minute interval is plotted for each group. CMS-treated animals showed significantly fewer beam breaks than controls during the test period.

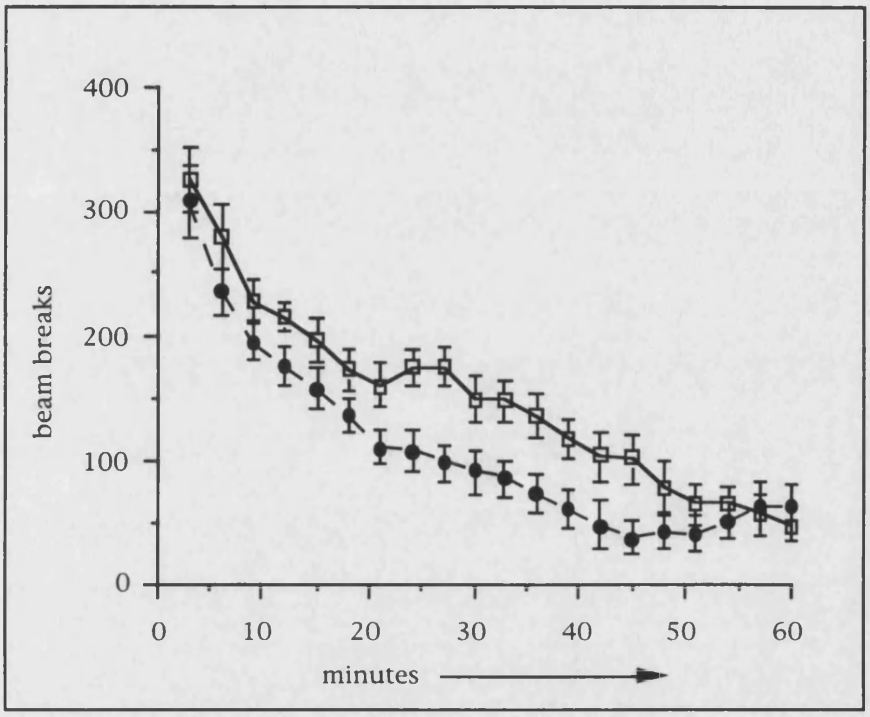


Figure 53. Timecourse of locomotor activity in CMS 2 animals, tested over a 60 min period in week 2 of the CMS regime. CMS 2 — ● — control —□— n=15 per group; values are mean±sem at each time point. CMS 2 animals were found to be significantly hypoactive compared to controls during the 60 min test period ($p \leq 0.05$).

Body weight

The starting weight of the control group animals was significantly less than that of pre-stressed animals (control 294±4.28 g vs CMS 316±3.75 g; $p \leq 0.001$: t-test). This weight deficit was reversed by CMS treatment, during which time the stress animals weighed significantly less than controls (treatment $F_{(1,28)}=10.75$; $p \leq 0.005$). This deficit increased on weekly exposure to CMS (week $F_{(4,112)}=78.913$; $p \leq 0.001$: Interaction of week and treatment $F_{(4,112)}=7.62$; $p \leq 0.001$). Control group animals gained weight at the rate of approximately 7.5% per week. CMS-treated animals gained weight at a slower rate (4% per week) and ended the treatment period (week 5)

at 123% of their initial weight compared to the control group animals which ended week 5 at 143% of their initial weight. The average weight gain per week for each group is illustrated in Figure 54. The deficit in body weight disappeared upon termination of the CMS procedure, at which point CMS animals gained weight more rapidly than controls (2.3% per week CMS vs 1.6% per week control), hence there was no significant difference during the extinction period between the two groups. CMS animals were sacrificed weighing 135% of their starting weight and controls weighed 149%.

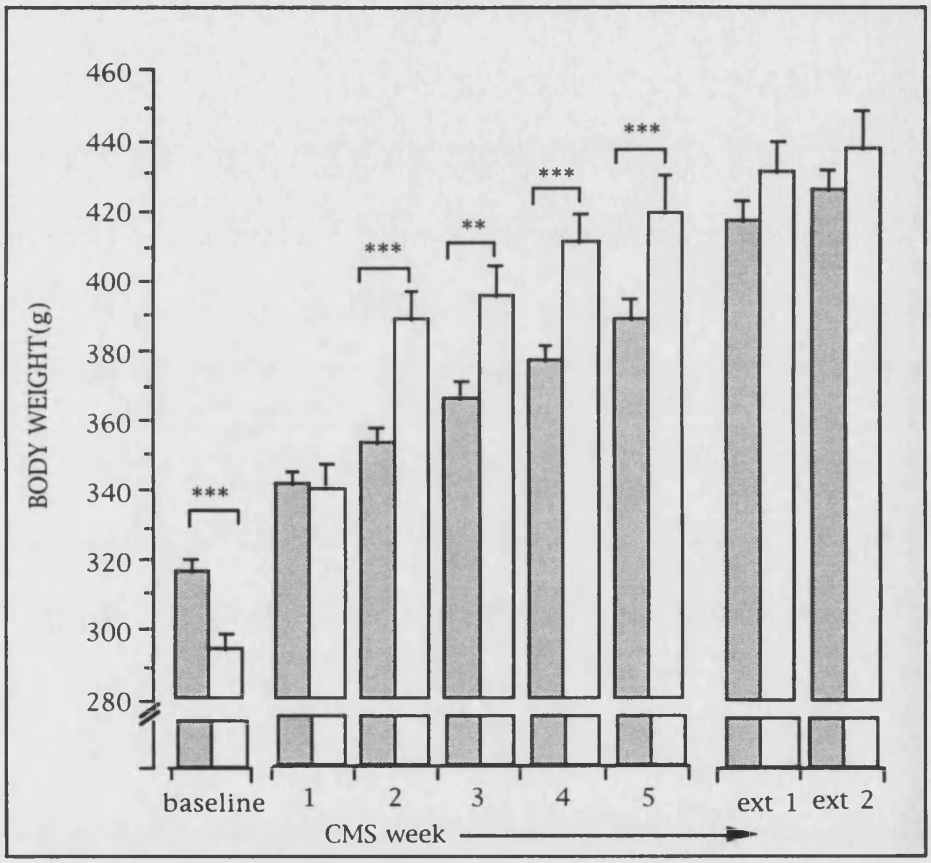


Figure 54. Change in body weight prior to and following five weeks CMS in CMS2 animals. CMS ▩ control □ values are mean ± sem; n=15 per group; *** $p \leq 0.001$; ** $p \leq 0.005$ vs. saline. Baseline = experimental starting weight.

DISCUSSION

The aim of this experiment was to assess whether the revised CMS protocol was able to induce a sucrose preference deficit in animals subjected to the regime. The protocol was altered to include two paired-housings per week, placing an animal with a different partner on each occasion. In addition, body weight and sucrose preference measurements were moved from mid-week to the end of the week to allow testing to cover a full week of CMS.

Within two weeks of exposure to the new regime, CMS-treated animals showed a reduced sucrose preference in the one-hour sucrose preference test. This deficit was maintained throughout the five-week exposure to CMS, but disappeared when CMS was stopped. Examination of the individual components of the sucrose preference test (water, sucrose and total fluid intake), revealed this deficit to be largely due to an increased water intake by stressed animals. These results differ from those of Muscat et al., (1992), who found the sucrose preference deficit to arise from a decrease in sucrose consumption by stressed animals compared to controls in which sucrose intake stayed the same. Furthermore, Willner et al., (1987) found that intake of the sweet solution stayed the same in stressed animals and increased in control animals, thus accounting for a decreased preference in CMS-treated animals. Hence, the validity of the sucrose preference deficit in the present study is questionable since it arose from an increase in the amount of water consumed by the animals, an alteration which other researchers propose to indicate a general decrease in consummatory behaviour, and not a specific decrease in the reward system (Willner et al., 1987; Muscat & Willner, 1992; Muscat et al., 1992; Willner et al., 1992).
The preference deficit elicited by the revised CMS regime lasted only for as long as the stressors were applied, and was seen to disappear within one week of CMS termination. This again is in contrast to the effects seen by other researchers, where the deficit lasted between two and three weeks after termination of the stress regime (Willner et al., 1987; Sampson et al., 1991; Muscat et al., 1988; 1990 & 1992; Moreau et al., 1993), and up to ten days where the effects of CMS measured using ICSS.

Animals exposed to the revised CMS regime also displayed reduced locomotor activity by the second week of exposure. However, when tested in subsequent weeks, the trend towards hypolocomotion in CMS animals was not statistically significant. This may be because repeated testing in the same environment gives rise to habituation, and to less exploratory behaviour as familiarity with the environment increases (Kant et al., 1980). To avoid the possibility of habituation masking the locomotor response, it was decided in future studies to test locomotor activity less frequently during the course of the experiment. Whilst it is accepted that simple

locomotor measurement does not correspond directly to the psychomotor changes seen in clinical depression, this deficit may never-the-less be a valuable marker in animal models. Katz (1982), based conclusions as to the effectiveness of CMS almost solely on deficits in locomotor activity during a 6 minute exposure to 'open field' apparatus. Moreover, other animal models of depression such as the Porsolt forced swimming test and separation techniques (Pare, 1994; Crawley et al., 1985) also rely on changes in locomotor activity as a behavioural marker. Importantly, changes in locomotor activity during exposure to CMS are not routinely measured by those researchers currently working with this model, since they concentrate on measurements of anhedonia (sucrose preference, ICSS or CPP). Results of locomotor activity changes could therefore contribute valuable information on the use of CMS as an animal model of depression.

Finally, stressed animals gained weight at a much slower rate than control animals in the present experiment, a deficit which was exaggerated with each weekly exposure to the regime. This reduced ability of CMS-treated animals to gain weight was not as severe as that reported by other groups. For example, Muscat et al., (1988), described control animals to gain weight at approximately 2% per week whilst stressed animals actually showed a small weight loss from baseline values to end the treatment period below their initial starting weight. The decreased body weight of stressed animals may reflect a decreased appetite, and it was therefore planned to monitor food consumption in a subsequent experiment to assess whether CMS-treated animals ate less than control animals, thus giving rise to a reduced weight gain.

In conclusion, the decreased preference for sucrose, decreased body weight and decreased locomotor activity recorded in animals subjected to the revised CMS regime were taken to indicate success of the depression model in eliciting two subsidiary symptoms and one major symptom representative of depressive illness. It was therefore decided to use this revised CMS protocol in all subsequent experiments with the single modification of testing locomotor activity only twice during the experimental period.

EXPERIMENT 3: PREVENTION OF CMS EFFECTS BY ANTIDEPRESSANT DRUG TREATMENT (CMS3)

The aim of this experiment was to determine whether the antidepressant drug paroxetine (a serotonin selective reuptake inhibitor) was able to prevent the CMS-induced decrease in sucrose preference, body weight and locomotor activity observed in the previous experiments. This is the first attempt to assess the *predicitive validity* of the CMS model with paroxetine.

SUBJECTS

Sixty male Hooded Lister rats (weighing 331±10g at the beginning of the experiment; supplied by Charles River, Margate, Kent) were individually housed as in CMS 1.

EXPERIMENTAL PROCEDURE

Following sucrose habituation week, the animals were divided into 4 evenly-matched groups (n=15), on the basis of their sucrose preference scores on day 3. Two of the groups entered the stress procedure whilst the other two groups served as control. One stress group and one control group were administered 6 mg/Kg/day paroxetine in the drinking water for the duration of the experimental period; the other control and stress groups received ordinary tap water throughout the experiment. Intake of paroxetine solution during the experimental period was monitored both in stress and control groups by measuring bottle weight. As in previous experiments, sucrose preference and body weight were monitored weekly, whilst locomotor activity was measured at the end of weeks three and four.

RESULTS

Sucrose Preference Testing

Baseline: There was no significant difference between the groups in the baseline sucrose preference test. The values of these intakes are shown in Table 10.

Table 10. Baseline intake values for CMS3 animals.

Mean intake (ml)	control/water	control/paroxetine	CMS/water	CMS/paroxetine
sucrose	6.69±0.63	7.59±0.52	8.09±0.59	7.41±0.55
water	1.33±0.14	1.32±0.19	1.40±0.21	1.28±0.17
total fluid	8.02±0.67	8.91±0.57	9.49±0.62	8.69±0.53
% sucrose preference	82.02±2.21	84.82±1.84	84.84±2.22	84.7±2.28

(n=15 for all groups)

CMS treatment:

Neither the stress procedure nor the chronic administration of paroxetine significantly affected sucrose intake (treatment $F_{(1,56)}=0.18$, NS; drug $F_{(1,56)}=1.17$, NS). Furthermore, there was no significant interaction of paroxetine and the stress procedure (treatment and drug $F_{(1,56)}=0.57$, NS). Within subjects, sucrose intake did vary significantly during the course of the experiment (week $F_{(3,168)}=5.34$; $p\leq0.002$), giving rise to the fluctuations illustrated in Figure 55a. These fluctuations were different between groups such that there was a significant week and treatment and week and drug interaction ($F_{(3,168)}=3.85$; $p\leq0.012$ and $F_{(3,168)}=3.17$; $p\leq0.028$, respectively). There was no combined week, treatment and drug interaction ($F_{(3,168)}=0.14$, NS).

Water intake was not significantly affected by either the CMS treatment or by chronic paroxetine administration (treatment $F_{(1,56)}=0.47$; drug $F_{(1,56)}=0.002$: NS), and there was no interaction of these two variables on water intake (treatment and drug $F_{(1,56)}=0.455$, NS). Water intake did not vary significantly within subjects during the course of the experiment (week $F_{(3,168)}=0.708$, 168), and was not significantly different within the treatment or paroxetine groups (week x treatment $F_{(3,168)}=1.02$; week and drug $F_{(3,168)}=1.74$: NS). Finally, there was no combined effect of treatment, paroxetine and week (week and drug and treatment $F_{(3,168)}=0.86$, NS) (Figure 55b).

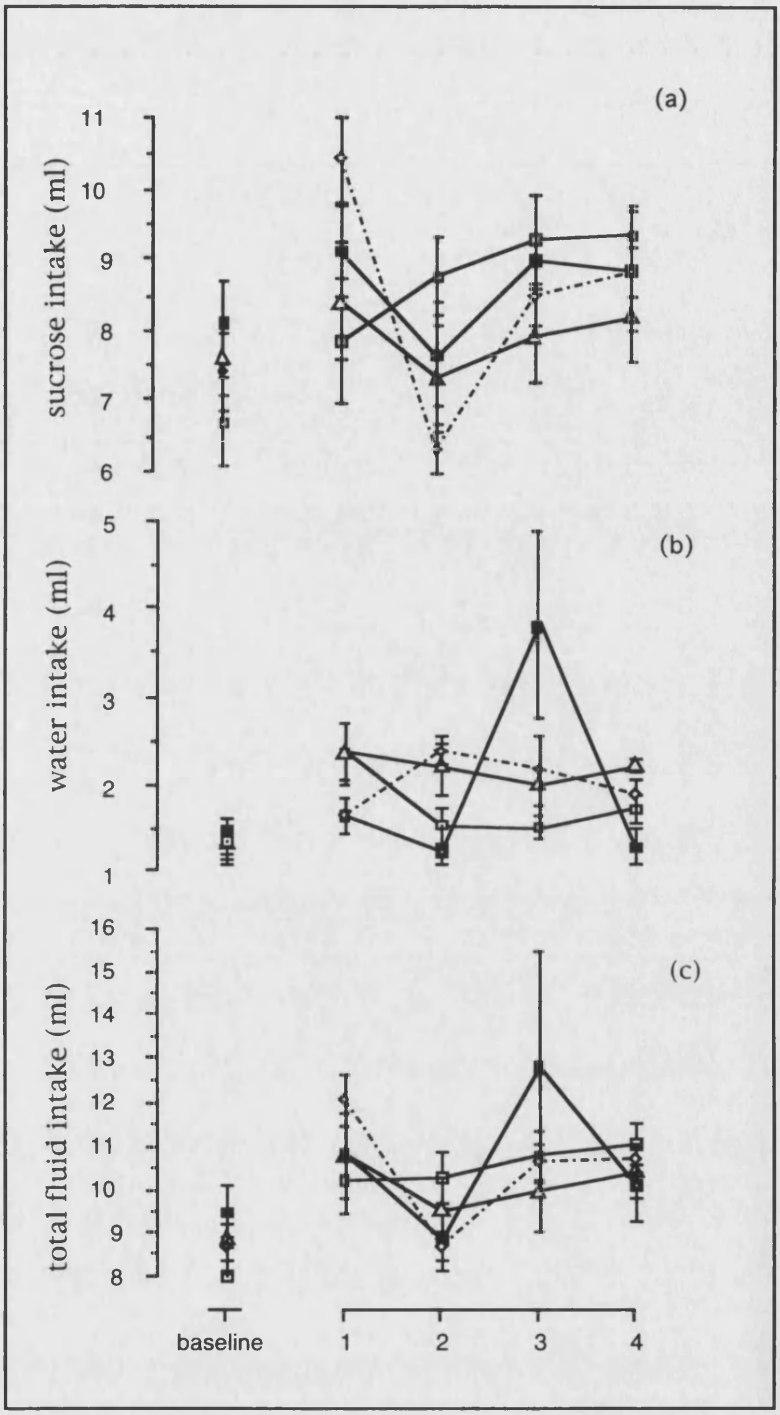


Figure 55a Sucrose intake, Figure 55b Water intake and Figure 55c Total fluid intake in CMS 3 animals during weekly sucrose preference testing. Values are mean ± sem. control/water control/paroxetine CMS/water CMS/paroxetine Paroxetine = 6 mg/Kg/day. Values are mean ± sem. n=15 per group. Baseline values are taken from week 0, habituation test 3.

Total fluid intake was not significantly different between control and stress groups (treatment $F_{(1,56)}$=0.24, NS), and paroxetine administration had no effect on the total

amount of fluid consumed (drug $F_{(1,56)}$=0.136, NS). There was no significant interaction of these two factors (drug and week $F_{(1,56)}$=0.055, NS). The total amount of fluid consumed at each test did vary significantly within individuals over the course of the experiment (week $F_{(3,168)}$=3.63; $p \leq 0.037$), but was not significantly different between the treatment or paroxetine groups (week and treatment $F_{(3,168)}$=1.53; week and drug $F_{(3,168)}$=1.95, NS). There was no combined effect of treatment, paroxetine and week (week and drug and treatment $F_{(3,168)}$=0.47, NS) (Figure 55c).

Although sucrose preference was not significantly affected by CMS treatment (treatment $F_{(1,56)}$=2.06, NS), it *was* significantly decreased by chronic administration of paroxetine to the drinking water (drug $F_{(1,56)}$=10.79; $p \leq 0.002$). Differences in sucrose preference between paroxetine-treated and water-only groups were greatest at week 2 CMS:

Table 11: Week 2 percentage sucrose preference in CMS 3 animals.

	Control	CMS
water	85.07±1.69	84.95±1.75
paroxetine	75.21±3.75	72.47±2.5

The paroxetine effect was independent of treatment as seen by the lack of a significant drug and treatment interaction (drug and treatment $F_{(1,56)}$=2.55, NS). Within subjects, there was no variation in sucrose preference during the course of the experiment (week $F_{(3,168)}$=0.93, NS), but the effect of paroxetine on sucrose preference varied significantly with time (week x treatment $F_{(3,168)}$=4.09; $p<0.013$; week x drug $F_{(3,168)}$=3.87; $p<0.017$). There was no combined week, drug and treatment interaction ($F_{(3,168)}$=0.303, NS). Figure 56 illustrates the sucrose preference test values for the CMS3 experiment.

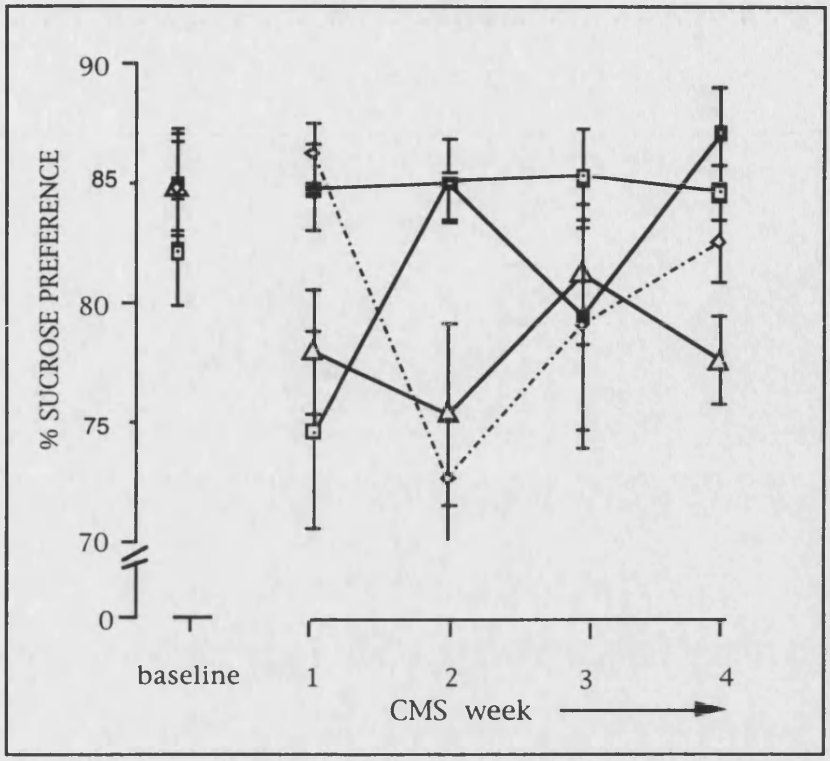


Figure 56. Sucrose preference test in CMS 3 animals prior to and during four weeks CMS.
control/water —□— control/paroxetine —△—
CMS/water —■— CMS/paroxetine -·-·◇-·-·
Paroxetine = 6 mg/Kg/day. n=15 per group; values are mean ± sem.

Locomotor Activity

Two-way ANOVA of locomotor activity results at the end of week three, revealed a significant effect of CMS on locomotor activity as in previous experiments ($F_{(1,56)}$ = 4.75; $p \leq 0.05$), i.e. the CMS group animals were hypoactive with respect to the control group animals during the 60 min test. Chronic administration of paroxetine did not significantly affect activity in either CMS-treated or control animals ($F_{(1,56)}$=2.146), although there was a tendency for paroxetine-treated animals to be less active in both groups. There was no treatment and drug interaction ($F_{(1,56)}$=0.001).

When tested at the end of four weeks, stress group animals were again found to have significantly lower levels of locomotor activity than control animals ($F_{(1,56)}$=7.88; $p \leq 0.01$) but again there was no significant effect of paroxetine on locomotor activity despite the lowered activity in paroxetine-treated animals ($F_{(1,56)}$=2.61). There was no treatment and drug interaction ($F_{(1,56)}$=0.046).

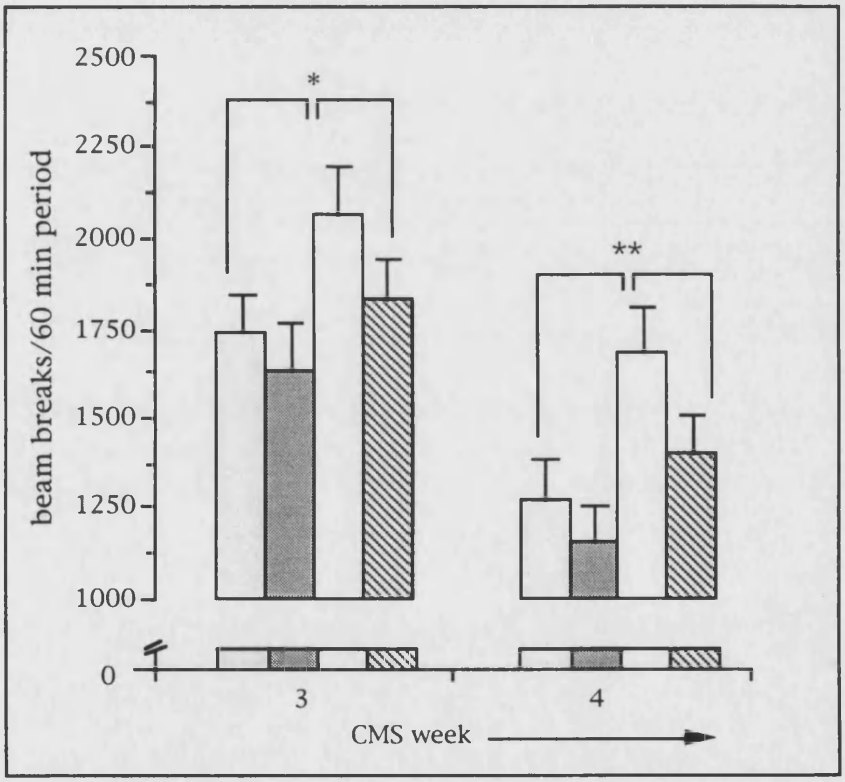


Figure 57. Locomotor activity of CMS 3 animals and controls measured in weeks 3 and 4 of the stress procedure. Values are mean±sem, n=15 per group. ☐ cms /water; ▩ cms/paroxetine; ☐ control/water; ▨ control /paroxetine. Paroxetine = 6 mg/Kg/day. * $p \leq 0.05$; ** $p \leq 0.01$ vs. control/water group.

Body weight

As in previous experiments, the stress procedure significantly decreased body weight in both water and paroxetine groups ($F_{(1,56)}$=18.2; $p \leq 0.001$). Despite the tendency of paroxetine-treated animals to weigh more than water-treated animals in both stress and control groups, this was not statistically significant ($F_{(1,56)}$=0.78; Figure 58). Stress group animals, irrespective of water/paroxetine treatment, gained weight at an average rate of 2% per week, ending the treatment period at 107% of their starting weight. Control group animals, again irrespective of treatment, gained an average of 4% per week, ending the treatment period at 117% of their starting weight.

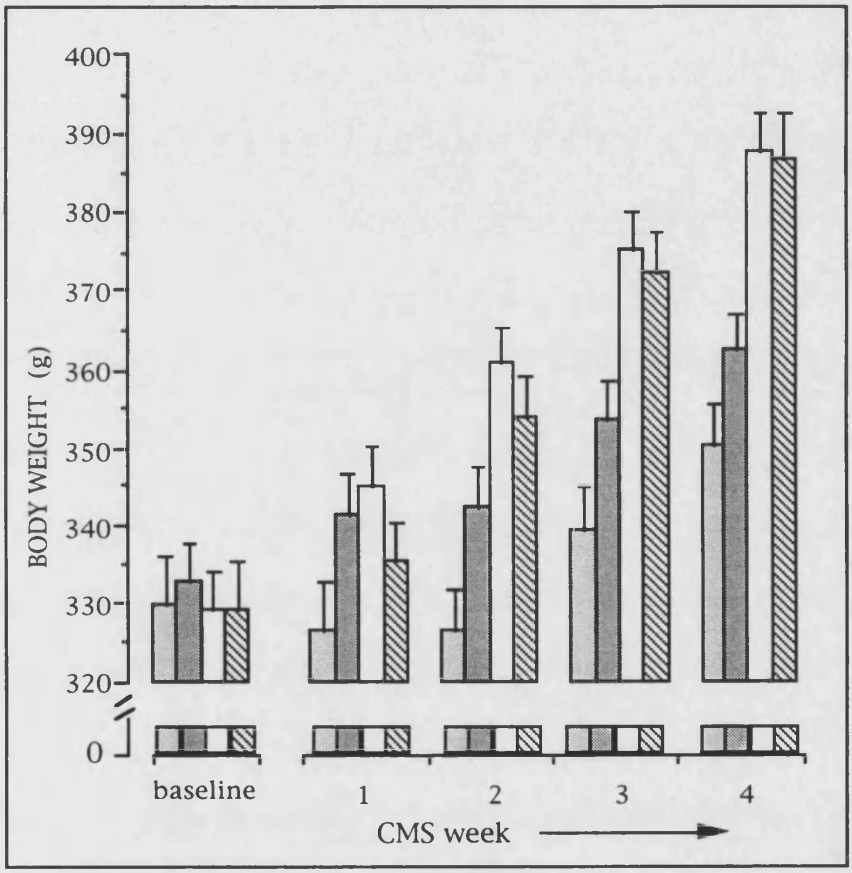


Figure 58. Changes in body weight in CMS 3 animals prior to and during four weeks CMS treatment. ☐ CMS/water ; ▩ CMS/paroxetine; ☐ control/water; ▨ control/paroxetine. Paroxetine= 6 mg/Kg/day Values are mean ± sem; n=15 per group.

DISCUSSION

The aim of this experiment was to determine whether the SSRI, paroxetine, was able to prevent the CMS-induced decreases in sucrose preference, body weight and locomotor activity observed in the previous experiment.

A decrease in sucrose preference was not seen in animals exposed to the CMS regime in the present experiment, and therefore reversal of this effect by paroxetine was not possible. Surprisingly, animals which had received chronic paroxetine treatment (both in the control and CMS-treated groups) exhibited a significant deficit in sucrose preference, an effect which is difficult to explain. It could be argued that during the hour-long sucrose preference test, the water for the two-bottle choice was paroxetine-free and this 'un-contaminated' water may have been more appealing to animals maintained on paroxetine-containing drinking water, resulting in an increased desire for the ordinary water over the sucrose solution. This possibility can be confirmed or refuted by repeating the experiment and providing paroxetine-treated animals with a sucrose versus paroxetine-solution during the preference test. This apparent ability of an antidepressant drug to generate, rather than alleviate, anhedonia adds to the speculation surrounding the credibility of this parameter as a measure of anhedonia (Matthews et al., 1995) (correction for body weight removes the significance factor on the sucrose preference measurement).

Animals exposed to the CMS regime showed a significant reduction in body weight compared to control animals, and although paroxetine-treated animals tended to weigh more in both the stress and control groups, paroxetine administration had no significant effect on the CMS-induced reduction in body weight. Similarly, CMS treatment caused a significant decrease in locomotor activity, and paroxetine administration had no effect on this decrease. It therefore appears that 6 mg/Kg/day paroxetine, despite being within the effective antidepressant dose range for this compound (Kleinlogel & Burki, 1987), was unable to prevent the effects of CMS on locomotor activity and body weight. It is possible that these responses were not true depressive behaviours, but were anxiety-related behaviours. This prospect has been tested by other groups working with a similar model of CMS. Muscat et al., (1992) found that the anxiolytic compound, chlordiazepoxide, did not reverse the sucrose preference deficit elicited by CMS, whereas the antidepressants maprotiline and fluoxetine did reverse the deficit. However, in the same study, the body weight of CMS and control animals administered chlordiazepoxide was over and above that of the vehicle- and antidepressant-treated groups, suggesting an anxiogenic component of the decreased body weight of CMS animals. To confirm or refute the suggestion that effects seen with the present version of the CMS model are anxious behaviours,

the experiment should be repeated with animals which are administered an anxiolytic compound.

In conclusion, despite the fact that different antidepressant drugs from distinct pharmacological classes have previously been shown to restore sucrose preference values to pre-stress baseline values (Muscat et al., 1988; Muscat et al., 1988; 1990; Muscat et al., 1990; Sampson et al., 1991; Willner et al., 1987), paroxetine did not demonstrate this activity in the present experiment.

EXPERIMENT 4: EFFECTS OF CMS ON THE FREE-RUNNING CIRCADIAN LOCOMOTOR ACTIVITY RHYTHM (CMS4)

The aim of the following experiment was to assess the effect of the CMS procedure on the free-running locomotor activity rhythm of the rat.

SUBJECTS

Thirty male Hooded Lister rats (Charles River, Margate, Kent), weighing between 360±5 g at the beginning of the experiment, were housed as in CMS 1.

APPARATUS

Details of the DATAQUEST III system used to measure circadian locomotor activity rhythms can be found in Chapter II. Briefly the apparatus comprises individual wire-bottomed activity cages (supplied by Mini-Mitter Co.; 56x38x18 cm) each containing a running wheel (345 mm diameter). Microswitch closures from each wheel revolution were read online and stored in 10-min bins on an IBM PC in an adjacent room using the Dataquest III data collection system (Mini-mitter, Sunriver, Oregon, USA). Activity was recorded continuously and printout actograms and periodograms provided the basis for circadian analysis. These experiments were conducted in a noise-restricted zone in a light-sealed room with independent lighting control, and access to the room was through another darkened room. Water and food were checked daily and waste trays were changed twice weekly, all at random times of day.

EXPERIMENTAL PROCEDURE

Following sucrose habituation week, animals were subjected to four weeks of the stress regime described for CMS 2 animals. Sucrose preference and body weight were monitored, and locomotor activity was measured in weeks 3 and 4. At the end of the four-week schedule, all animals were transferred to individual cages containing an activity wheel and allowed to free-run under constant dim red light (Encapsulite Safelight, λ =610nm). Transferral to wheels took place at the light-dark transition point (19.00h).

DATA ANALYSIS

For this particular experiment, actograms were printed without applying limits or a threshold value to the activity data. This is because curtailing activity counts for this set of actograms detracted from, rather than clarified, the pattern of wheel-running activity. The following parameters were determined from each actogram, and all analysis was made blind to treatment code.

i) The number of days to form a continuous activity band was calculated.
ii) The number of animals with abnormal free-running rhythms was assessed.

Periodograms were plotted for each animal across the range 0 to 36 hours to allow easy visual inspection of recurring periodicities. The following parameters were determined from these computer-plotted periodograms:

i) The period of the free-running activity rhythm
ii) The presence of 12-hour and ultradian rhythms.
iii) The mean amount of activity per 10 minute bin per average cycle.

RESULTS.

Sucrose Preference Testing

Baseline:

Analysis of baseline values revealed no significant difference between control and stress-group animals in terms of sucrose, water and total fluid intakes. The values of these intakes were, in ml: sucrose 10.61 ± 0.87 vs 10.74 ± 0.81; water 4.55 ± 0.57 vs 5.34 ± 0.70 and total fluid 15.32 ± 0.56 vs 16.08 ± 0.50 for the CMS and control groups, respectively. There was no significant difference between the two groups in the sucrose preference test prior to CMS treatment (69.04 ± 4.14 % CMS and 66.43 ± 4.63 % control; $t=0.42$, df 28; $p=0.68$).

CMS treatment:

There was no significant effect of the stress procedure on sucrose intake (treatment $F_{(1,28)}=0.74$, NS), although intake did vary significantly within subjects during the course of the experiment (week $F_{(5,140)}=5.06$; $p \leq 0.007$) (fluctuations in sucrose intake can be seen in Figure 59a). The fluctuations in sucrose intake were similar for both groups such that there was no significant interaction of week and treatment ($F_{(5,140)}=1.37$, NS).

CMS treatment significantly affected water intake (treatment $F_{(1,28)}=20.08$; $p \leq 0.001$), and this varied significantly within subjects during the course of the experiment (week $F_{(3,84)}=49.41$; $p \leq 0.001$). The most pronounced difference occurred in week 3 ($p \leq 0.001$), when CMS group animals consumed 8.75 ± 0.85 ml compared to 3.45 ± 0.67 ml for the control group (the fluctuations in intake can be seen in Figure 59b). This variation in intake by the CMS group gave rise to a significant interaction of week and treatment ($F_{(3,84)}=19.45$; $p \leq 0.001$).

Total fluid intake was not significantly different between the two groups during the stress period (treatment $F_{(1,28)}=1.76$, NS), although the volume of intake did vary significantly within subjects during the experimental period (week $F_{(3,84)}=30.79$;

$p \leq 0.001$). The fluctuation was significantly different between the groups (week x treatment $F_{(3,84)}=8.04$; $p \leq 0.001$) (Figure 59c).

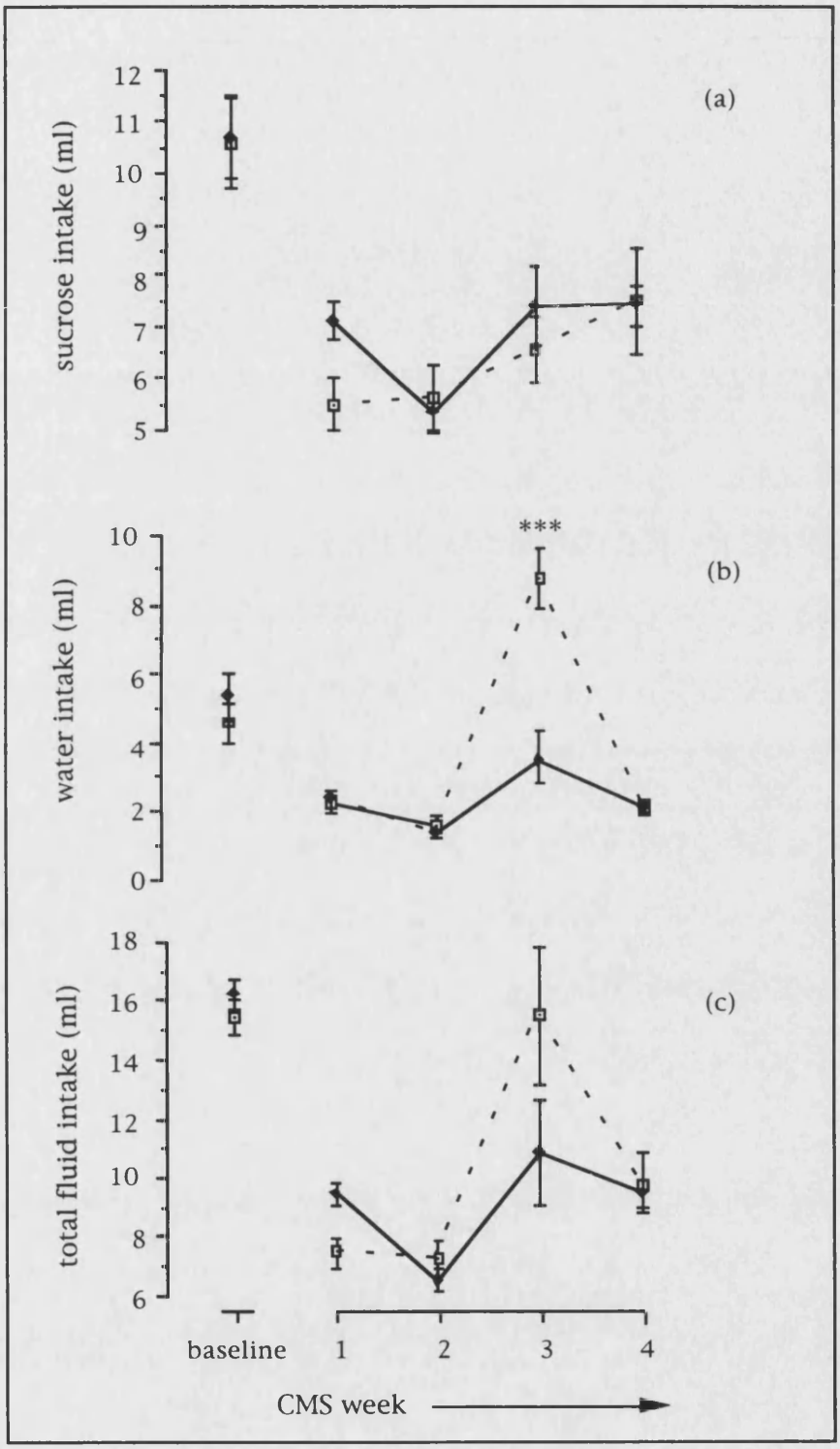


Figure 59a Sucrose intake, Figure 59b Water intake and Figure 59c Total fluid intake in CMS4 animals during weekly sucrose preference testing. Values are mean ± sem. Stress group – – ◻ – – control group ——◆—— n=15 per group. *** $p \leq 0.001$ vs control. Baseline values are from week 0, 3rd habituation test.

CMS treatment did not significantly affect sucrose preference (treatment $F_{(1,28)}=0.98$, NS). However, preference varied significantly within subjects during the the course of the experimental procedure (week $F_{(3,84)}=5.33$; $p\leq 0.005$) in a similar manner in both groups (week x treatment $F_{(3,84)}=0.055$, NS). Figure 60 displays the sucrose preference test values.

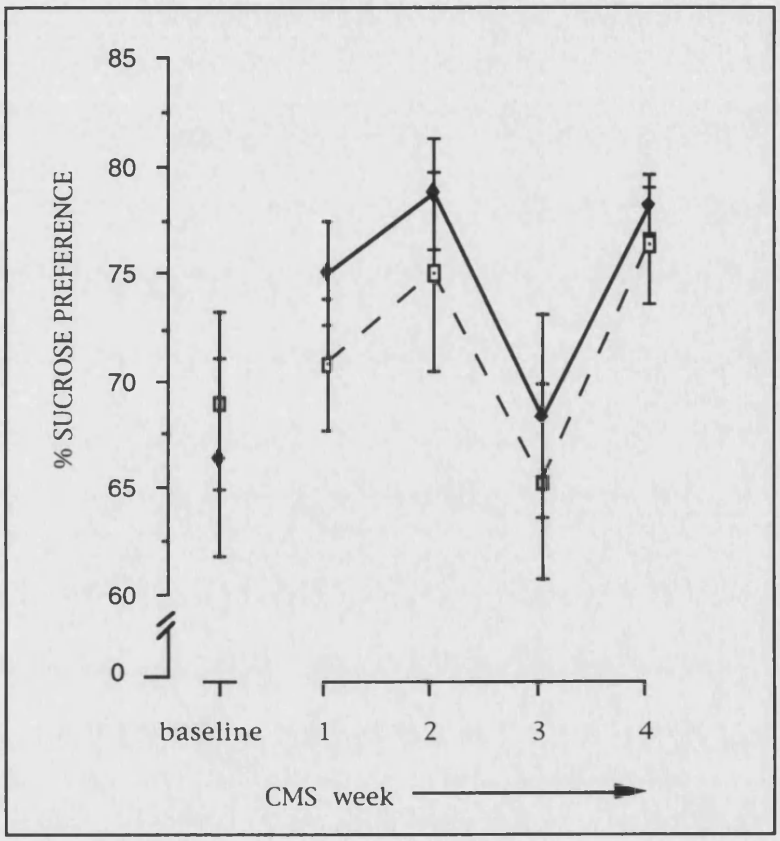


Figure 60. Sucrose preference test in animals subjected to four weeks CMS 4 — □ - and control —◆— animals n=15 per group; values are mean ± sem.

Locomotor Activity

Locomotor activity testing at the end of CMS week 3 and week 4 revealed CMS group animals to be significantly less active compared to controls: week 3 beam breaks, 1947.2±181.3 CMS and 3040±235.8 control; t=-3.58, df 28; p=0.001: week 4 beam breaks, 2066.13±143.54 CMS and 2563.47±148.99 control; t=-2.4, df 28; p=0.023 (Figure 61).

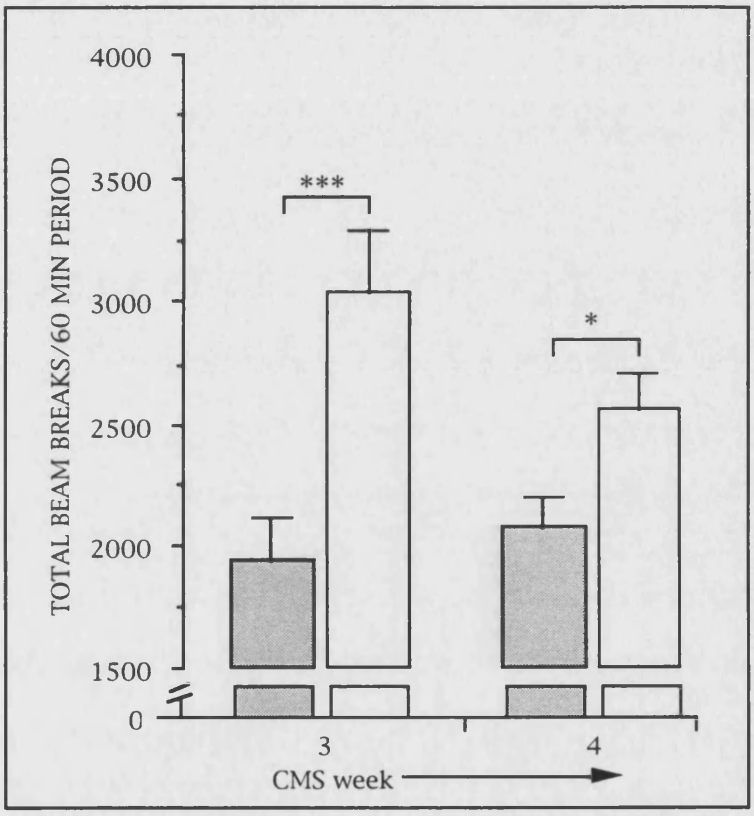


Figure 61. Locomotor activity measured during 60 min exposure to a novel cage in CMS 4 ▨ and control □ rats at the end of weeks 3 and 4 of the CMS regime. Values are means ± sem; n=15 per group; ***p≤0.001 * p≤0.05 vs. control.

Body weight

There was a significant body weight difference between the two groups (treatment $F_{(1,28)}=15.64$; $p\leq0.001$) which increased on weekly exposure to CMS (week $F_{(3,84)}=283.33$; $p<0.001$; interaction of week and CMS $F_{(3,84)}=10.032$; $p\leq0.001$). Control group animals gained weight at the rate of approximately 3% per week. CMS-treated gained weight at a slower rate (1.8% per week) and ended the CMS treatment period (week 4) at 107% of their initial weight compared to the control group animals who ended week 4 at 113% of their initial weight. The average body weight per week for each group is illustrated in Figure 62.

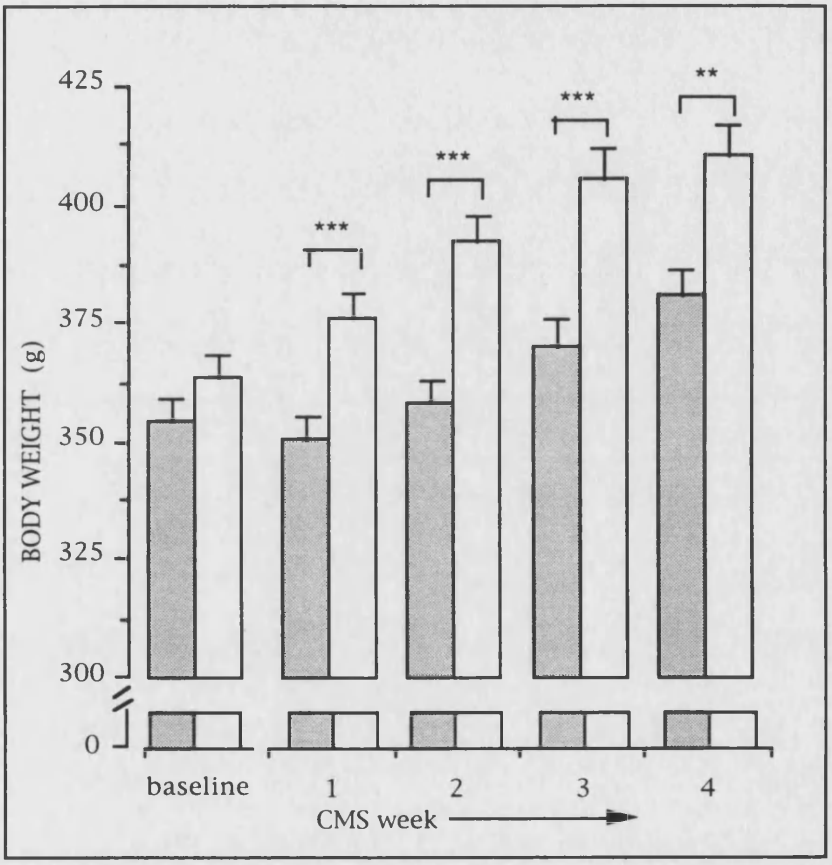


Figure 62. Differences in body weight between ▩ CMS 4 and □ control animals prior to and during four consecutive weeks exposure to chronic mild stress. Values are mean±sem n=15 per group. *** $p\leq0.001$; ** $p\leq0.005$ vs control. Baseline = experimental starting weight.

CIRCADIAN RHYTHM RESULTS

Table 12: Actogram assessment of the circadian free-running rhythms in control and CMS-treated animals.

CMS animal No.	W-shaped rhythm disturbance (Y/N)	No. days to form continuous activity	Control animal No.	W-shaped rhythm disturbance (Y/N)	No. days to form continuous activity
4	n	5	2	arrhythmic	-
12	y	9	6	n	12
14	n	6	62	n	7
18	n	7	10	n	5
20	y	15	16	n	13
22	y	11	26	n	8
24	n	6	32	n	6
28	y	16	34	n	11
30	y	12	38	n	5
36	n	5	42	n	?
40	y	13	44	n	8
50	n	7	46	n	6
52	arrhythmic		48	y	17
54	n	7	56	n	17
58	y	13	60	n	9
	mean ± sem	9.43 ± 1.02		mean ± sem	9.54 ± 1.16
		where n=14			where n=14

Analysis by t-test showed there to be no significant difference between the two groups in the time taken to form a continuous, single band of locomotor activity (t= -0.07, 26 df, p=0.9).

After discounting the first four days of activity due to the 'settling-in' of the animals, 7 out of the 15 CMS-treated animals displayed a distinctive pattern of activity (No's 12, 20, 22, 28, 30, 40, 58), which lasted on average approximately 9 days. The distinctive pattern generally appeared to involve two bands of free-running activity, one of which delayed whilst the other advanced. Visually, this activity pattern is seen as a W-shape (illustrated in Figures 63a and 63b). In each case, the free-running activity bands coalesced after a certain number of days to produce one band. Only one control animal was scored as having the same type of W-shaped rhythm disturbance (No. 48). Other animals, although displaying disrupted activity, did not exhibit the same distinctive pattern. One animal from each group was arrhythmic in its locomotor activity rhythm throughout the test period. Fischers exact test revealed the number of stressed animals displaying an abnormal rhythm compared to controls to be significant; p=0.035.

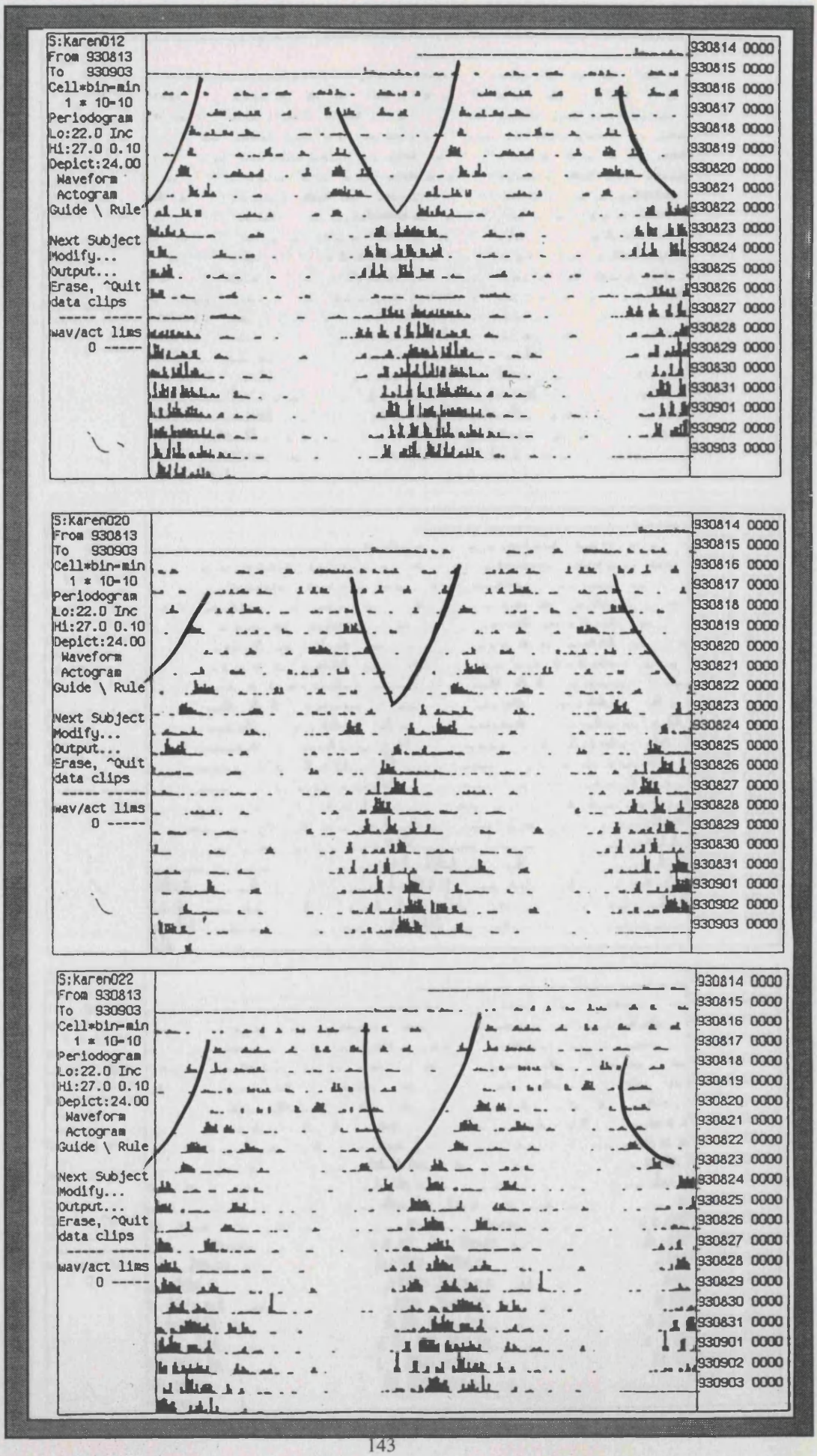
Figures 63a and b show actograms from CMS-treated animals displaying the W-shaped rhythm. Figure 63c shows actograms from control animals. The first of these control actograms is that animal No. 48, which recorded as displaying the same W-shaped rhythm seen in CMS-treated animals.

Figure 63a. Actograms from animals in experiment CMS 4.

CMS-TREATED

CMS-TREATED

CMS-TREATED



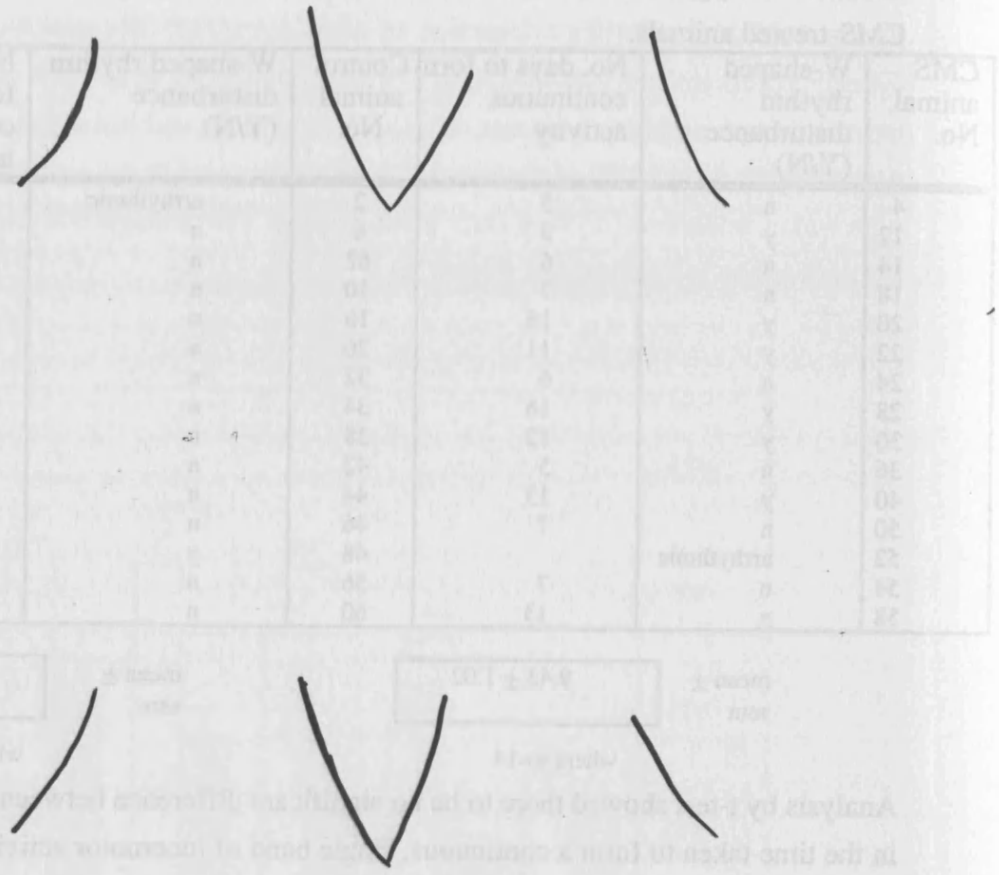


Figure 63a. Actograms from animals in experiment CMS 4.

CMS-TREATED

CMS-TREATED

CMS-TREATED

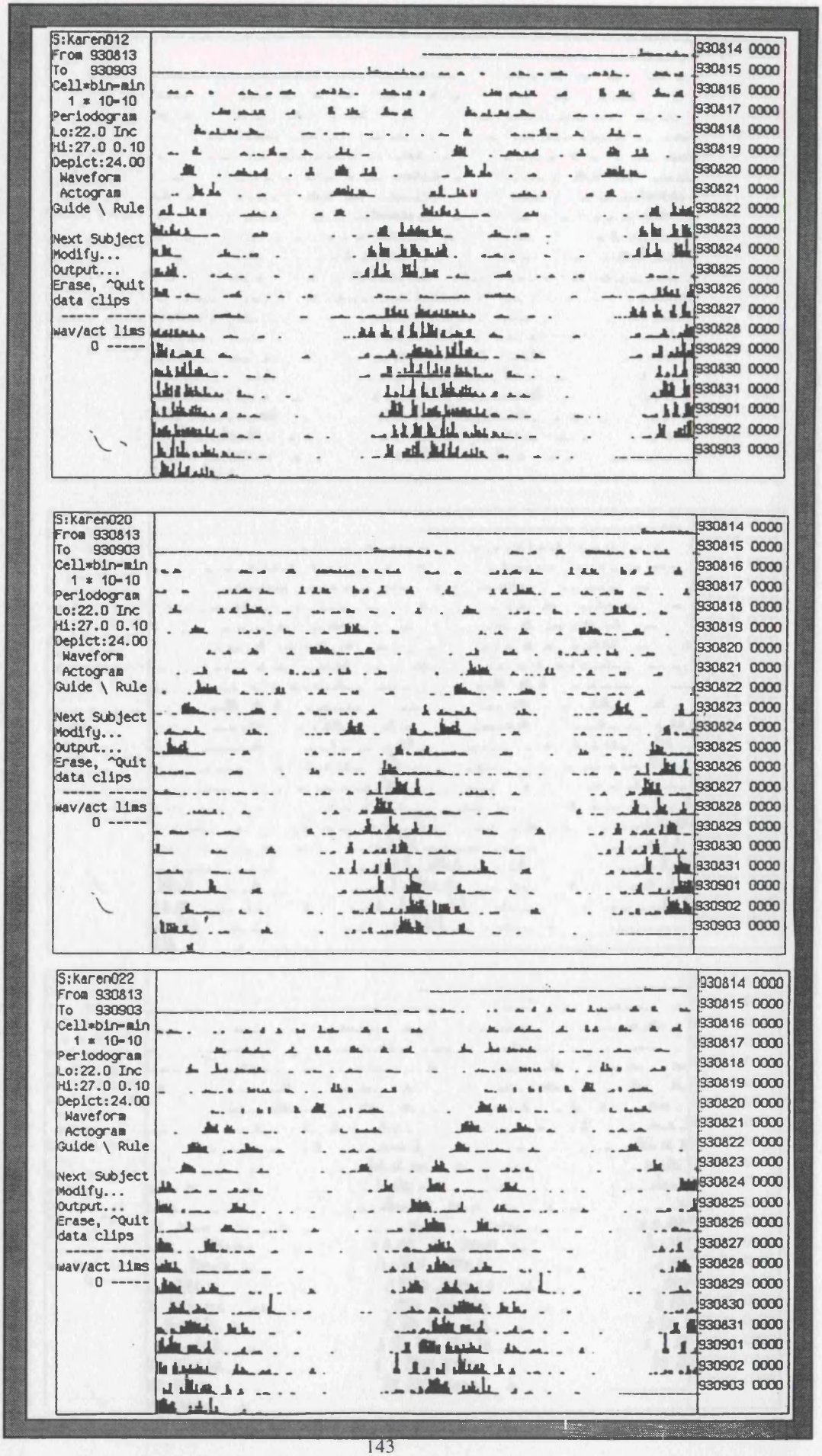


Figure 63b. Actograms from animals in experiment CMS 4.

CMS-TREATED

CMS-TREATED

CMS-TREATED

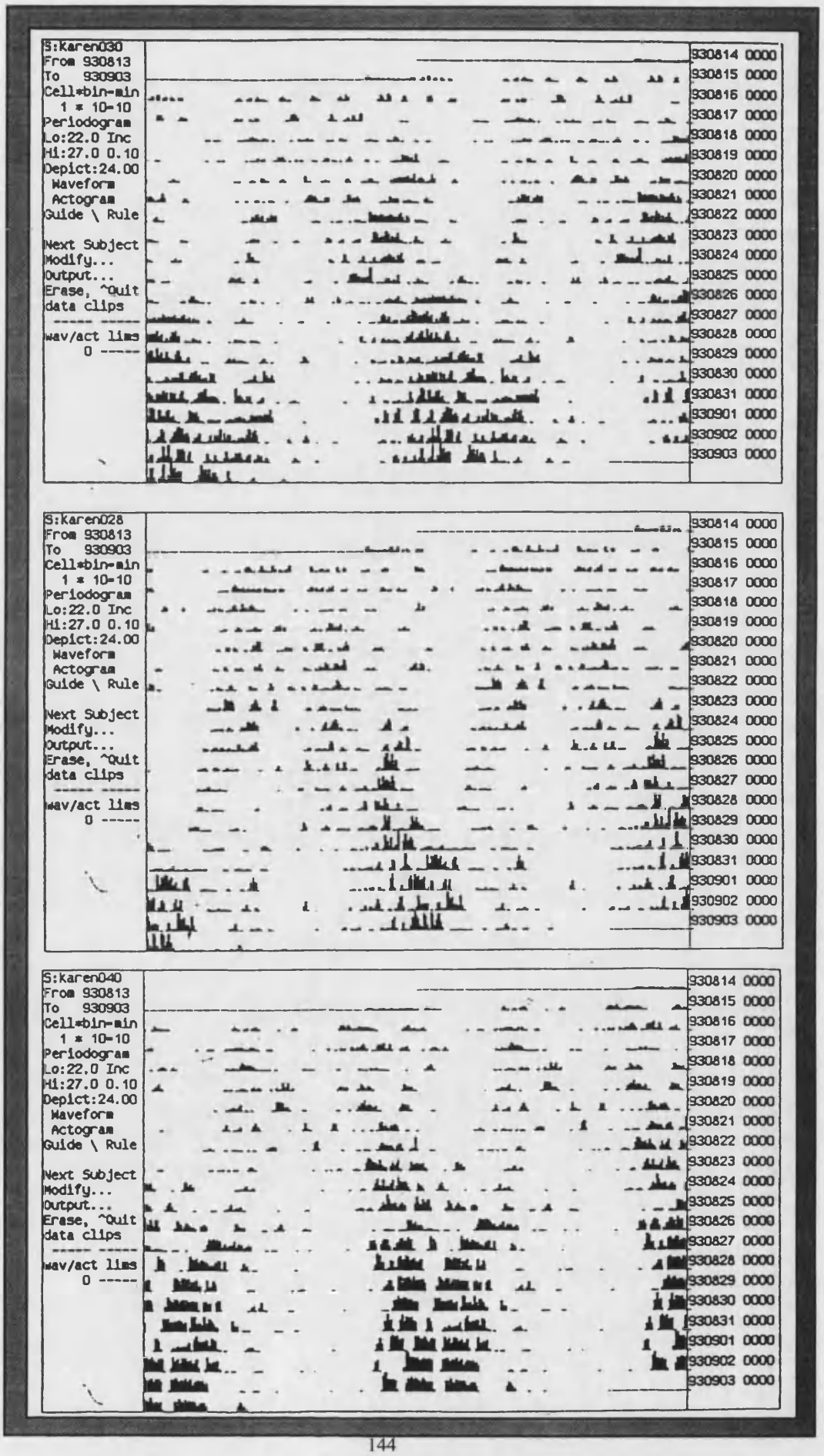
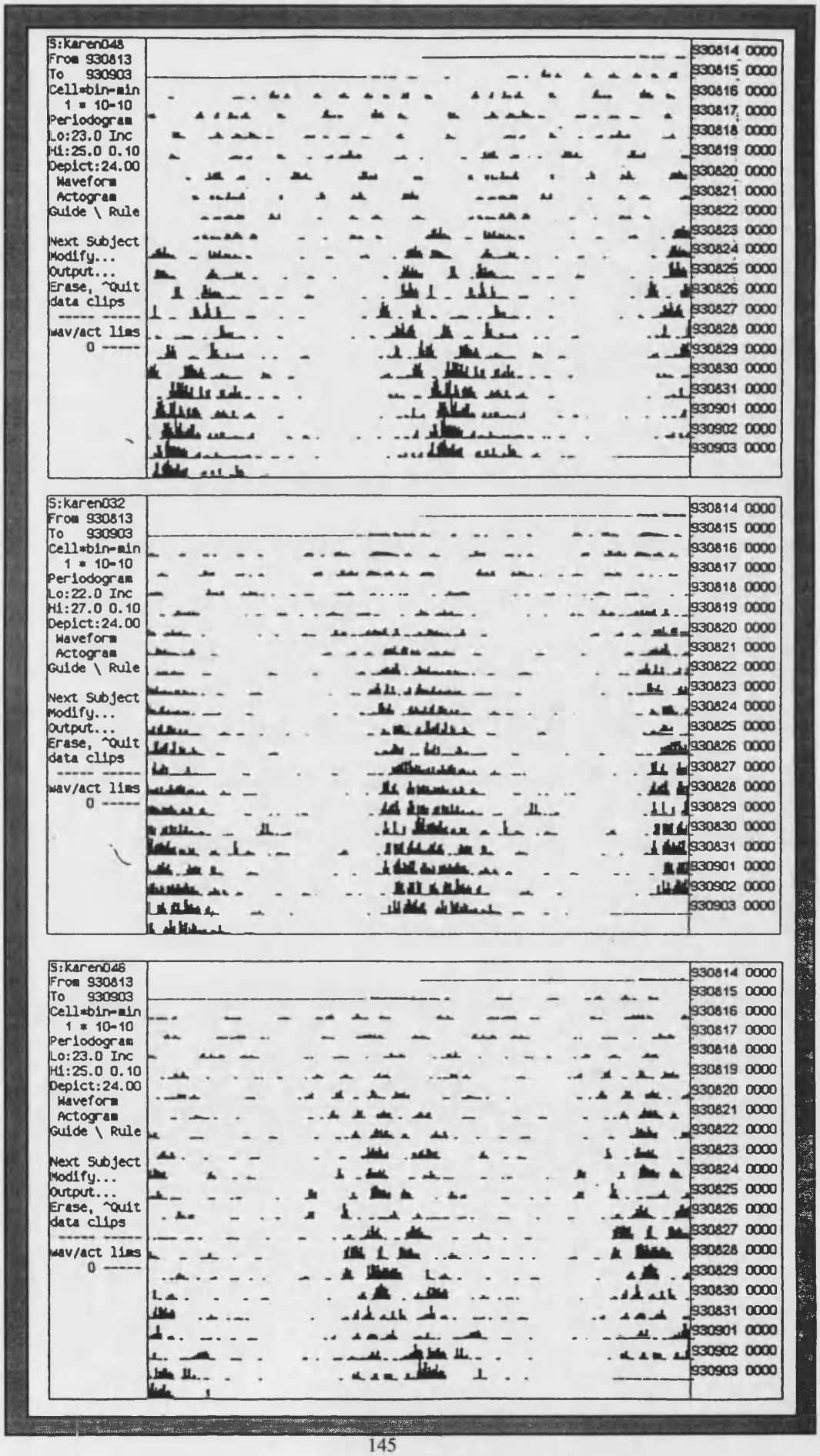


Figure 63c. Actograms from animals in experiment CMS 4



Periodogram and waveform analysis

Each actogram was divided into two halves for further period and activity analysis. The first half (part A), covered a 7-day span (day 5-11), beginning four days after animals were put into wheels. The second (part B), covered the activity until the end of the experiment (10 days; day 12-21). These divisions are illustrated in Figure 64.

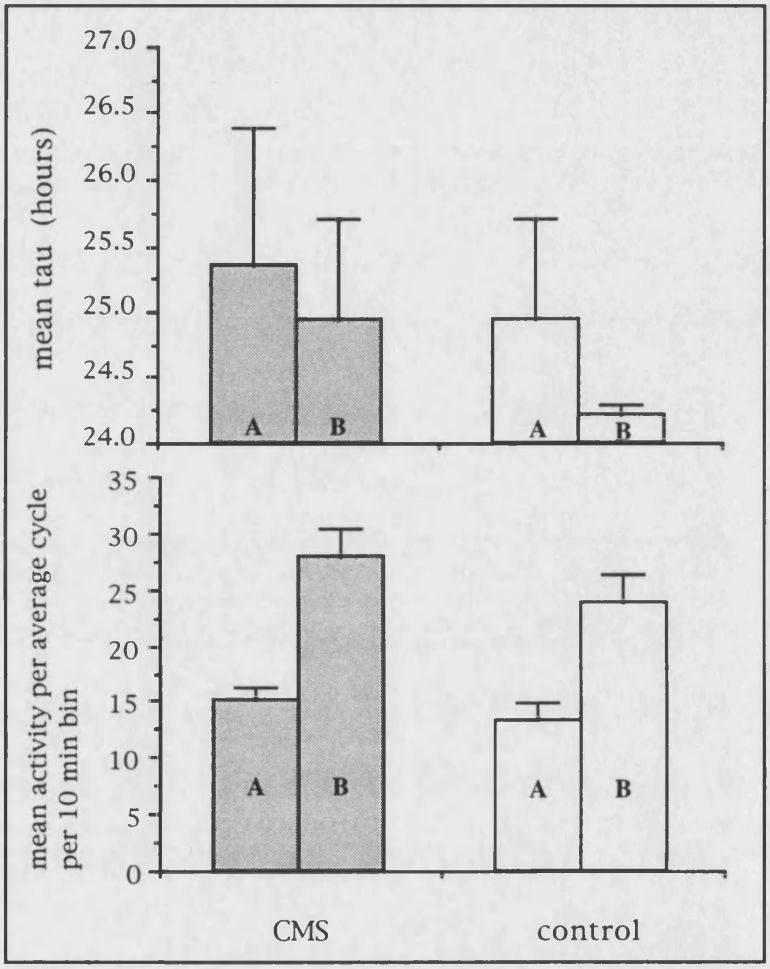


Figure 64. Periodogram analysis of free-running rhythms in CMS 4 ■ and control □ animals. The analysis is based on division of actograms into two parts:
part **A** = days 5-11
part **B** = days 12-21 (n=15 per group)

Analysis revealed no significant difference between the two groups in the computer-estimated tau for the first half of actogram analysis. CMS-treated animals had an average tau of 25.35±1.05 hours, compared to 24.95±0.75 hours for control animals. Similarly, there was no significant difference in tau between the groups in the second half of the actogram analysis (CMS: 24.95±0.76; control: 24.22±0.07 hours).

Activity

Analysis by t-test revealed no significant difference between the two groups in the mean amount of locomotor activity during either portion of the actogram (see Figure 64).

All animals exhibited rhythms with an approximate 24 hour period at the 95% significance level in both halves of the actogram analysis. Furthermore, all but three animals exhibited 24 hour periodicities at the 99% significance level. The three animals with a circadian rhythm which did not reach this significance level were CMS numbers 2 and 52 and control number 20.

Some animals from both the control and CMS group also elicited rhythms with an approximate 12 hour component which reached 99% significance. These harmonics occurred in four of the CMS-treated animals (numbers 24, 28, 30 and 40), and two control animals (2, 48).

Three CMS-treated animals (20, 22 and 28) plus one control animal (26), displayed rhythms with an approximate 99% significant 8 hour component. Finally, three control animals exhibited rhythms with an approximate 99% significant 6 hour component (2, 44 & 48). This 6 hour harmonic was not seen to a 99% significance level in any of the CMS-treated animals. These results are summarised in Table 13.

Table 13. Distribution of rhythm periodicities in control and CMS-treated animals

Periodicity at the 99% significance level	Control	CMS
24 hour	14/15	13/15
12 hour	2/15	4/15
8 hour	1/15	3/15
6 hour	3/15	0/15

A sample of the periodograms obtained is illustrated in Figures 65a and 65b. *Part A* and *Part B* refer to the division of the original actogram for the purpose of analysis. Figure 65a shows periodograms from a CMS-treated animal displaying a significant 12 and 24 hour peak in the first half of analysis, with the 12 hour significant peak disappearing in the second half of the analysis. Also illustrated as the bottom trace in Figure 65a is a periodogram from a CMS-treated animal displaying a significant 8, 12 and 24 hour rhythm. The 8 and 12 hour periodicities disappeared in the second half of the actogram analysis (top trace, Figure 65b). The lower two traces in Figure 65b illustrate periodograms from a control animal displaying a significant 6 hour rhythm which disappears in the second half of the analysis.

Figure 65a. Periodgrams from animals in experiment CMS 4.

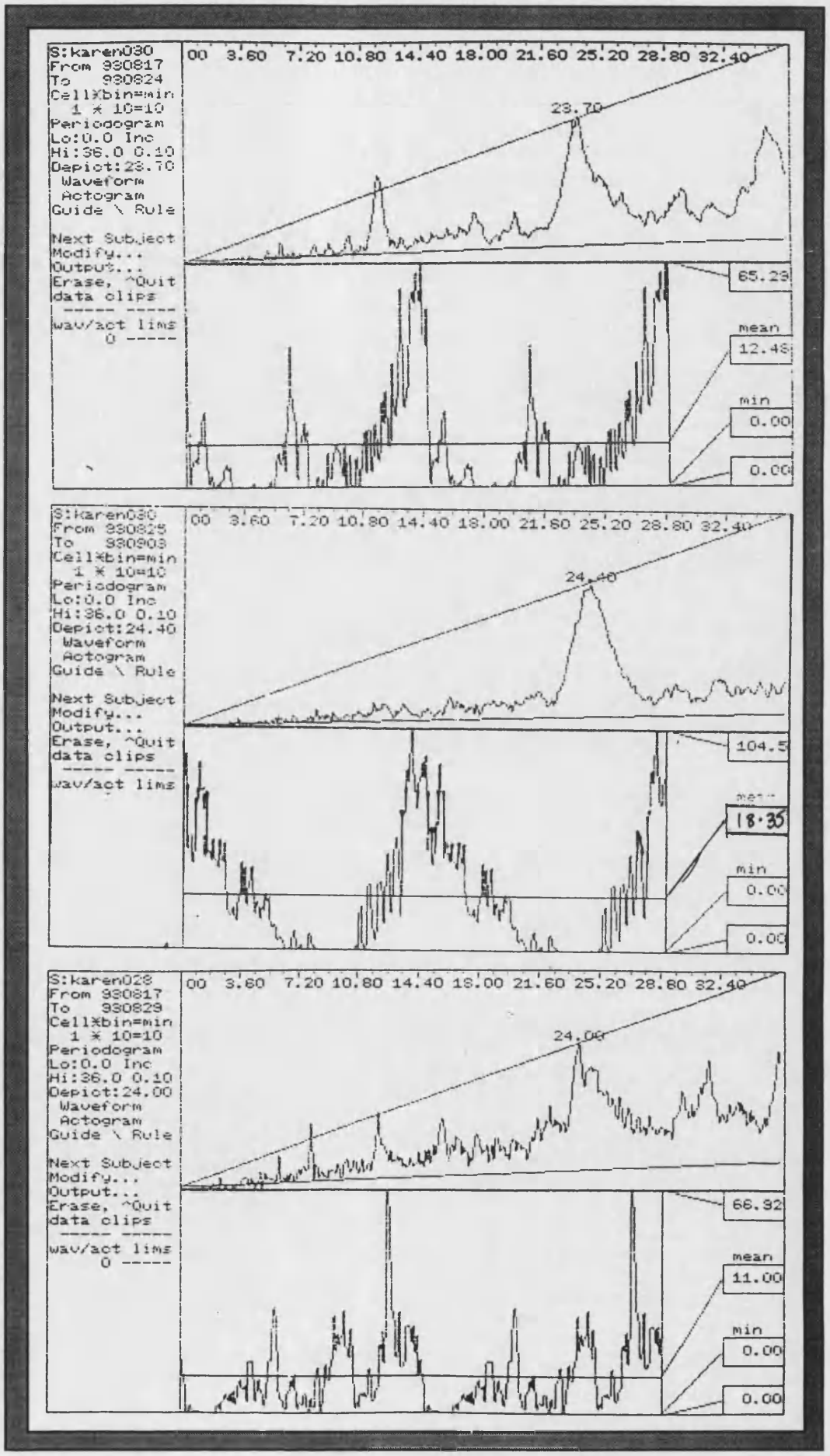


Figure 65b. Periodgrams from animals in experiment CMS 4

CMS-TREATED: part B analysis

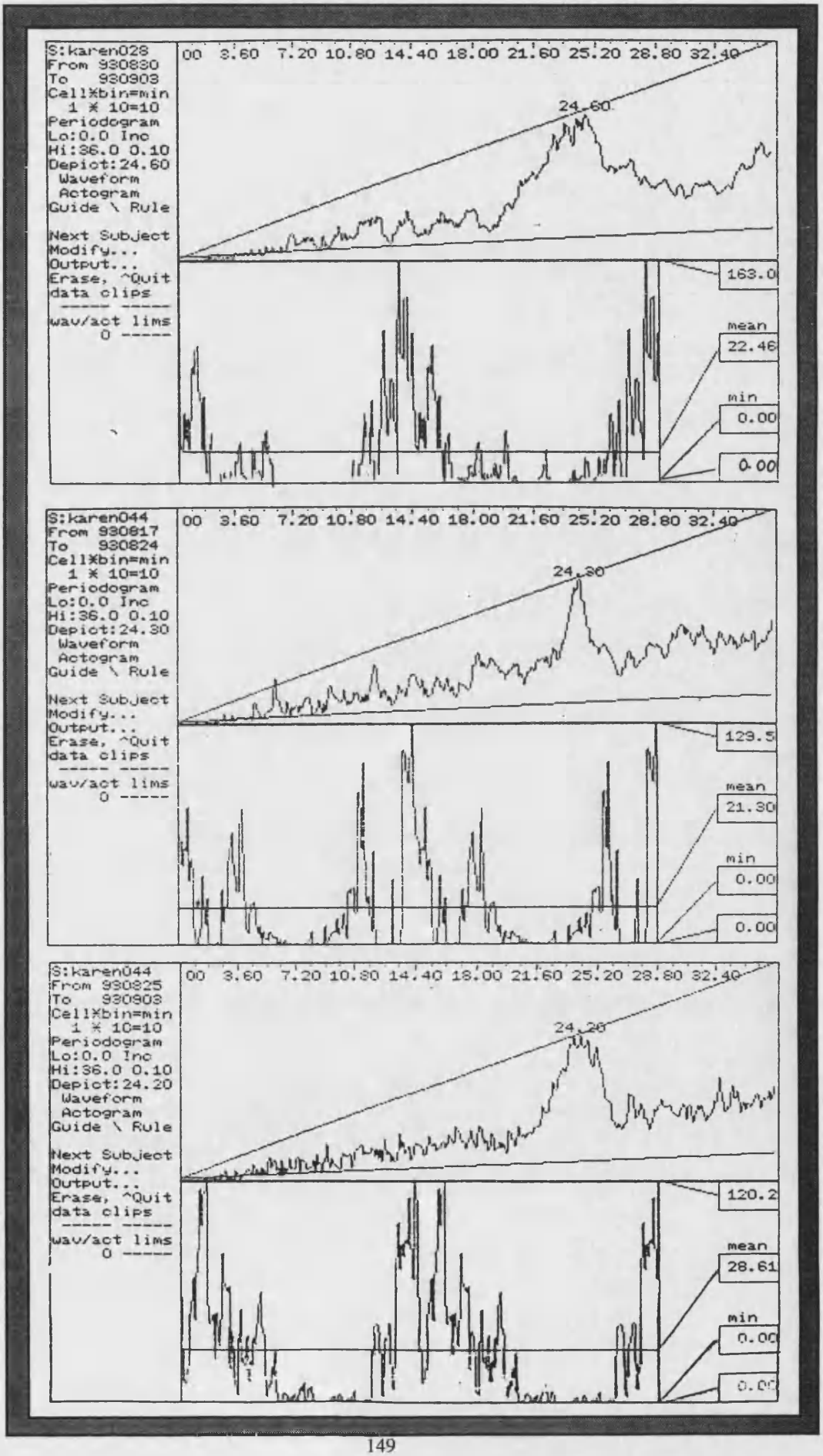


Table 14 shows a summary of the results obtained from actogram and periodogram analysis. It lists the identification numbers of the animals displaying the W-shaped rhythm abnormality and displaying signficant 6, 8 and 12 hour harmonic rhythms in the first 7 days of analysis.

Table 14. Summary of circadian rhythm results from experiment CMS4

	CMS	Control
Rhythm abnormality	12, 20, 22, 28, 30, 40, 58	48
12 hour periodicity	24, 28, 30, 40	2, 48
8 hour periodicity	20, 22, 28	26
6 hour periodicity		2, 44, 48

DISCUSSION

The objective of this experiment was to assess the effects of the CMS procedure on the free-running locomotor activity rhythm of the rat. Firstly, it had to be established that exposure to the CMS regime was successful in inducing a decrease in sucrose preference and in causing alterations in locomotor activity and body weight.

Animals which had been exposed to four weeks of the CMS regime did not express a decreased preference for sucrose. This is inspite of the fact that animals in this experiment were subjected to the same stressors as animals in Experiment 2 where a decreased sucrose preference was recorded. Furthermore, like the animals in Experiment 2, CMS-treated animals in the present experiment showed a significant increase in water intake during the one hour bottle tests, but this did not give rise to a significant sucrose preference deficit in this experiment. Because CMS-treated animals did not exhibit the major symptom of the model (decreased sucrose preference), they could have been considered less than ideal subjects in which to assess circadian free-running rhythms; however, it must be remembered that results from the CMS3 experiment and the work of Matthews et al., (1995) are beginning to cast doubt on the credibility of this parameter as a measure of anhedonia. Locomotor activity testing at the end of weeks three and four showed CMS-treated animals to be significantly less active than controls, a feature which was also characteristic of CMS treatment in Experiments 1 and 2. Furthermore, the significant difference in body weight between control and stress group animals seen in the previous experiment was again recorded in this experiment. Therefore, on the basis of alterations in locomotor

activity and body weight in CMS-treated animals, it was decided to continue with circadian rhythm analysis.

CMS treatment had a significant effect on the circadian free-running rhythm. Seven out of the 15 CMS animals exposed to the regime were seen to exhibit a distinctive W-shaped rhythm compared to 1 out of 15 control animals which showed the same type of disturbance. The pattern of activity displayed may be described as a type of splitting. The 'splitting' phenomenon is seen in several species of nocturnal rodents during prolonged exposure to constant light conditions (Meijer et al., 1990), and it was Pittendrigh and Daan (1976e) who were amongst the first to advance the theory that a two-oscillator pacemaker must underlie the circadian rhythms of these animals, since it is difficult to see how a single oscillator could simultaneously produce two such differing frequencies. Evidence for the existence of more than one oscillator was outlined in the Introduction to this thesis (Satinoff & Prosser, 1988; Reppert et al., 1981; Wever, 1979). However, the location of oscillators outside the SCN has not been established. The SCN itself has been proposed to be the site of multiple oscillators. Partial lesions of the SCN (Davis & Gorki, 1984; Pickard & Turek, 1982; 1985; Rietveld, 1984) as well as recordings from SCN slices or cultures (Watanabe et al., 1993) have indicated that separate groups of cells within the SCN are independently capable of producing a circadian rhythm.

According to the two oscillator hypothesis, the onset of running-wheel activity is regulated by the evening (E) oscillator and the offset of activity is regulated by the morning (M) oscillator. Under normal conditions E and M are mutually coupled to each other in a stable phase relationship, with E phase-leading M by several hours. This relationship depends on the spontaneous, independent frequencies of E and M and, in turn, the period of the coupled system (τ), determines the length of activity time (α). The two-oscillator model was mathematically sustained by Daan and Berde (1978), and more support for the theory came from the work of Honma et al., (1985). These researchers examined the rat PRC for activity rhythms, and found that the PRCs for activity onset and offset were different in shape, suggesting an independent source of control for rhythm onset and offset. More recently, the E and M model was found to be consistent with a number of characteristics in the rat pineal N-acetyltransferase (NAT) rhythm, where evening rise and morning decline behave as though controlled by two separate oscillators (Illnerova, 1991; Elliot & Tamarkin, 1994). Therefore, if the coupling between the two oscillators is altered, for example by exposure to constant light conditions for a number of days (Pittendrigh & Daan, 1976e), by pinealectomy (Aguilar-Roblero & Vega-Gonzalez, 1993) or by administration of testosterone (Morin & Cummings, 1981), this is revealed as a splitting of the rhythm being monitored.

One interpretation of the present results is that CMS treatment is also capable of altering the coupling between the E and M oscillators in susceptible animals. Therefore, instead of locomotor activity existing as a continuous band in these animals, it is reduced to a form of splitting (W-shaped rhythm formation). This theory is further supported by the periodogram analysis, in which clear 12 and 8 hour as well as 24 hour rhythms were seen in the first 7 days of actogram analysis. These ultradian frequencies may represent a change in status of the oscillator possibly due to a decrease in coupling between its E and M component parts. Importantly, periodogram analysis offers a non-subjective method of assessing the effects of CMS treatment, since those animals with disturbed rhythms were also those likely to show an increase in the power of ultradian components. Furthermore, the only control animal to have demonstrated a similar rhythm disturbance also displayed significant ultradian harmonics. Thus it seems that CMS treatment increases the natural tendency of rhythms to split. A potential correlate with human clinical depression may be drawn from the work of Daimon et al., (1992) who suggested that the fundamental rhythm disturbance seen in 62 patients with major depressive disorder may be due to a weakening of the coupling processes between internal pacemakers and an abnormal sensitivity to environmental information. Spectral analysis of deep body temperature revealed that there were significantly more ultradian rhythm components in these depressed patients than in normal subjects.

Although the most common type of splitting reported in the literature is that in which the two components show similar periods in approximately 180° anti-phase, in their original work, Pittendrigh & Daan (1976e) also reported on splitting in which the two components have different periods and never achieve a stable phase relationship, then coalesce after many days. This is a similar picture to that obtained in the present study. Eventually, the split rhythms of the animals coalesce, and this may represent a waning of the effects of CMS and the re-establishment of stronger coupling between the E and M oscillators to form a continuous activity band. Again, this hypothesis is backed-up by periodogram analysis, because the ultradian rhythms disappeared in the second half of the analysis and only circadian peaks remained.

It must be remembered that, as with all E and M data, the evidence is largely phenomenological, since no such oscillators have yet been anatomically discovered and the theory is based on dividing the split components of the rhythm into an onset and offset (Boulos & Morin, 1985; Pittendrigh & Daan, 1976e). However, much of the data generated in the present experiment can be accounted for using this model.

Waveform analysis showed running wheel activity to be lower in both groups during the first 7 days in the wheels (excluding the 4-day settling-in period) than in the last 10 days. Although decreased locomotor activity was used as a behavioural marker for the effects of CMS, CMS-treatment had no after-affect on the amount of circadian

locomotor activity measured in running-wheels. This may not be surprising given the results in the CMS2 experiment in which the deficit in locomotor activity seen during CMS treatment disappeared within one week of stopping CMS.

Finally, assuming sucrose preference to be an appropriate measure of anhedonia, this experiment generates an interesting issue concerning circadian rhythm alterations and coping mechanisms in stress. Stewart et al., (1990), proposed that successful behavioural adaptation to stress may be associated with changes in circadian rhythmicity. These researchers observed that lengthening of circadian period in rats exposed to footshock or handling was positively correlated with escape performance. This rationale could be applied to the results from the present experiment in which animals exposed to the stress regime did not exhibit anhedonia (and therefore did not succumb to the stress regime), but did display alterations in circadian rhythms. Is rhythm plasticity therefore a mechanism for adapting to a chaotic environment?

EXPERIMENT 5: EFFECTS OF CMS ON THE FREE-RUNNING CIRCADIAN LOCOMOTOR ACTIVITY RHYTHM (CMS 5)

The aim of the following experiment was to demonstrate that the effects of CMS on free-running rat locomotor activity rhythms were reproducible.

SUBJECTS

Thirty male Hooded Lister rats (Charles River, Margate, Kent), weighing between 285±5 g at the beginning of the experiment, were housed as in CMS 1.

EXPERIMENTAL PROCEDURE

Animals were divided into two equally matched groups (n=15 per group) on the basis of their final sucrose preference test score in habituation week, and subjected to four weeks of CMS or the appropriate control procedure. At the end of the four week stress period, the animals were moved to the circadian rhythm apparatus at the light-dark transition point (19.00h) and allowed to free-run under constant dim red light conditions. This procedure is the same as that used for the CMS4 experiment.
In addition, the average weekly intake of rat chow in control and stressed animals was monitored during the course of this experiment. Five animals were selected at random from each group, and the amount of rat chow in each animal's food hopper was weighed at the end of each week to give an approximate indication of the amount consumed.

RESULTS

Sucrose Preference Testing

Baseline:

Analysis by t-testing of baseline values revealed there to be no significant difference between control and pre-stressed groups in terms of sucrose and total fluid intakes. The values of these intakes were, in ml: 11.13±0.87 vs 11.99±0.44 and 12.65±1.02 vs 13.66±0.4 for the CMS and control groups, respectively. However, the pre-stressed animals did drink significantly more water than controls on this final test day (2.59±0.24 vs 1.67±0.14 ml respectively; t=3.31, df 28; p=0.003). Overall, there was no significant difference between the two groups in the sucrose preference test prior to CMS treatment (69.12±5.18 % CMS and 75.02±2.61 % control; t=-1.02, df 28; p=0.32).

CMS treatment:

There was no significant effect of the stress procedure on sucrose intake (treatment $F_{(1,28)}=0.073$, NS), although intake varied significantly within subjects during the course of the experiment (week $F_{(3,84)}=2.805$; $p\leq0.045$), fluctuations in sucrose intake can be seen in Figure 66a. The fluctuations in intake were sufficiently similar between groups not to elicit a significant week x treatment interaction ($F_{(3,84)}=1.49$, NS).

CMS treatment significantly increased water intake (treatment $F_{(1,28)}=8.16$; $p\leq0.008$), and this parameter varied in the same manner in both groups during the course of the experiment (week $F_{(3,84)}=12.76$; $p\leq0.001$: week x treatment $F_{(3,84)}=1.68$, NS; Figure 66b).

Total fluid intake did not vary significantly between groups (treatment $F_{(1,28)}=0.26$, NS) or within subjects (week $F_{(3,84)}=2.59$, NS) and there was no significant week x treatment interaction ($F_{(3,84)}=1.39$, NS) (Figure 66c).

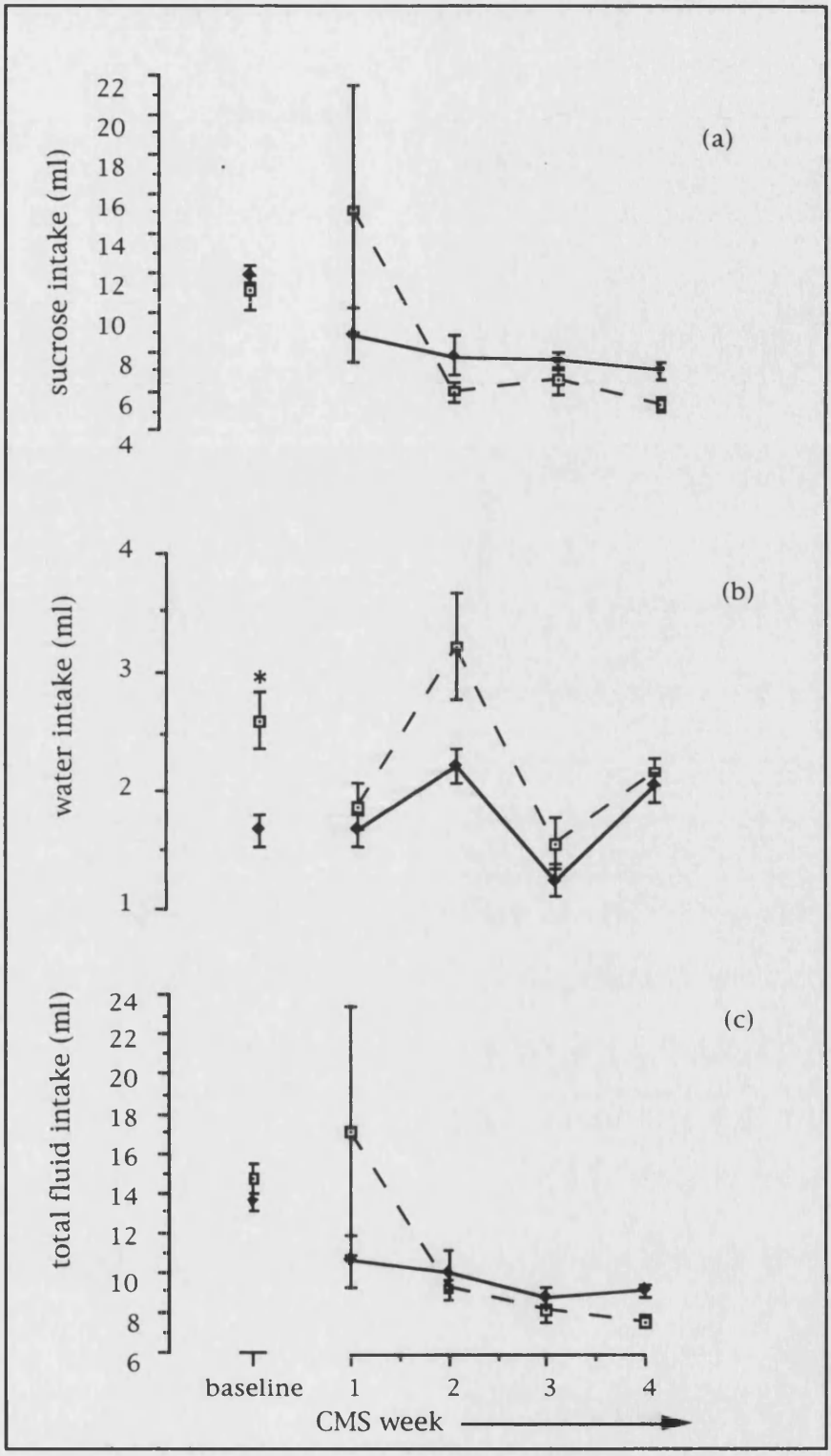


Figure 66a Sucrose intake, Figure 66b Water intake and Figure 66c Total fluid intake in CMS 5 animals during weekly sucrose preference testing. Values are mean ± sem. Stress group — □ - control group ——♦—— n=15 per group. $p \leq 0.05$. Baseline values are from week 0, 3rd habituation test.

CMS-treatment significantly affected sucrose preference (treatment $F_{(1,28)}=5.97$; $p\leq0.021$), which varied significantly within subjects during the experimental procedure (week $F_{(3,84)}=11.08$; $p\leq0.001$). There was no significant week and stress interaction ($F_{(3,84)}=1.167$, NS). Figure 67 displays the sucrose preference test values for the CMS 5 experiment.

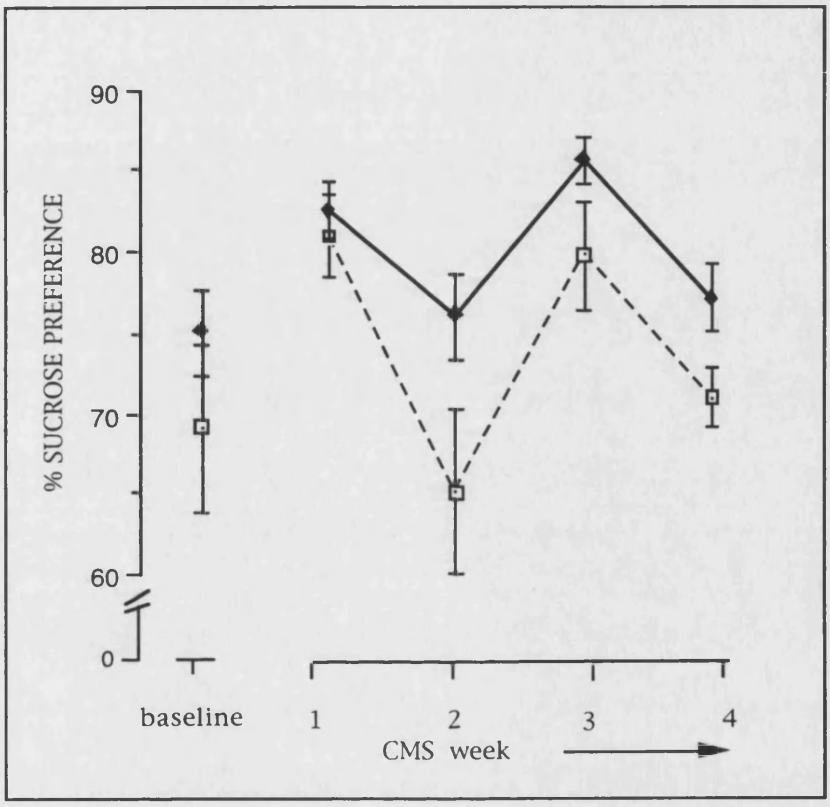


Figure 67. Sucrose preference test in animals subjected to four weeks CMS. Control —◆— CMS 5 — □ - n=15 per group; values are mean ± sem.

Locomotor Activity

Locomotor activity testing at the end of stress week 3 revealed CMS animals to be significantly less active than controls (treatment=2794.2±94.7 and control=3356.6±116.27 beam breaks, respectively; t-test $p \leq 0.05$). This is illustrated in Figure 68.

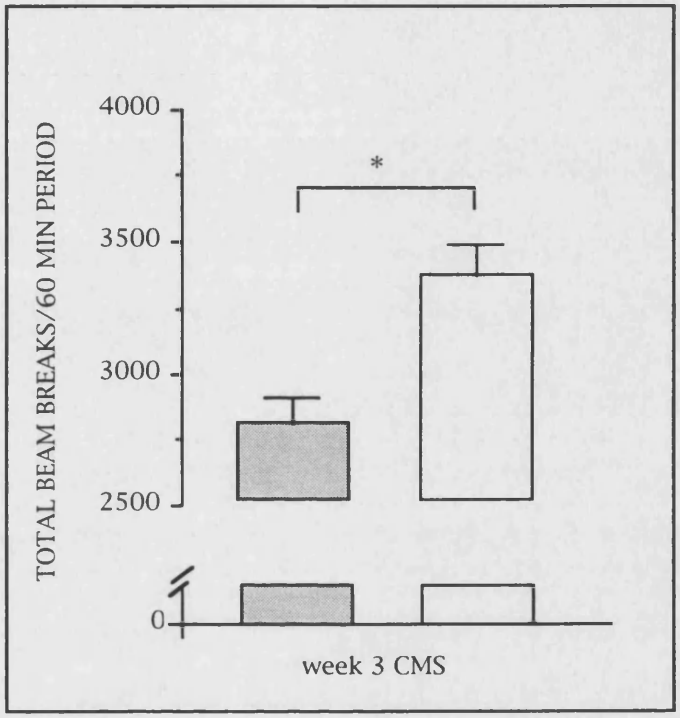


Figure 68. Locomotor activity test at week 3 CMS in CMS 5 animals. control ☐ CMS ▩ Values are mean±sem; n=15 per group. *$p \leq 0.05$ vs control.

Body weight

Stressed animals had a significantly lower body weight than control animals during the CMS procedure (treatment $F_{(1,28)}$=29.61; $p \leq 0.001$). This deficit in weight was significant at each weekly time-point (week $F_{(3,84)}$=452.4; $p \leq 0.001$) and increased with continuing exposure to the stress regime (week x treatment $F_{(3,84)}$=7.1; $p \leq 0.001$). The deficit was greatest at week 3 CMS when stressed animals weighed on average 35 grams less than control animals. Control group animals gained weight at the rate of approximately 8% per week whilst CMS-treated gained weight at 6% per week. Stressed animals ended the CMS treatment period (week 4) at approximately 126% of their initial weight compared to the control group animals who ended week 4 at approximately 135% of their initial weight. The average body weight per week for each group is illustrated in Figure 69.

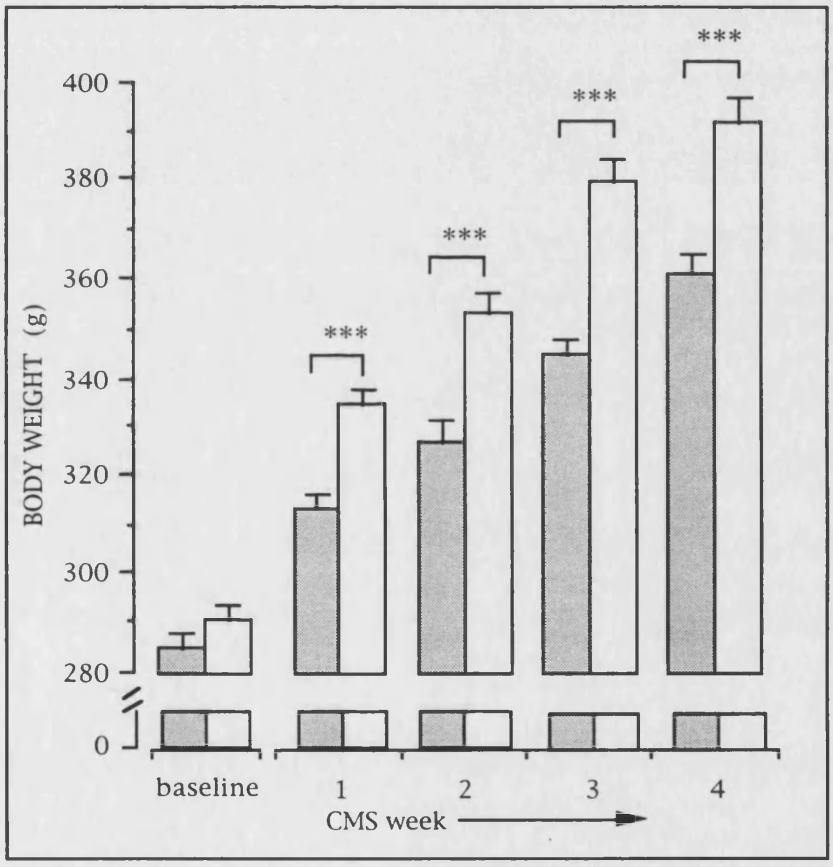


Figure 69. Differences in body weight between CMS 5 ■ and □ control animals prior to and during four consecutive weeks exposure to chronic mild stress. Values are mean+sem n=15 per group. *** $p<0.001$ vs saline. Baseline = experimental starting weight.

Food Consumption

CMS-treated animals consumed significantly less rat chow per week than control animals during the experimental period (treatment $F_{(1,8)}=9.68$; $p\leq0.05$) (Figure 70). There was significant variation within subjects during the course of the experiment ($_{week}F_{(3,24)}=8.66$; $p\leq0.005$), but no significant stress and week interaction ($F_{(3,24)}=0.135$; NS).

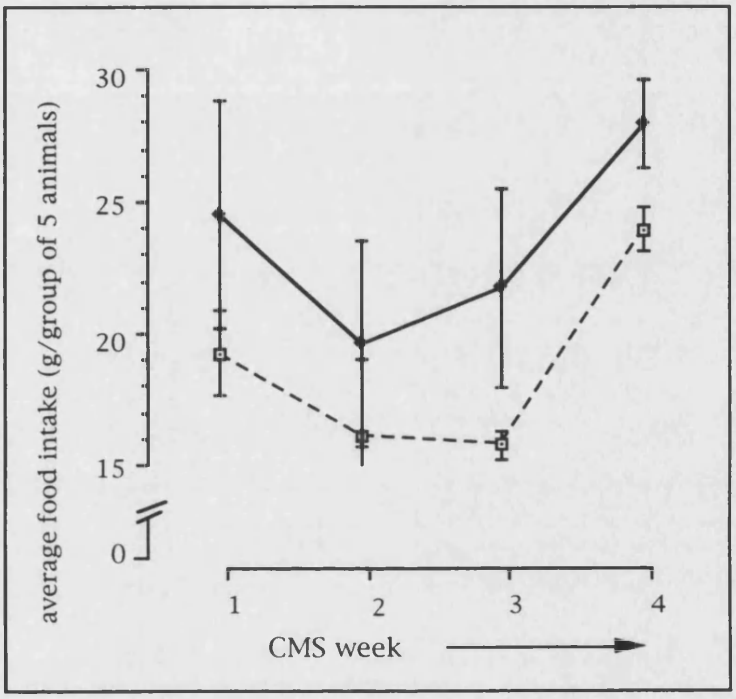


Figure 70. Food consumption during CMS treatment in CMS 5 - - -□- - - and control —◆— animals. Values are mean±sem; n=5 per group. Each point represents the average food intake for that week.

CIRCADIAN RHYTHM RESULTS

Table 15: Actogram assessment of the circadian free-running rhythms in control and CMS-treated animals.

CMS animal No.	W-shaped rhythm disturbance (Y/N)	No. days to form continuous activity	Control animal No.	W-shaped rhythm disturbance (Y/N)	No. days to form continuous activity
2	n	7	4	-	-
6	n	immediate	62	-	-
10	n	5	12	n	12
14	n	-	16	n	immediate
22	n	immediate	18	n	5
24	n	immediate	20	n	immediate
30	y	14	26	n	immediate
32	n	7	28	n	immediate
34	n	8	36	n	immediate
40	n	6	38	n	12
42	n	9	44	-	-
50	n	5	46	n	3
52	n	-	48	n	immediate
58	y	15	54	n	4
60	n	immediate	56	n	9
mean ± sem		5.85±1.39	mean ± sem		3.75±1.38

Where 'immediate' is taken as 0 days to the formation of a continuous rhythm

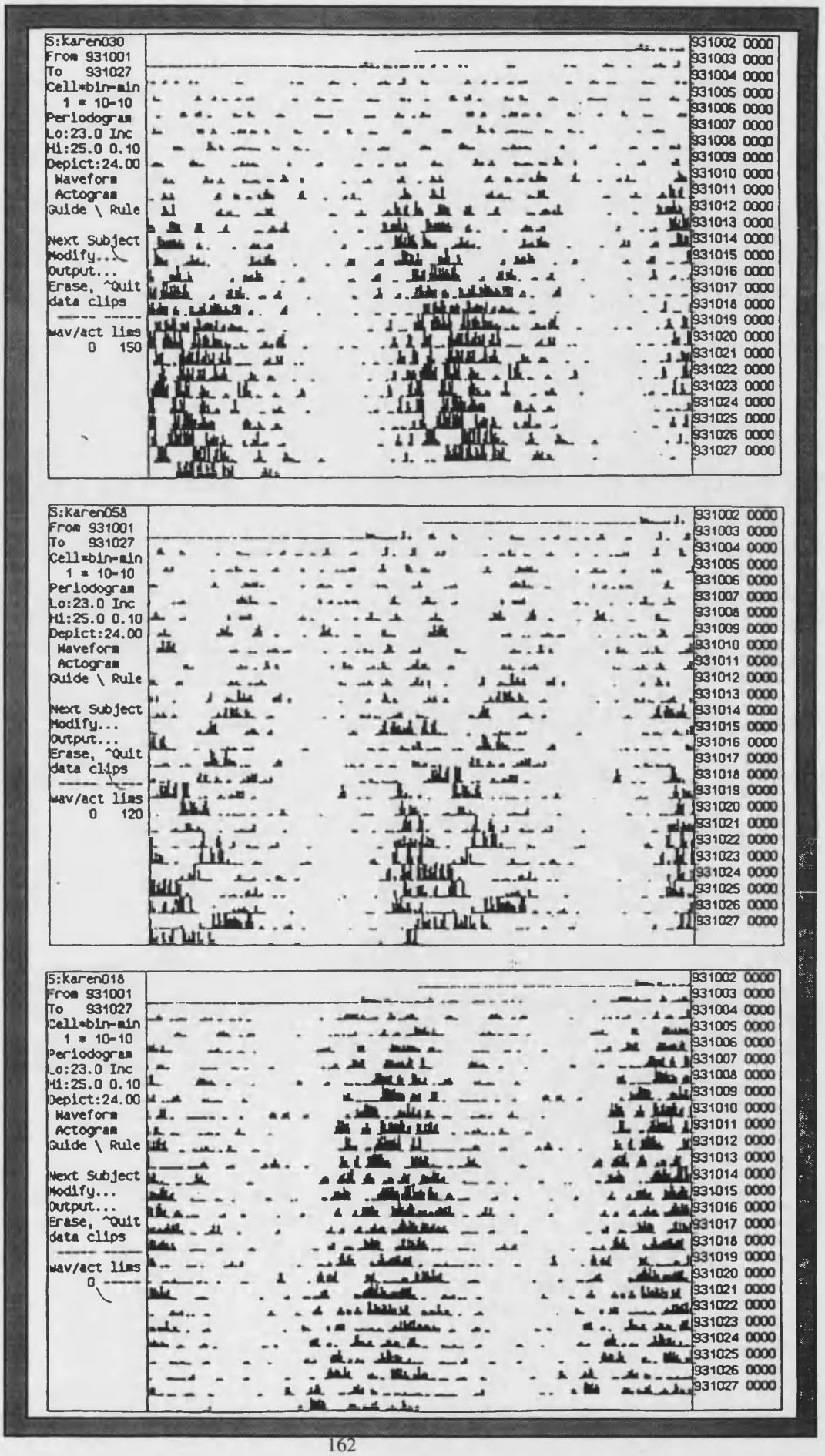
where n=13 where n=12

Analysis by t-test revealed there was no significant difference between the two groups in the time taken to form a continuous, single band of locomotor activity (t=1.26, df 24, p=0.2).

Actogram inspection revealed two out of 15 CMS 5 animals exhibited a similar type of circadian rhythm disturbance as animals in the CMS4 experiment. The affected animals were numbers 30 and 58. None of the control animals exhibited the W-shaped activity pattern. This difference between the two groups in the tendency to exhibit abnormal patterns of activity was not statistically significant when tested using Fischer's exact test.

Actograms from the two CMS-treated animals which exhibited an abnormal pattern of activity, plus an actogram from a control animal, are shown in Figure 71.

Figure 71. Actograms from animals in experiment CMS 5.



Periodogram and waveform anlaysis

Again, each actogram was divided into two sections. The first covered ten days of 'post-settling-in' activity, and the second half was the continuation of activity until the end of the experiment. As in the previous experiment, the periodicity and the mean activity level was determined from a periodogram and waveform plot for each animal in each half of the actogram. Analysis by t-test revealed no significant difference between the two groups in the computer-estimated tau for the first half of actogram analysis. CMS-treated animals displayed an average tau of 24.02±0.04 hours, compared to 23.98±0.15 hours for control animals. Similarly, there was no significant difference in tau between the groups in the second half of the actogram analysis (CMS: 24.95±0.79; control: 25.57±3.32 hours).

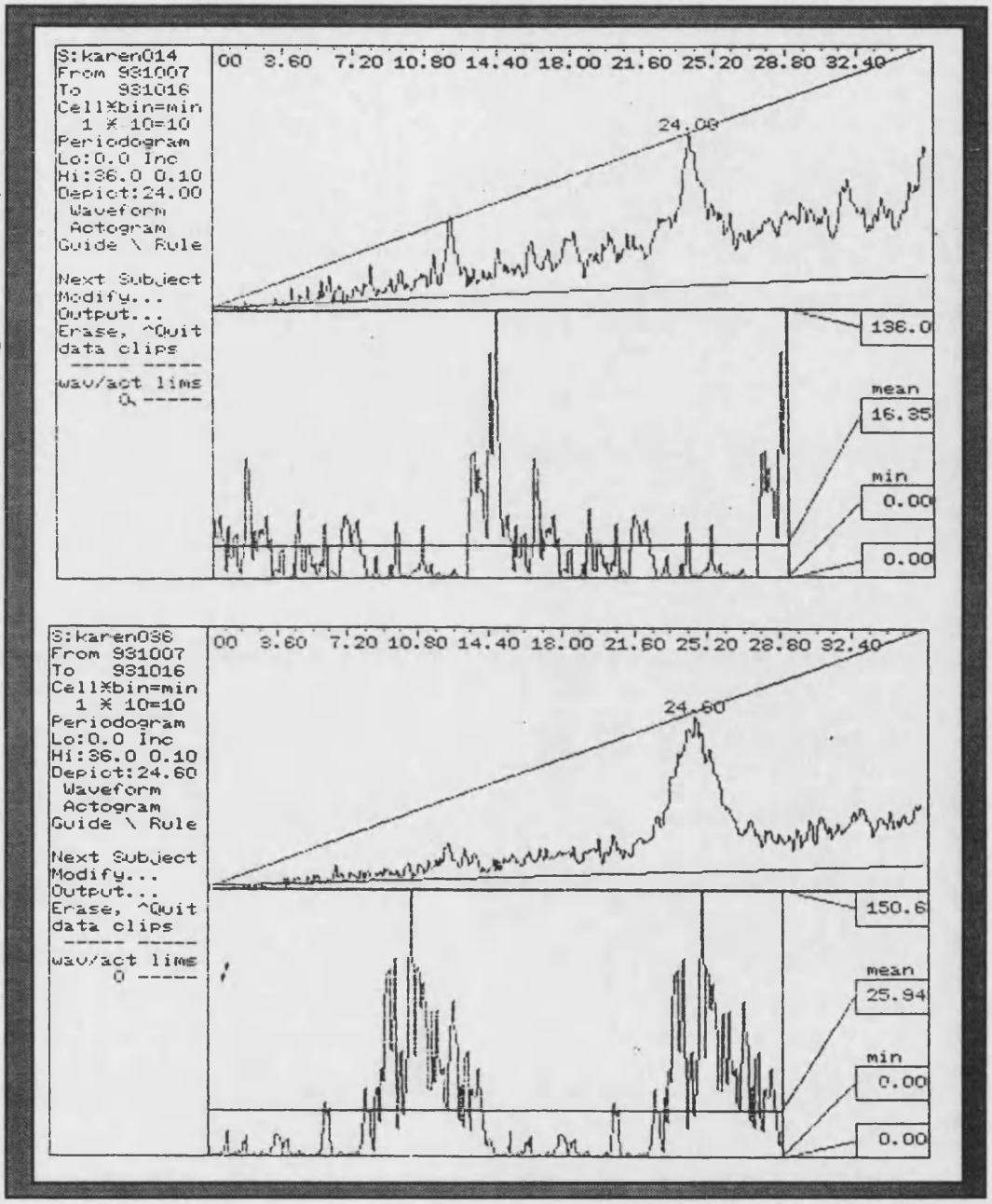
12 hour and ultradian analysis

As in the previous experiment, all animals (except CMS No. 24) exhibited rhythms with a 24 hour periodicity at the 95% significance level in both halves of the analysis. However, although many of the animals displayed approximate 12 hour harmonic rhythms greater than 95% significance, this was significant in only one animal (CMS No. 14). No animals exhibited significant 6 or 8 hour periodogram peaks.
Periodograms from a CMS-treated and control animal depicting the first half of the actogram data are shown in Figure 72. It can be seen that the CMS-treated animal displayed a significant 12 hour harmonic during this time period whereas the control animal did not.

Activity

Analysis by t-test revealed no significant difference between the two groups in the mean amount of locomotor activity (wheel revolutions per average cycle per 10 minute bin), during either portion of the actogram (first half of actogram analysis: CMS 24.39±4.35 and control 19.94±2.54; second half of actogram analysis CMS 26.49±3.33 and control 25.57±3.32).

Figure 72. Periodograms from animals in experiment CMS 5.



DISCUSSION

The aim of this experiment was to determine if the effects of CMS exposure on free-running rat locomotor activity rhythms seen in CMS4 were reproducible. As in the previous experiment, it was important that CMS-treated animals displayed symptoms of the depression model sufficient to warrant examination of their free-running rhythms.

Animals subjected to four weeks of the CMS regime exhibited all three symptoms indicative of exposure to the animal model of depression. Firstly, CMS-treated animals displayed a decreased preference for sucrose compared to control animals. This deficit may have arisen from the significant increase in water consumpton by stress animals during the one hour bottle tests. This result parallels that seen in the CMS 2 animals, where stress-treated animals also drank significantly more water than controls and exhibited a decreased preference for sucrose. Secondly, locomotor activity in a novel environment was significantly decreased by CMS-treatment in the present experiment. This effect has so far been recorded in every group of animals exposed to the regime, making the CMS-induced locomotor deficit a more robust index of CMS-treatment than decreased sucrose preference. Finally, body weight was significantly decreased by CMS treatment in the present experiment. As with decreased locomotor activity, the decrease in body weight gain also appears to be a more persistent index of CMS exposure than sucrose preference deficit.
The decreased body weight may be accounted for by the significant decrease in food intake of CMS-treated animals recorded during the four week experimental period. Whilst reports in the literature concur that exposure to the CMS regime causes weight loss, some authors advise that food intake should not be decreased by CMS treatment, because this would represent a decrease in general consummatory behaviour and not a specific reward-related deficit (Papp et al., 1991; Muscat & Willner 1992). Food intake has been shown to be unaffected or even increased in CMS animals (Papp et al., 1991), but there are no reports in the literature of decreased intake. The CMS procedure has also been assessed using the conditioned place preference (CPP) test, in which food is the natural reward. CMS-induced anhedonia in CPP is represented by a decrease in the desire for food (Papp et al., 1991; Muscat et al., 1992). It therefore seems parodoxical that when assessing anhedonia within the CMS model, a decrease in food intake acts as the behavioural marker in one type of study, but must not occur when measuring anhedonia via sucrose preference in another type of study. What is the cause of the decreased food intake? Stress in the form of restraint has been shown to reduce food intake in adult male rats (Marti et al., 1993; 1994). These workers describe a positive relationship between decreased food intake and the intensity and duration of the restraint applied. Whilst it would be interesting to further examine the

components of the CMS procedure which affect food intake, this is not the purpose of the present study. The purpose is to generate animals which show symptoms of exposure to the CMS model of depression so that their circadian rhythms can be analysed. In the present experiment, CMS-treatment induced all three symptoms of the model and all animals were transferred to activity for rhythm analysis under constant dim red light conditions.

It must be emphasized that no animal from the present experiment exhibited such a distinct W-shaped rhythm as those in experiment CMS4. Even in the two animals classified as having abnormal rhythms, the changes were not as robust as in the previous experiment. Similarly, periodogram analysis showed that the ultradian frequencies associated with rhythm disturbance in the CMS4 experiment did not exist in the actograms of CMS5 animals. Why then did the CMS regime not produce the same alterations in free-running rhythms in this experiment? Stressed animals did exhibit the behavioural signs indicative of exposure to CMS, and perhaps the theory of rhythm plasticity suggested in experiment 4 can also be applied here. Successful behavioural adaptation to stress is associated with alterations in circadian rhythmicity (Stewart et al., 1990). In the present experiment the circadian rhythm changes were minor, and this coincided with a marked sucrose preference deficit as a behavioural indicator of stress, suggesting that the animals did not adapt to the CMS regime. However, this theory can only be generalised to the group as a whole in both this and the previous experiment, because individual animals which displayed abnormal rhythms were not specifically correlated with higher levels of sucrose preference than those which did not display rhythm alterations. Future work specifically correlating physiological markers of stress with CMS-induced changes in circadian rhythms may clarify the issue of whether plasticity of circadian rhythms is associated with the ability to adapt to stressful environmental changes.

EXPERIMENT 6: EFFECTS OF HANDLING AND LIGHTING COMPONENTS OF THE CMS PROCEDURE (CMS 6)

The aim of this experiment was to determine the contribution of factors such as changes of the light/dark cycle and stimulation of animals by handling, to the alterations seen in circadian free-running rhythms in the previous two experiments.

SUBJECTS

Thirty male Hooded Lister rats (weighing 275±10g at the beginning of the experiment; supplied by Charles River, Margate, Kent) were housed as in CMS 1.

EXPERIMENTAL PROCEDURE

Animals were randomly divided into three groups (n=10 per group) to undergo either lighting-only (Appendix 4), handling-only (Appendix 5) or control-only (Appendix 6), aspects of the CMS regime for a 4 week period. Handling-only occurred at a time when the animal would have been transferred to confinement box, taken for weighing or moved for group housing under the normal CMS regime. Handling lasted for approximately one minute both at the beginning and at the end of the allotted period. Strobe-lighting was the same as that used for the full CMS procedure (300 flashes per minute). The sucrose habituation week and weekly sucrose preference testing were omitted from the schedule since these involve periods of food and water deprivation which may have confounded attempts to measure effects of light and/or handling. Body weight was measured weekly and locomotor activity was measured in week 3. At the end of the four week regime, the animals were transferred to activity-wheel cages at the LD transition point and maintained under constant dim red light for 21 days as in CMS4.

RESULTS

Locomotor Activity

There were no significant differences between any of the three groups ($F_{(2,27)}$=2.9). The average number of beam breaks over the 60 min test period was 2795.7±351.1 for the lighting-only group, 2049.5±167.81 for the handling-only group and 2811.2±210.6 for the control group.

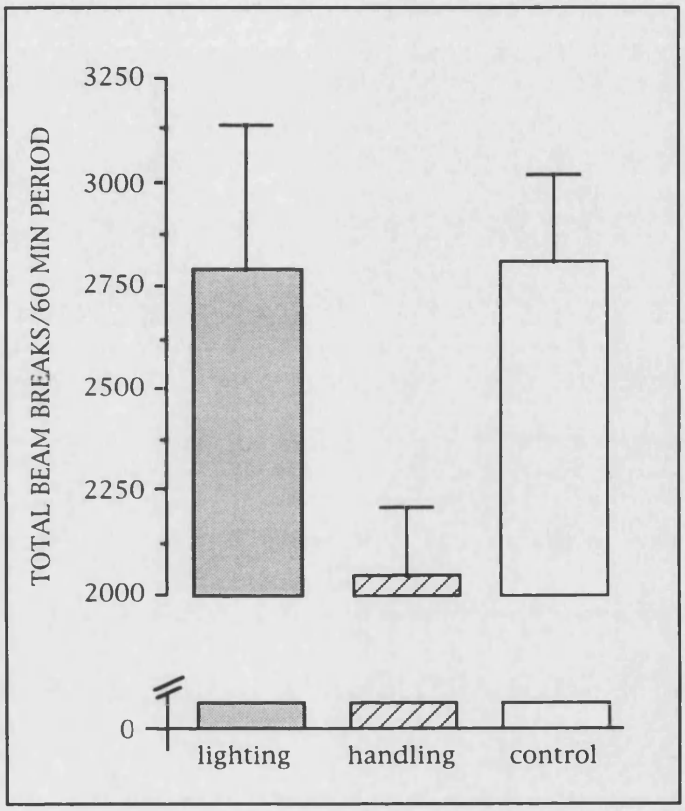


Figure 73. Locomotor activity in CMS 6 animals at week 3 of CMS treatment. Values are mean±sem; n=10 per group.

Body weight

There was no significant effect of any treatment on body weight in the three groups of animals ($_{treatment}$ $F_{(2,27)}$=0.081, NS). All animals gained weight each week ($_{week}$ $F_{(3,81)}$=1069; $p \leq 0.001$) at an approximately equal rate of 12% per week ($_{treat\ x\ treat}$ $F_{(6,81)}$=0.615, NS), and ended the treatment period at approximately 140% of their initial weight. Figure 74 illustrates the body weight changes of the three groups of animals during the experimental procedure.

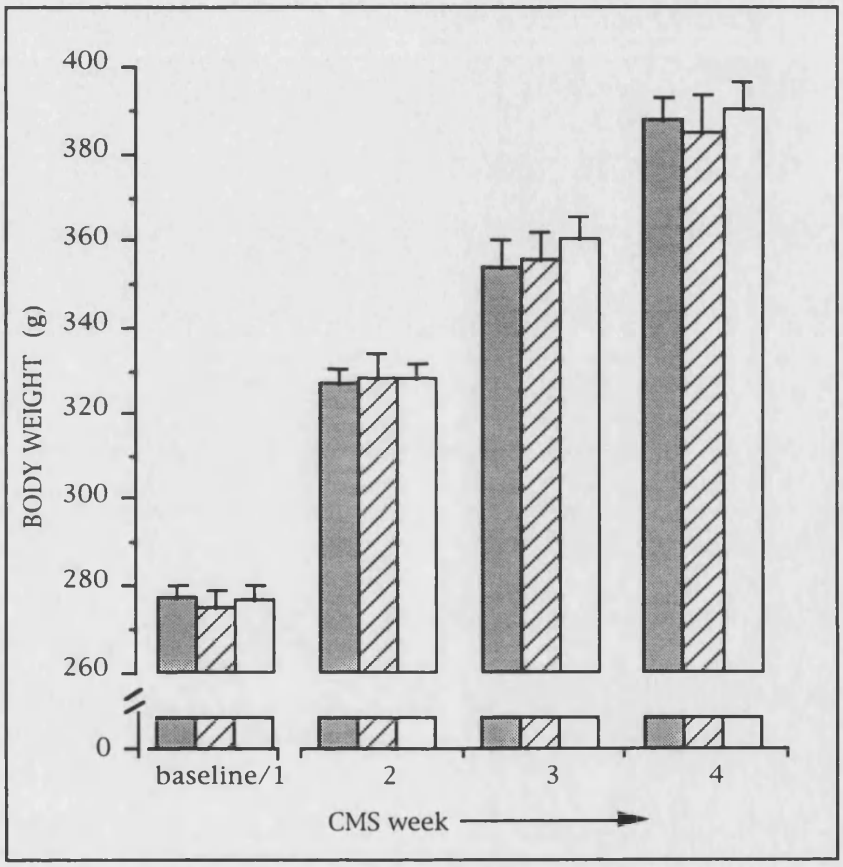
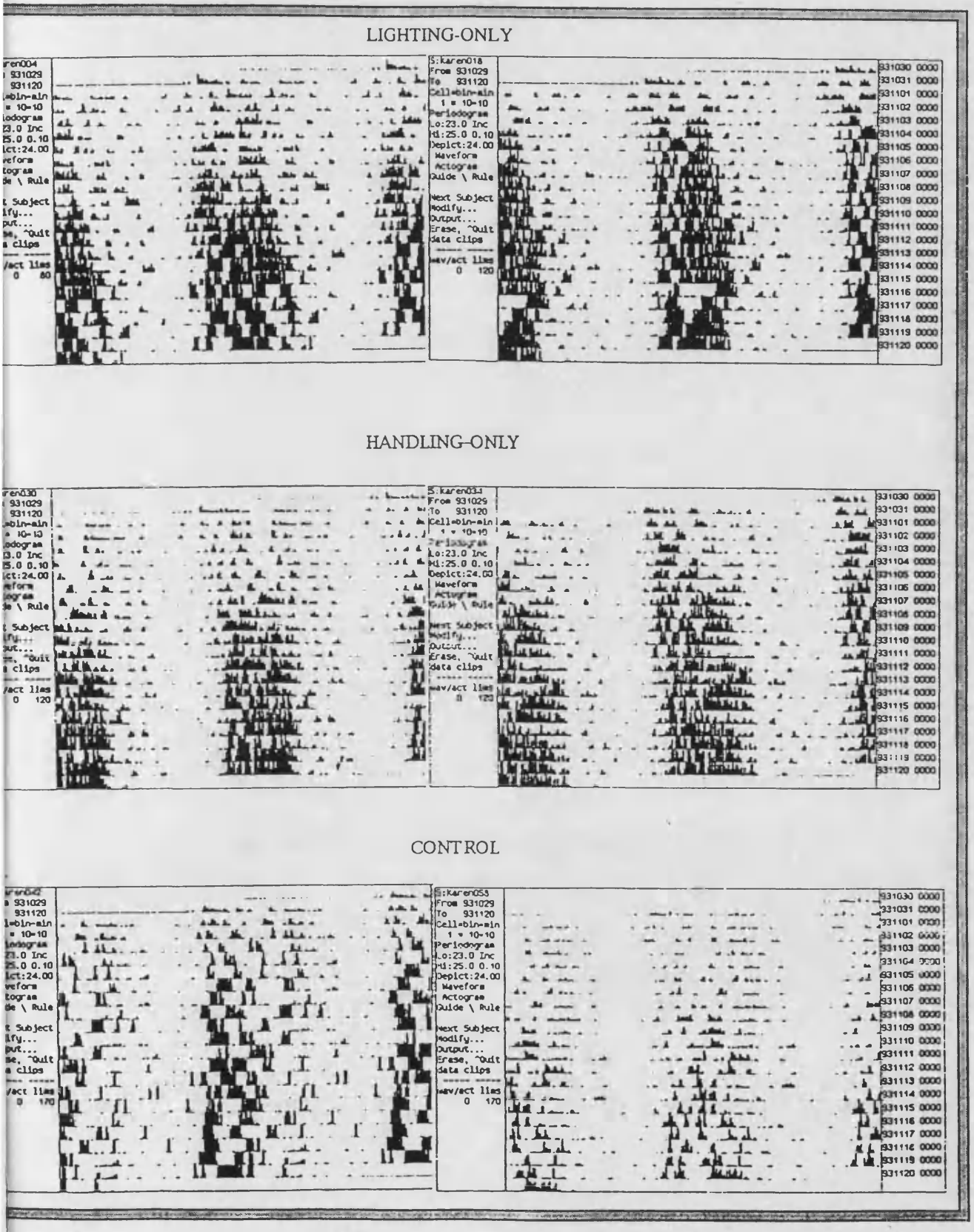


Figure 74. Changes in body weight of animals subjected to the handling- ▩ and lighting-only ▨ aspects of the CMS procedure, plus control group □ Values are mean ± sem; n=10 per group. Baseline value is the experimental starting weight of the animals.

CIRCADIAN RHYTHM RESULTS

Strobe-lighting and handling had no effect on the pattern of circadian locomotor activity. No animal from any group exhibited the W-shaped disturbance observed in experiments CMS4 and CMS5. Figure 75 illustrates actograms from each of the three groups.

ıre 75. Actograms from animals in experiment CMS6.



DISCUSSION

The CMS regime has previously been dissected to determine which were the key elements in inducing a sucrose preference deficit (Muscat & Willner, 1992). Similarly, it was decided to assess which elements were responsible for the W-shaped abnormality seen in nine out of the thirty animals so far exposed exposed to the full CMS procedure (experiments CMS4 and CMS5). The ability of both photic (Pittendrigh & Daan, 1976a-e) and non-photic (Mrosovsky et al., 1989) pertubations to affect circadian rhythms is well known, and it was these elements of the regime, in the form of strobe-lighting and animal handling, respectively, which were analysed for their effects on the free-running locomotor activity rhythm.

Handling and strobe-lighting are major components of the CMS protocol. An animal may be handled anything up to six times per day during procedures such as transferral to a confinement box, being placed in paired housing, cage change or body weight measurement. In addition, animals exposed to the CMS regime experience disturbances of the light/dark cycle when they are exposed to a strobe-light during an otherwise dark period. Two groups of animals were therefore subjected to the handling-only or the lighting-only aspects of the CMS regime, with one group acting as control.

The results of the present experiment show that exposure to handling-only and strobe-lighting-only did not significantly affect body weight gain, relative to control animals, throughout the experimental period. Neither did the treatments affect locomotor activity during a 60 minute exposure to a novel environment. Animals in the handling-only group recorded the lowest activity in this test (reminiscent of the hypolocomotion seen previously in groups exposed to the full CMS procedure), although this deficit was not statistically significant. Recordings of the circadian free-running locomotor activity rhythms revealed that neither of the treatments resulted in the W-shaped rhythm abnormality characteristic of some of the animals exposed to CMS in previous experiments. Finally, it must be mentioned that although individual housing, a funamental element of the CMS regime, has been shown to affect the circadian rhythm of hypothalamic catecholamines and their metabolites in the rat (Gambardella et al., 1994; Greco et al., 1992), this can not be the element responsible for the rhythm disturbances seen in CMS 4 and CMS5, since control animals were also singly housed and did not show similar rhythm disturbances. Furthermore, all animals in the present experiment were also singly housed.

It can therefore be concluded that the decreased body weight and hypolocomotion, plus the alterations in circadian free-running rhythm recorded in CMS-treated animals are due to more than just the handling and lighting components of the CMS regime.

EXPERIMENT 7: EFFECTS OF CMS ON THE ENTRAINED CIRCADIAN LOCOMOTOR ACTIVITY RHYTHM (CMS 7)

The aim of this experiment was to determine the effects of the CMS procedure on the 24-hour light/dark entrained circadian locomotor activity rhythm.

SUBJECTS

Thirty male Hooded Lister rats (Charles River, Margate, Kent), weighing between 330±5 g were housed as previously described prior to the start of the experiment.

EXPERIMENTAL PROCEDURE

Following the sucrose habituation week and four subsequent weeks of CMS treatment, animals were placed in individual activity wheels and allowed six days to acclimatise (n=15 CMS; n=15 control). The lighting schedule of 07.00h-19.00h used for the CMS procedure was continued for the first six days in the activity wheels. On day 7, the animals received a 6 hour phase advance of the LD cycle. This was achieved by shortening the dark phase as follows: after lights off at 19.00h on day 6, the lights were switched on at 01.00h on day 7 and switched off again at 13.00h of the same day. This cycle of lights on 01.00h and lights off 13.00h was then continued for 21 days.

At the end of the 21 day phase advance period, animals were exposed to a 6 hour phase delay of the light/dark cycle. This was achieved by lengthening the light phase as follows: instead of lights off at 13.00h on day 21, the lights remained on until 19.00h, and were switched on again at 07.00h the next morning. This cycle of lights on 07.00h and lights off 19.00h was continued for 20 days. Illumination was provided by two anglepoise lamps projecting white light of approximately 100 lux at cage level. Food and water were maintained daily at random times.

CIRCADIAN DATA ANALYSIS

The following parameters were measured from each animal's actogram: i) number of days to reentrain, ii) direction of reentrainment, iii) phase angle of reentrainment and iv) the continuity of the alpha band. Measurements were made based on steady-state and not immediate phase shifts, and were made blind to the treatment code.

i) *Days to reentrain.*

The number of days required for an animal to reentrain to the new LD cycle was counted from the date of the phase shift to the beginning of the steady-state of the reentrained rhythm, (i.e. the number of transient cycles was counted).

ii) *Direction of reentrainment*

The direction (advance or delay) of shift in the activity rhythm in response to the two LD phase shifts was recorded for each animal.

iii) *Phase angle of entrainment.*

The phase angle of entrainment of both the onset and offset of activity following a phase shift of the LD cycle was measured in each animal. Following a phase shift of the LD cycle, when the activity rhythm had reached steady-state, the position of onset and offset of activity relative to lights off and lights on, respectively was determined.

iv) *Continous α band.*

The continuity of the band of activity (α) during the subjective night was assessed. If the period of activity from lights off to lights on was broken by a rest period of at least 30 minutes, the animals was recorded as having a disrupted α band.

RESULTS

Sucrose Preference Testing

Baseline:

Analysis by t-testing of baseline values revealed there to be no significant difference between the two groups in terms of sucrose, water and total fluid intakes. The values of these intakes were, in ml: sucrose 8.09±0.59 vs 6.69±0.63; water 1.40±0.21 vs 1.33±0.14 and total fluid 9.49±0.62 vs 8.02±0.67 for the CMS and control groups, respectively. Consequently, there was no significant difference between the two groups in the sucrose preference test prior to beginning CMS treatment (84.84±2.22 % CMS and 82.02±2.21 % control; t=0.9, df 28; p=0.37).

CMS treatment:

There was no significant effect of the stress procedure on sucrose intake (treatment $F_{(1,28)}=0.055$, NS), and intake did not vary significantly within subjects during the course of the experiment (week $F_{(3,84)}=0.99$, NS) (Figure 76a). There was no significant interaction of week x treatment ($F_{(3,84)}=1.26$, NS).

CMS treatment had no significant effect on water intake (treatment $F_{(1,28)}=0.15$, NS), and intake did not vary significantly within subjects with week (week $F_{(3,84)}=0.927$, NS) thus there was no significant interaction of week and treatment ($F_{(3,84)}=1.344$, NS). (Figure 76b).

Total fluid intake was not significantly different between the two groups during the CMS period (treatment $F_{(1,28)}=0.007$, NS). There was no significant variation in total

fluid intake within subjects during the experimental period (week $F_{(3,84)}$=1.47, NS)and no significant interaction (week x treatment $F_{(3,84)}$=1.08, NS) (Figure 76c).

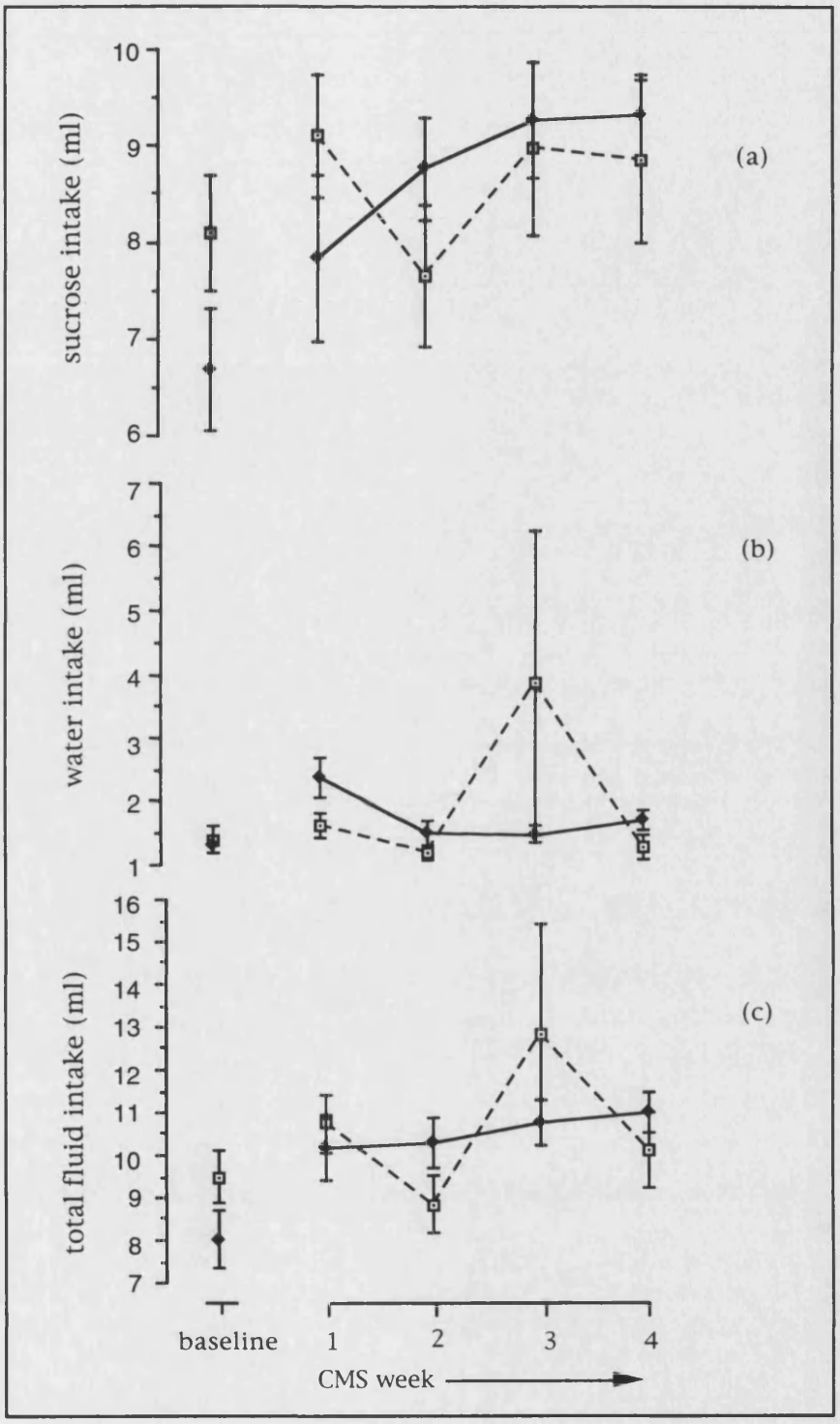


Figure 76a Sucrose intake, Figure 76b Water intake and Figure 76c Total fluid intake in CMS 7 animals during weekly sucrose preference testing. Values are mean ± sem. Stress group – – ⊡ – – control group ——●—— n=15 per group. vs control. Baseline values are from week 0, 3rd habituation test.

Overall, CMS treatment did not significantly affect sucrose preference (treatment $F_{(1,28)}=1.049$, NS), and there was no significant difference in this preference ratio within subjects during the experimental period (week $F_{(3,84)}=1.73$, NS). The lack of variation in sucrose preference within and between the groups is reflected in the non-significant interaction (week x treatment $F_{(3,84)}=0.055$, NS). Figure 77 displays the sucrose preference test values for the CMS7 experiment.

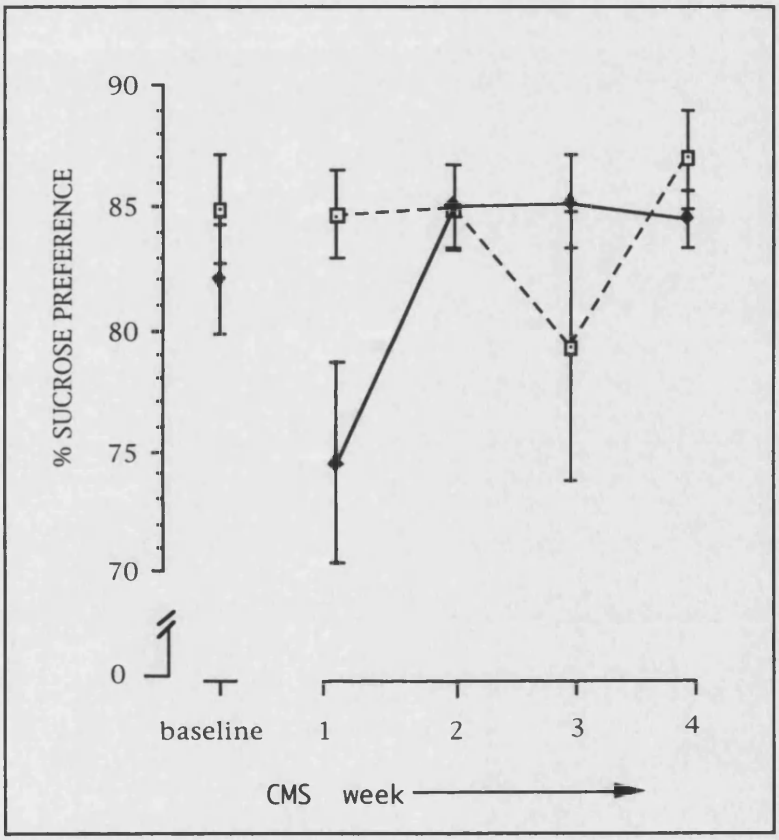


Figure 77. Sucrose preference test in animals subjected to four weeks CMS 7 — □ - and control —◆— animals n=15 per group; values are mean ± sem.

Locomotor Activity

Analysis by t-test of the locomotor activity counts at the end of week 3 revealed there to be no significant difference in locomotor activity between the groups. However, when tested at the end of week 4, stress group animals were found to have significantly lower levels of locomotor activity than the control goup ($p \leq 0.05$).

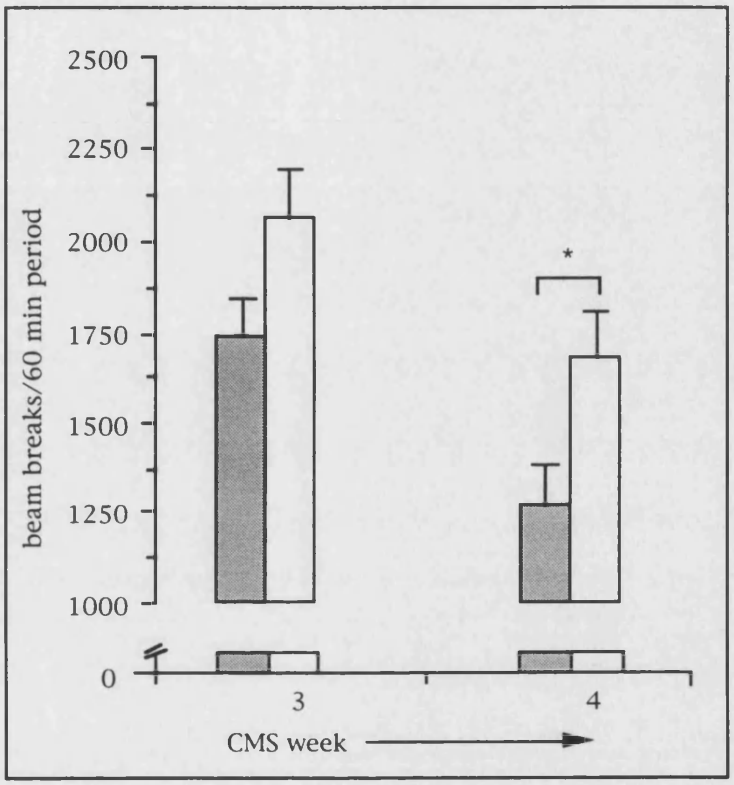


Figure 78. Locomotor activity of CMS 7 animals and controls measured in weeks 3 and 4 of the stress procedure. Values are mean±sem, n=15 per group. ▩ cms ☐ control * $p \leq 0.05$ vs. control group.

Body weight

CMS treatment caused a significant decrease in body weight (treatment $F_{(1,28)}=18.66$; $p\leq0.001$), an effect which increased with weekly exposure to CMS (week $F_{(3,84)}=244.55$; $p\leq0.001$) giving a significant week and treatment interaction (treatment x week $F_{(3,84)}=23.09$; $p\leq0.001$).

Stressed animals lost weight during the first two weeks of treatment and the average body weight for the group declined from 330.13±5.87 to 326.73±5.189 grams. In the second two weeks, the animals increased their body weight by an average of 3% per week, ending the treatment period at 106% of their starting weight. Control animals gained weight steadily during the experimental period at an average rate of 5% per week, ending the treatment period at 117% of their starting weight.

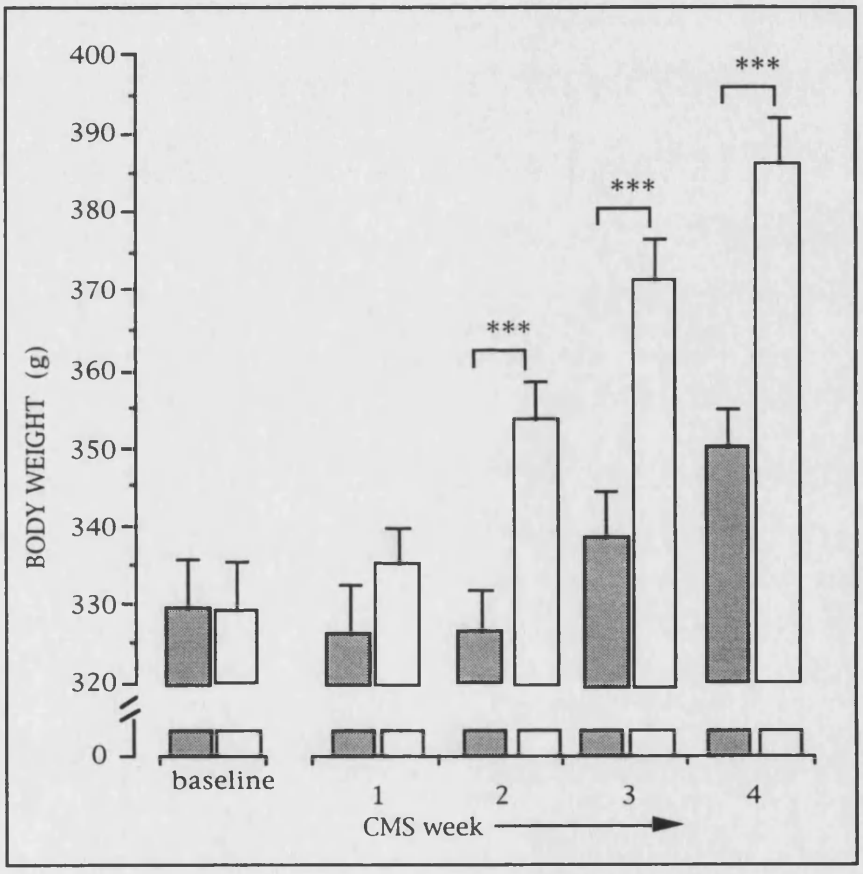


Figure 79. Changes in body weight in CMS 7 animals prior to and during four weeks CMS treatment. ▩ CMS; □ control. Values are mean ± sem; n=15 per group. *** $p\leq0.001$ Baseline = experimental starting weight.

CIRCADIAN RHYTHM RESULTS

1. ***Days to reentrain***

CMS animals took significantly longer (18.13±0.87 days) to reentrain to the advance phase shift of the LD cycle than did control animals (12.20±1.17 days) (t= 4.07; $p \leq 0.001$). The length of time taken to reach a steady-state of reentrainment to the delay phase shift revealed no significant difference between groups (CMS=10.93±1.37 days; control=8.54±0.97 days). (Figure 80). Actograms from a CMS-treated and a control animal illustrating the difference in time taken to reentrain are shown in Figure 81. The upper actogram is from a control animal which took 8 days to reentrain to a phase advance, compared to the lower actogram from a CMS-treated animal which took 21 days to reentrain to the same phase shift.

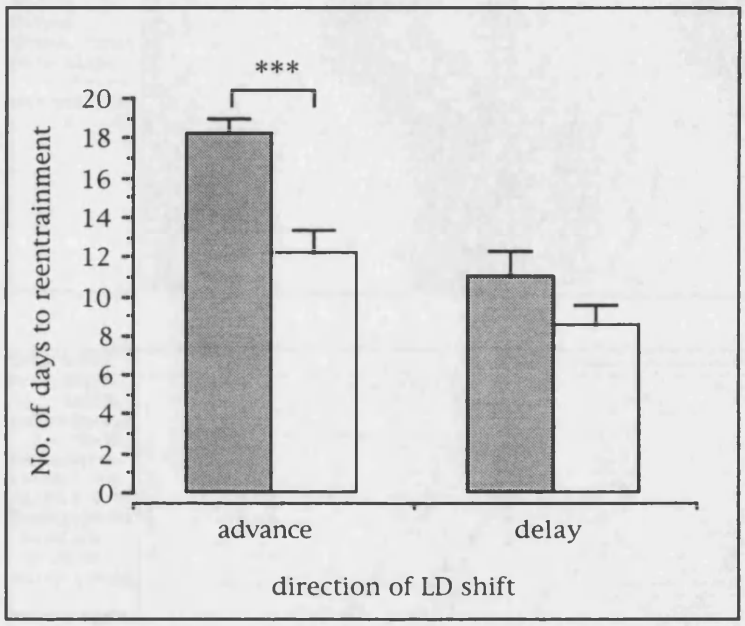
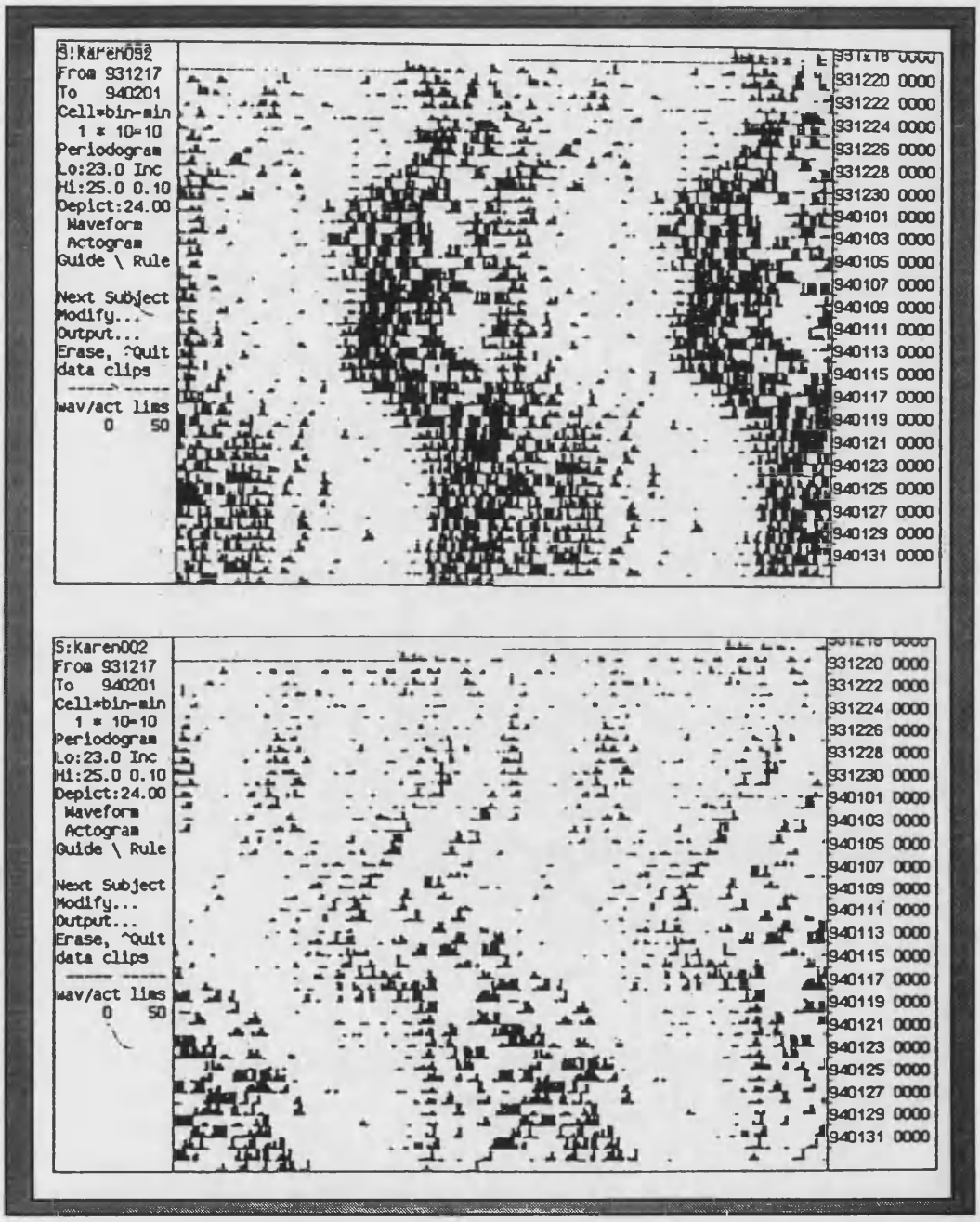


Figure 80. Number of days taken to reentrain to a phase shift of the light/dark cycle in CMS ▨ and control □ animals. Values are mean ± sem where n=15 per group. *** $p \leq 0.001$

Table 16. Number of days to reentrainment to a shift of the LD cycle.

Treatment	days to reentrain to advance shift	days to reentrain to delay shift
CMS	18.13±0.87	10.93±1.37
control	12.2±1.17	8.54±0.97

Figure 81. Actograms from animals in experiment CMS7



2. ***Direction of reentrainment***

Seven out of 15 CMS-treated animals reentrained to the advance shift of the LD cycle by delaying their activity pattern, thus moving in the opposite direction to the shift in the LD cycle. This is compared to 1 out of 15 of control animals which reentrained in this direction. All other animals advanced their rhythm in response to the advanced light cycle. Fischer's exact test showed this difference to be significant (p=0.035).

Following a delay of the light.dark cycle, all animals in both groups reentrained to the shift by delaying their activity rhythm. The phase shifts are illustrated graphically in Figure 82.

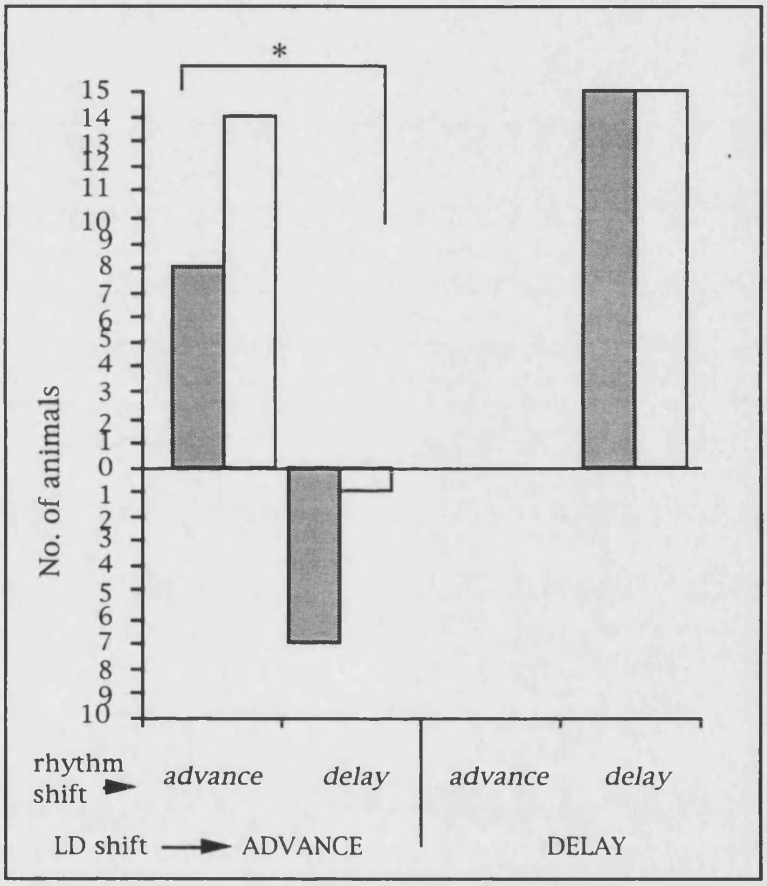
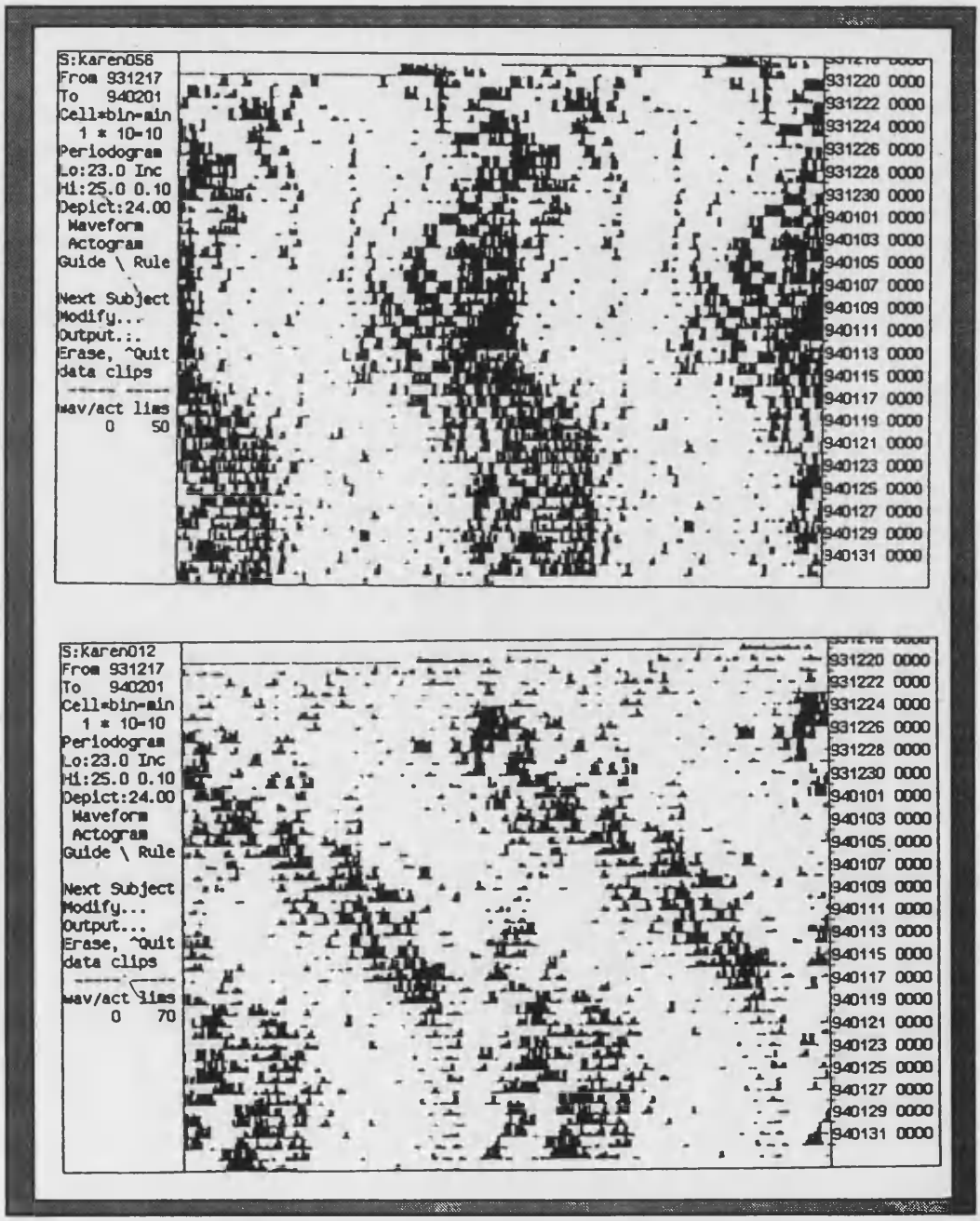


Figure 82. Direction of shift in the locomtor activity rhythm (indicated in *italics*) of animals exposed to a 6 hour shift of the light dark cycle. CMS ▨ control □ * $p \leq 0.05$

The actograms from a control and CMS-treated animal are illustrated in Figure 83. The upper actogram shows that the control animal first advances and then delays its activity rhythm, following the direction of the two phase shifts in the LD cycle. The lower actogram shows that the CMS-treated animal delays its locomotor activity rhythm in order to reentrain to the advanced LD cycle, and again delays its locomotor activity rhythm to reentrain to the delayed LD phase shift.

Figure 83. Actograms from animals in experiment CMS7



3. ***Phase angle of entrainment***

Figure 84 shows that following a phase advance of the LD cycle, the phase angle of entrainment did not differ significantly between the two groups of animals when either onset or offset was used as the phase marker. When they had reached steady-state, all animals began locomotor activity at approximately the point of lights off, and ended activity at approximately the point of lights on. However, following a phase delay of the LD cycle, there was a significant difference between the two groups when offset was used as the phase reference ($t=3.04$ with 21 deg. freedom; $p \leq 0.01$). The end of activity occurred on average 129 ± 16.5 minutes into the returning light phase in CMS animals compared to 60.4 ± 15.2 minutes for control animals. When onset was taken as the phase reference, there was no significant difference in phase angle of entrainment between the two groups.

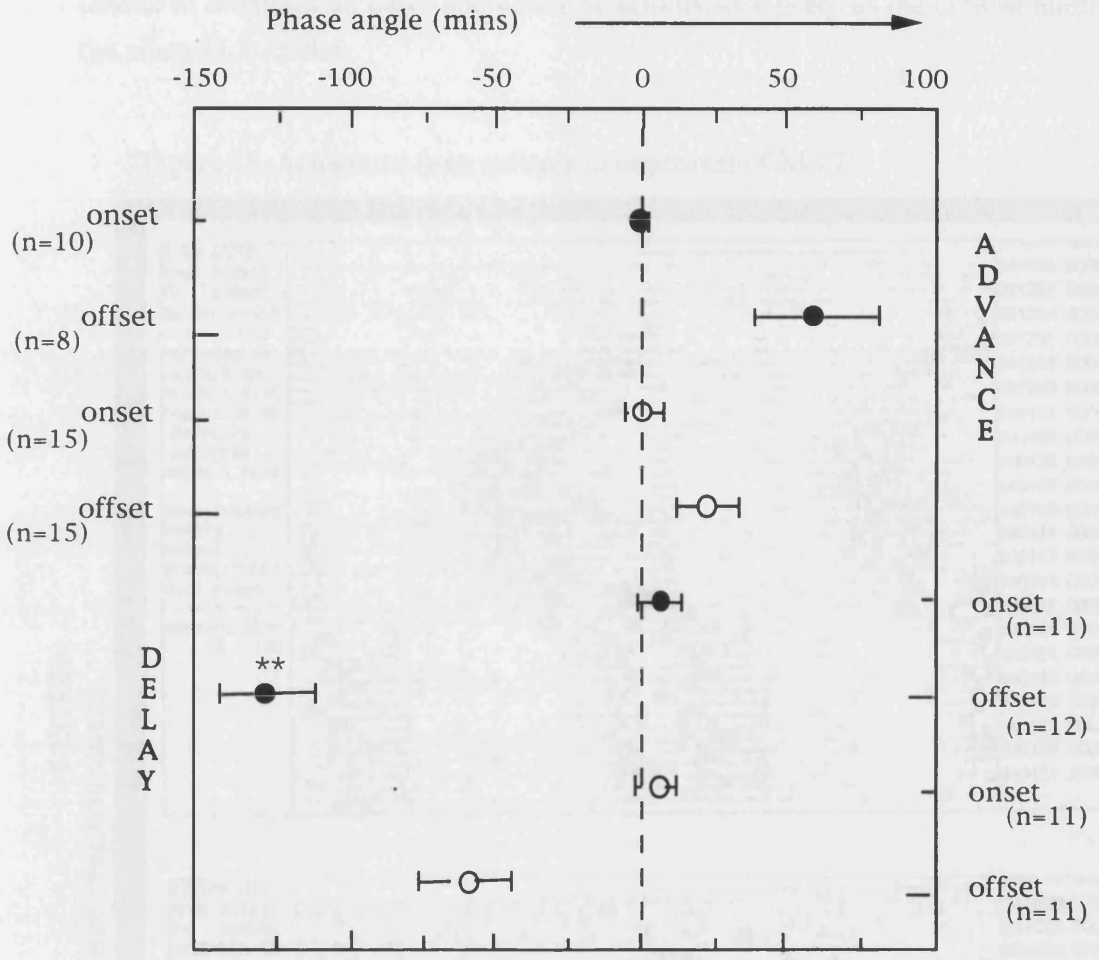
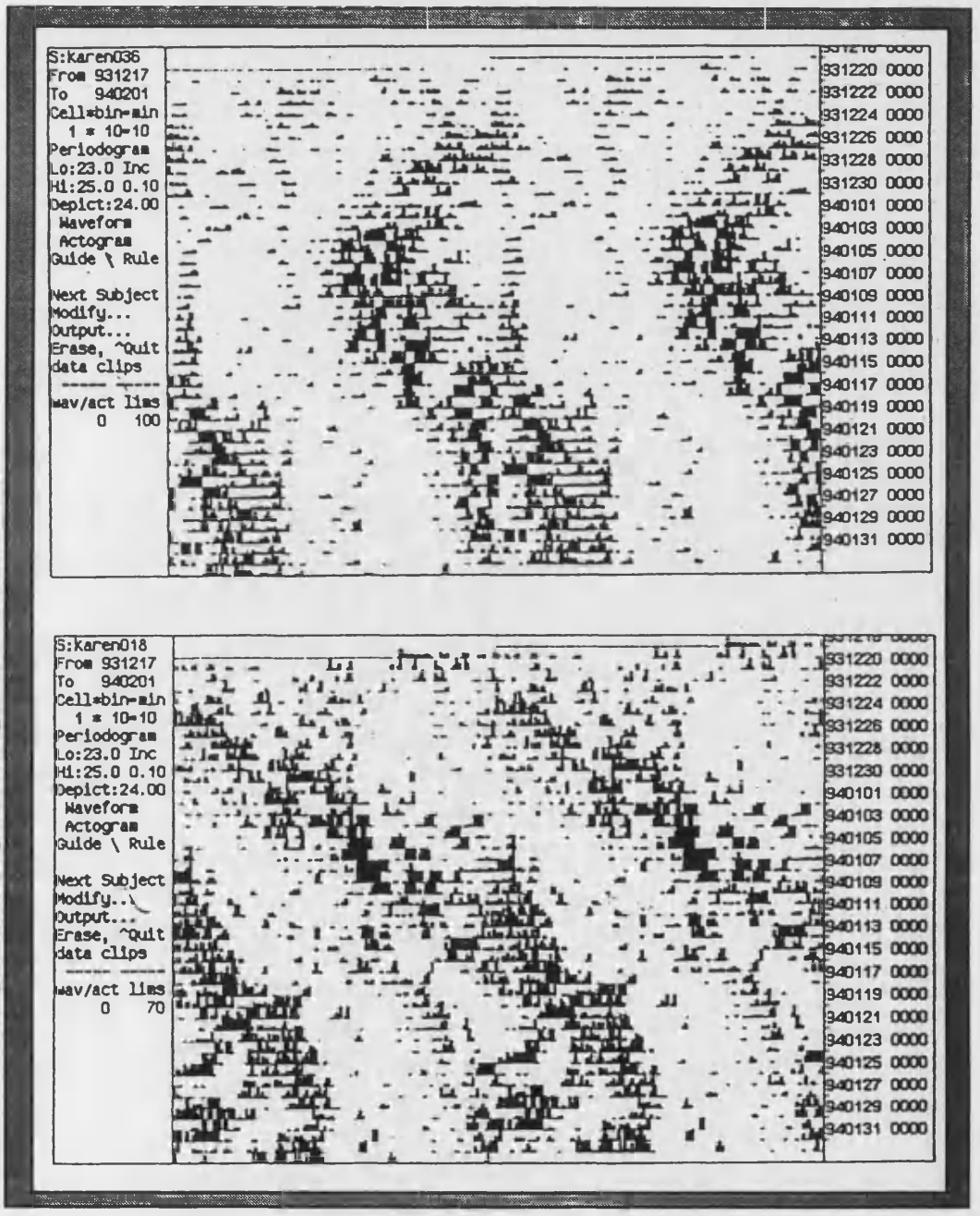


Figure 84. The mean phase angles of activity onset and offset following 6 hour phase shifts of the LD cycle. CMS treatment ● control ○
The mean phase angle of entrainment is given in minutes ± one sem of the mean value. All measurements were made relative to lights off (onset) or lights on (offset) as the time cue, which is represented by the dotted line at time 0. Reentrainment was measured at steady-state.
A negative value indicates that the rhythm lagged behind its light-cue, and a positive value indicates that the rhythm was ahead of its light cue. For example, the offset of activity should theoretically occur at the point of lights on. The offset in CMS-treated animals, following an advance of the LD cycle, occurred 59.83±21.36 minutes before lights-on (positive sign); whereas the offset following a delay of the LD cycle occurred 129.14±16.55 minutes after lights on (negative sign). ** $p \leq 0.01$

The upper actogram in Figure 85 illustrates the activity of a control animal which was able to reentrain its onset and offset of activity closely to the shifting light and dark cues. The lower actogram illustrates the activity of a CMS-treated animal which was unable to reentrain its onset and offset of activity as closely as the control animal to the altered LD cycles.

Figure 85. Actograms from animals in experiment CMS 7



4. ***Continous α band***

Following a phase advance of the LD cycle, 11 out of 14 (78.5%) CMS animals displayed a non-continuous pattern of activity, compared to 7 out of 15 (46.6%) control animals which had a similar pattern. Following a delay of the LD cycle, a non-continuous activity band was seen in 10 out of 14 (71.4%) CMS animals, compared to 4 out of 14 (28.5%) control animals. These differences were found not to be statistically significant using Fischer's exact test (Figure 86).

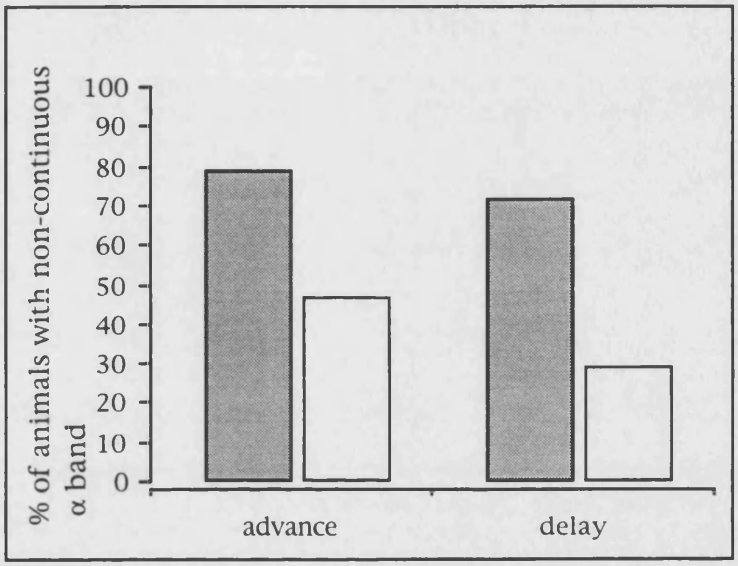
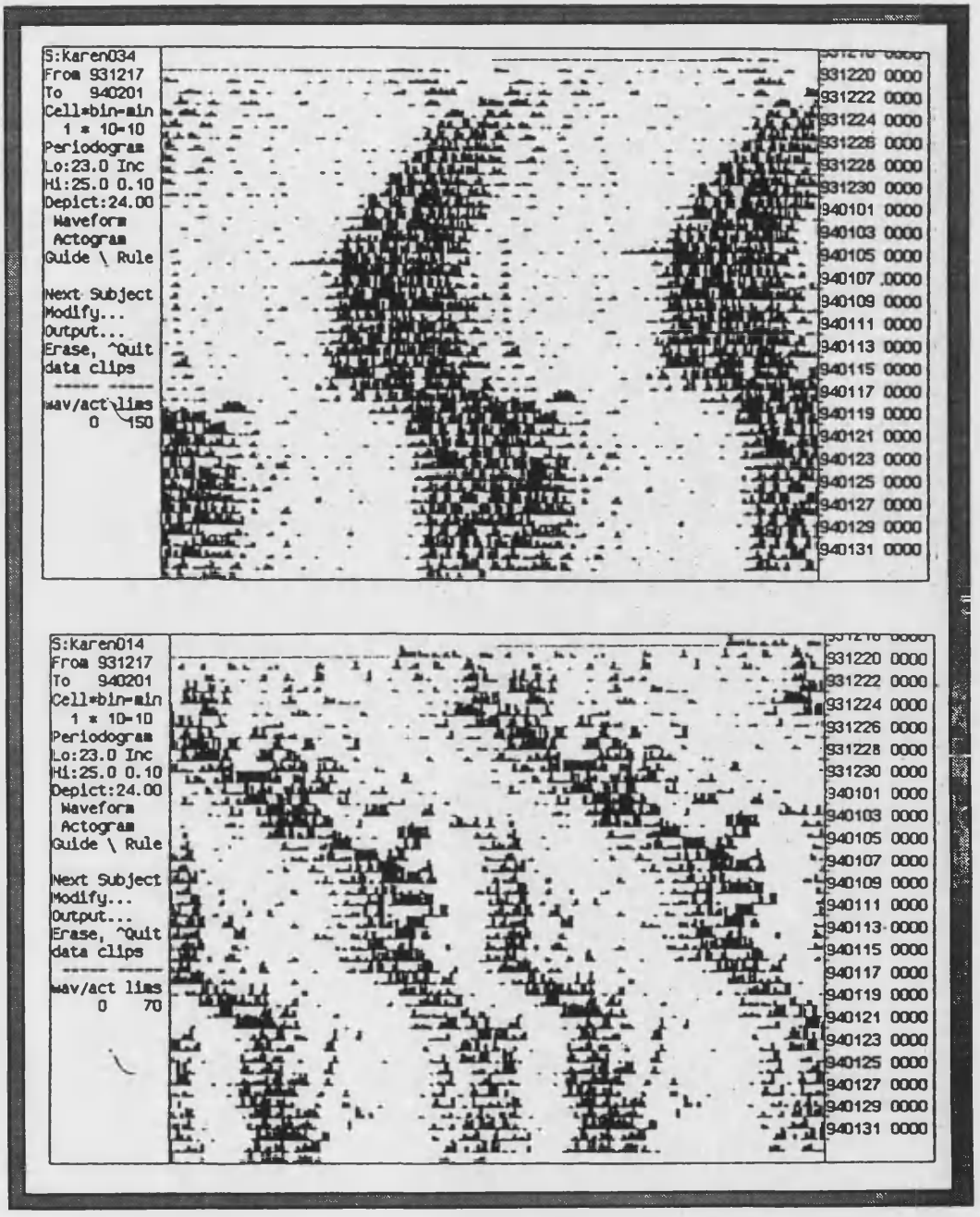


Figure 86. Proportion of animals showing a non-continuous activity band following a shift of the light/dark. A non-continuous band is one in which activity is stopped for > 1 hour.
CMS ■ control □

Actograms from control and CMS-treated animals showing continous and non-continuous α band activity, respectively, are illustrated in Figure 87.

Figure 87. Actograms from animals in experiment CMS 7

CONTROL:
continuous α band



CMS-TREATED:
non-continuous α band

DISCUSSION

Before the effects of CMS on entrained locomotor activity rhythms could be determined, the effectiveness of exposure to the stress regime had to be assessed. Whilst CMS exposure resulted in a significant decrease in body weight gain and in locomotor activity in a novel environment, CMS-treated animals did not exhibit a decreased sucrose preference. However, based on the results of the previous experiments in this study, the decreased body weight gain and locomotor activity were deemed to be sufficient criteria to forward the animals to circadian analysis.

Prior exposure to the CMS regime significantly affected reentrainment of the circadian locomotor activity rhythm to phase shifts of the LD cycle in a number of ways. Firstly, CMS-treated animals took significantly longer to reentrain to an advance of the LD cycle than control animals. In order to achieve this advance, approximately half of the CMS animals delayed, rather than advanced their rhythm. This proportion is significant when compared with the control group in which all animals, except one, advanced the activity rhythm to the advanced LD cycle. Secondly, following both an advance and a delay of the lighting cycle, more CMS animals than control animals displayed a non-continuous activity band. Finally, the phase angle of entrainment following a phase delay of the LD cycle was significantly affected in CMS-treated animals when offset was taken as the phase marker. How can these differences be explained in terms of the circadian system?

If a zeitgeber that is entraining a circadian rhythm is abruptly phase-shifted by several hours, there will be several cycles of transients while the circadian rhythm resynchronizes with the new phase of the time cue (Pittendrigh & Daan, 1976a). The rate at which this resynchronization occurs is said to be a measure of the coupling strength between the zeitgeber and the circadian system (Moore-Ede et al., 1982). In the present experiment, it follows that since the zeitgeber was the same for control and CMS-treated animals (a 6 hour shift of the LD cycle), but their rate of reentrainment was different, the difference must lie within the circadian system of one of the groups of animals. As in the free-running CMS experiments, the two-oscillator theory of rhythm control proprosed to account for CMS-induced rhythm abnormalities may also go some way to explaining the effects of CMS on entrained rhythms.
If E and M correspond to the onset and offset of activity respectively, then due to the temporal spacing of the two oscillators, each oscillator would be at a slightly different phase when a single light pulse hits the coupled system. This would result in a different effect of the light pulse on the phases of E and M, and this effect has been demonstrated in the Golden hamster by Pittendrigh (1981). He showed that the advance portion of the PRC was shifted more than the delay portion. As a result of

this it was hypothesized that the M oscillator is able to advance immediately, but the E oscillator requires several cycles before it regains a steady-state phase relation with the M oscillator. This concept, that the E oscillator lacks the ability to phase advance, was further supported by the work of Honma et al., (1985). When measuring immediate phase shifts to a light pulse in the onset and offset of rat locomotor activity, the advance area was absent from the onset (E) PRC, but present in the offset (M) PRC. Furthermore, the delay area was evident in both onset and offset PRCs. This theory has recently been reiterated for the evening rise (E) and morning fall (M) in N-acetyl transferase activity in the rat pineal gland (Illnerova et al., 1989), and for the rise and fall of pineal melatonin levels, which are thought to be under the control of separate oscillators (Elliot & Tamarkin, 1994).

It is thought that under normal circumstances the two oscillators are coupled and a 'push-pull' system operates (Boulos & Rusak, 1982b). This means that an advanced M oscillator is able to push the E oscillator into a phase advance. This hypothesis is said to account for the phenomenon of α compression followed by decompression (Pittendrigh & Daan 1976e). Therefore, the immediate PRC is thought to represent the separate reaction of each oscillator to the given stimulus, whilst the steady-state PRC represents the culmination of a push-pull system between the two oscillators over several cycles. If the coupling force between the E and M oscillators changes (a theoretical suggestion which may acount for the effects of continuous light-induced splitting, and presently for CMS-induced rhythm changes), and the animal is exposed to a phase advance, the E oscillator is no longer linked to the M oscillator and this could result in a longer time taken to reentrain (i.e. an increased number of transient cycles before steady-state) as was the case in the present experiment. Furthermore, since both oscillators are thought to be able to delay independently, this may explain why there was no difference between the CMS and control groups in the time taken to reentrain to a phase delay. In addition, this hypothesis may also explain why almost half of the CMS-treated animals delayed their rhythm to a phase advance of the LD cycle - because a delay was an easier option for the oscillators when the coupling strength relied upon for an advance was weakened. Finally, a weakened coupling of the two parts of the activity band may be said to be visually represented by the larger number of CMS-treated animals which exhibited disruptions of this α band compared to controls.

Alternatively, it may be suggested that no difference between groups was seen following a phase delay, not because this direction of shift is easier to accomodate in an uncoupled E-M system, but simply because the effects of CMS had 'worn-off' by this time that the delay of the LD cycle was presented. This suggestion gains experimental support from experiments CMS2 and CMS4. In experiment CMS2, the CMS-induced deficits in sucrose preference, locomotor activity and body weight were

no longer present one week after stopping CMS treatment. In experiment CMS4, animals which displayed the abnormal W-shaped rhythm coalesced to a normal, single activity band, rhythm within 15 days of stopping the CMS regime. At the time of measuring reentrainment to a phase delay in CMS7 animals, CMS treatment had been stopped 25 days previously. The question of whether the reentrainment to a phase delay of the LD cycle was in fact measured when the effects of CMS had disappeared, could be answered by repeating the experiment and performing the delay shift before the advance shift.

Results from the measurement of phase angle of rentrainment may contradict the latter suggestion that the effects of CMS had disappeared by the time of delay shift. The phase angle of entrainment was found to be significantly different between the two groups when offset was used as the phase reference and following a delay shift only. It appears, therefore, that CMS-treatment still had an effect on the circadian system by this stage of the experiment. To explain this altered angle of reentrainment, one could return to the E and M oscillator theory. Although it is said that both oscillators possess the ability to phase delay (Pittendrigh & Daan, 1976e; Illnerova et al., 1989; Elliot & Tamarkin, 1994), Honma et al., (1985) pointed out that the presence of the delay area in the offset PRC, may not necessarily indicate the phase-delaying ability of the M oscillator. Honma's measurements were made in normal rats in which the E and M oscillators were supposedly coupled, and the apparent delay of the M oscillator could therefore have been due to it being pushed to delay by the E oscillator. This explanation is dependent on the immediate PRC being influenced by the interaction of the two oscillators, whereas it has been proposed to represent largely the direct effect of light on each (Pittendrigh & Daan, 1976e). An M oscillator which could not phase delay correctly without being linked to the E oscillator may explain the differences in angle of reentrainment in the present experiment.

Finally, leaving the E-M coupling issue aside, if CMS-treated animals possessed a longer free-running period than controls, this could instead explain observations such as the longer time taken to reentrain to a phase advance shift. A 6 hour phase shift for an animal with a tau of 25 hours would require a 7 hour advance of its rhythm for reentrainment. If this is compared to an animal with a shorter tau of 23 hours, only a 5 hour shift would be required for the latter to reentrain. This would explain not only the slowed reentrainment to a phase advance in CMS treated animals, but would also explain the tendency of CMS animals to delay their rhythm to a phase advance of the LD cycle. However, measurement of free-running period in experiments CMS 3 and CMS 4, showed that tau was *not* significantly different from control in the first days after putting the animals into wheels.

In conclusion, the present observations of the effects of CMS on the entrained circadian locomotor activity rhythm support the observations made in free-running conditions, namely that this animal model of depression is associated with disruptions in circadian rhythmicity. In addition, these results add weight to the hypothesis that locomotor activity is controlled by two separate oscillators, and it is proposed that the coupling of these oscillators is changed by exposure to the CMS regime.

GENERAL CONCLUSION

In these studies, a CMS animal model of depression was successfully adapted to yield rats displaying behaviours representative of clinical depression, following only four weeks exposure to the regime. The results can be summarised as follows:
Of the four separate groups of animals exposed to the revised (CMS2) regime, CMS-treated animals in all four groups displayed a reduced weight gain and a decreased locomotor activity in a novel environment compared to controls. A decreased preference for sucrose over water, said to be indicative of a defective reward system and representative of anhedonia, was seen in only two out of these four groups. Circadian rhythm analysis revealed CMS treatment to significantly affect both the free-running and 12:12 hour light/dark entrained locomotor activity rhythm. Approximately one third of all animals exposed to the regime displayed alterations of the circadian locomotor activity rhythm in free-running, constant dim red light conditions. These alterations were found to be independent of the handling and lighting conditions used in the regime. In a separate experiment, animals were housed under 12:12 hour light/dark conditions. Following an advance shift of the light dark cycle by 6 hours, CMS-treated animals were found to take significantly more days to reentrain to the new light/dark cycle. In addition, approximately half of all CMS-treated animals were found to delay their activity rhythm in order to reentrain to the advanced light/dark. This response was significant when compared with the control group in which all, except one animal, advanced the activity rhythm to an advanced lighting cycle. Finally, CMS-treated animals displayed an altered phase angle in the offset of entrainment following a 6 hour phase delay of the light/dark cycle.
Many of the observed rhythm disturbances can be explained in terms of the established two oscillator model theory - a theory which has traditionally grown from data on splitting of activity rhythms. Whereas splitting has previously been shown to be caused by agents such as continuous lighting or hormone administration, this is the first time that it has been demonstrated to be caused by exposure to a depression model. Importantly, this study is one of only a small number to date which have researched the field of depression models and circadian rhythms, and more specifically, this study is novel in that it is the first account of circadian rhythm disturbances due to exposure to the CMS model of depression.

This set of experiments should therefore be viewed as a foundation for novel work in the area of circadian rhythm research. Suggested targets for future study include testing the ability of antidepressant drugs to prevent or reverse the rhythm changes seen in CMS-treated animals. In addition. the concurrent monitoring of a separate circadian rhythm, such as the body temperature rhythm, would reveal whether the effects of CMS on the circadian system were widespread, or whether they were confined to the desynchronisation of the locomotor activity rhythm.

In conclusion, this experimental combination is a novel one, and one which will provide a unique means of testing the hypothesis that abnormality of circadian rhythms is causally implicated in the aetiology of depression, and that the clinical efficacy of antidepressant drugs is dependant on their ability to restore rhythm normality.

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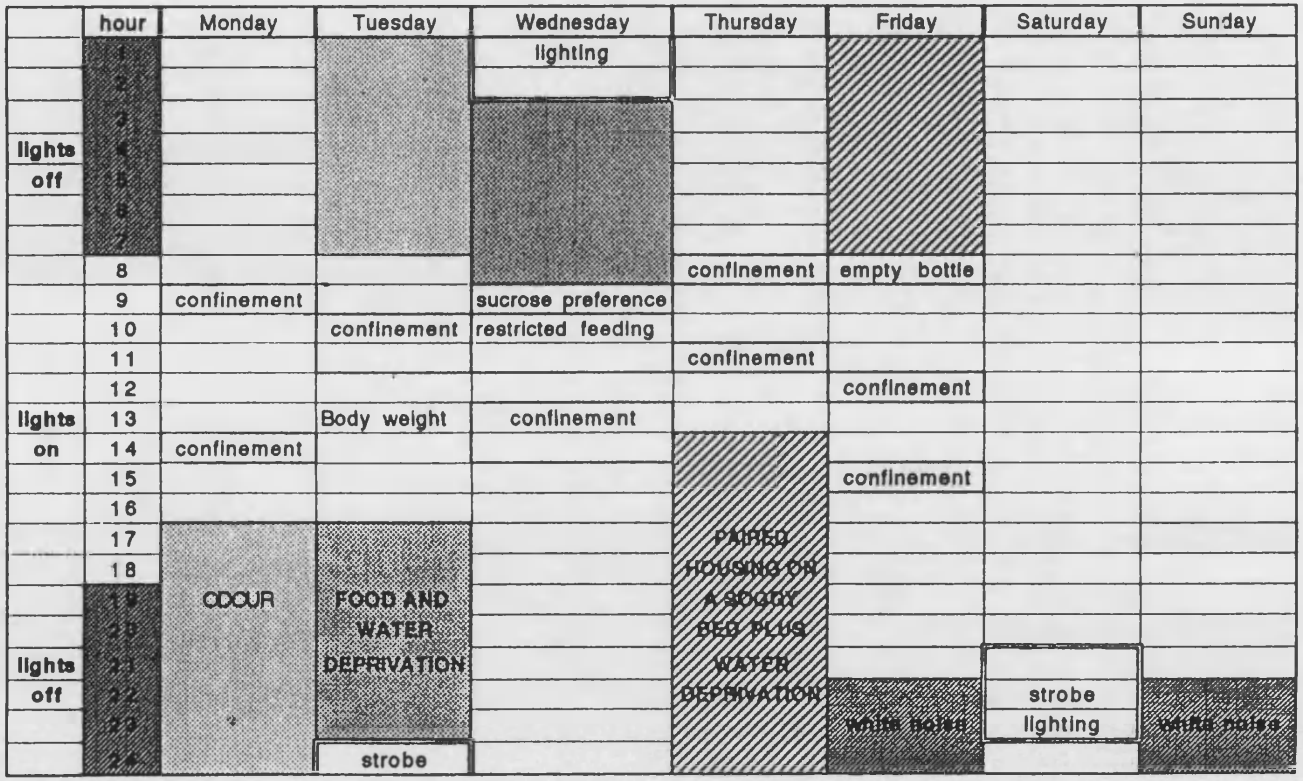
APPENDIX 1



Timetable for the application of chronic mild stress to CMS 1 animals. No more than three stressors were applied at any one time. For details of confinement, strobe lighting and white noise see Chapter V. The 'odour' was a set of 3-4 air-freshners attached to the cage battery. Restricted feeding on Wednesday morning involved scattering 4 pellets of rat chow on the cage floor, so that the animals could feed and not become satiated. On Thursday, the 'soggy bed' was the ordinary sawdust bedding soaked in sufficient water to be damp to the touch. Animals were paired with the *same* partner each week. The timetable was adapted from that of Willner *et al.*, (1987) with the addition of confinement periods from Moreau et al., (1993a; 1993b).

APPENDIX 2

	hour	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
	1							
	2							
	3							
lights	4							
off	5							
	6							
	7							
	8							
	9			sucrose preference				
	10		cage change					
	11							
	12							
lights	13		Body weight					
on	14							
	15							
	16							
	17							
	18							
	19		FOOD AND WATER DEPRIVATION					
	20							
lights	21							
off	22							
	23							
	24							

Timetable for the treatment of control-group animals. Control animals were weighed, and received a clean cage once per week. Animals were also food and water deprived beginning on Tuesday evening to enable sucrose preference testing once per week.

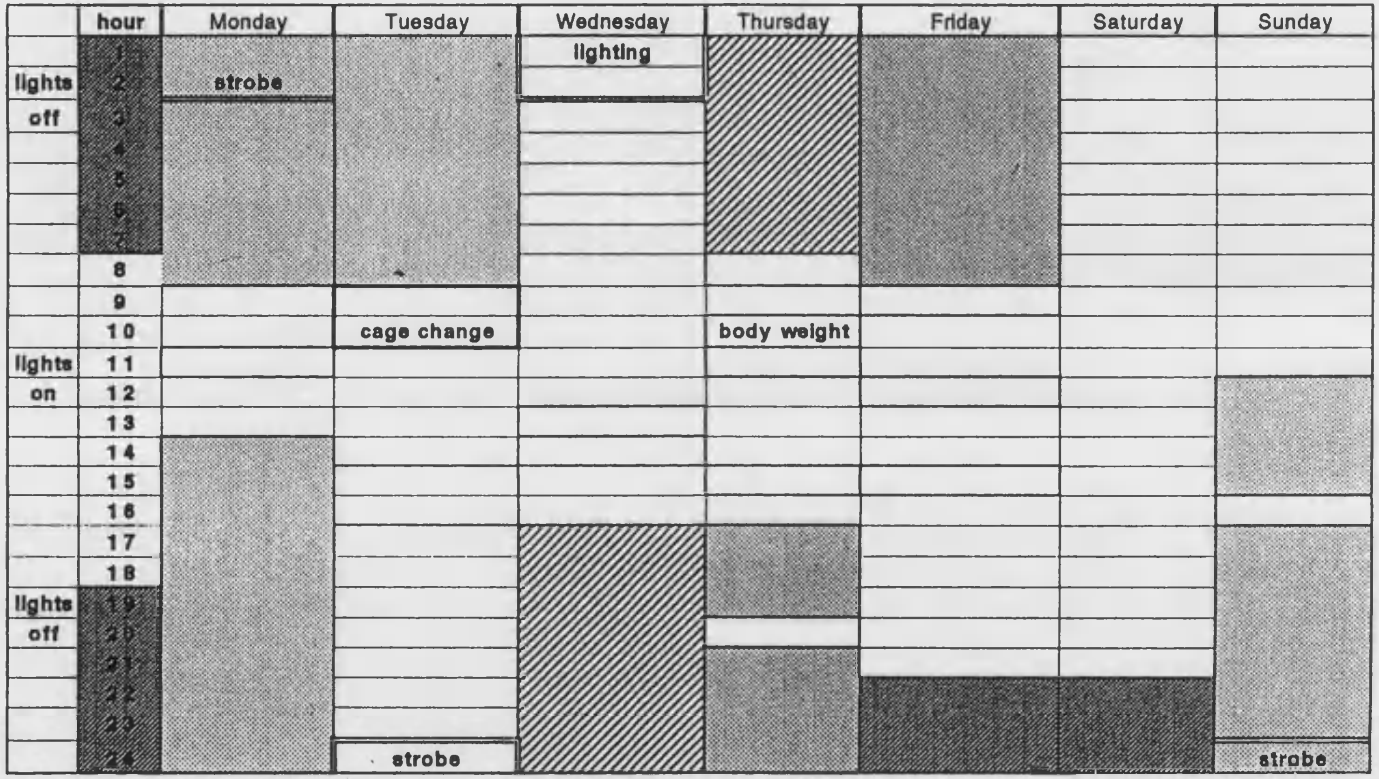
APPENDIX 3

	hour	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
	1	strobe		lighting				
	2							
lights	3	grouped housing on a soggy bed			water deprivation			
off	4							
	5							
	6							
	7							
	8	confinement			empty bottle			
	9		food scatter			sucrose preference		
	10		plus cage change		body weight			
	11	confinement			confinement			
lights	12					confinement		grouped housing on a soggy bed
on	13			confinement				
	14	food and water deprivation plus odour						
	15		confinement		confinement	confinement		
	16							
	17			water deprivation	grouped housing plus food and water deprivation			
	18							
	19							
lights	20							
off	21							
	22					white noise	white noise	
	23							
	24		strobe					strobe

Revised timetable for application of chronic mild stress to CMS2 animals. No more than three stressors were applied at any one time. Details of the confinement, strobe lighting and white noise procedures are the same as for experiment CMS 1 animals. The number of paired housings was increased to 2 per week and animals were paired with a *different* partner on each occasion. Sucrose preference testing took place at the *end* of each stress week.

Control-group animals were not subjected to any procedure other than a weekly cage change, body weight measurement Thursday evening food and water deprivation plus subsequent Friday morning sucrose preference testing.

APPENDIX 4



Timetable for the application of strobe lighting-only aspects of chronic mild stress. The shaded areas represent times at which the animal would have received other aspects of the CMS procedure, but instead received no treatment. Strobe-lighting was the same as for the revised CMS2 procedure, i.e. 300 flashes/minute continuously for 3 hours over Sunday and Tuesday night. Animals were weighed and received a clean cage once per week.

APPENDIX 5

	hour	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
	1							
	2							
lights	3							
off	4							
	5							
	6							
	7							
	8							
	9				body weight			
	10		cage change		HANDLING			
	11	HANDLING			HANDLING			
lights	12					HANDLING		
on	13			HANDLING				
	14			HANDLING				
	15		HANDLING		HANDLING	HANDLING		
	16							HANDLING
	17							
	18							
	19							
lights	20				HANDLING			
off	21							
	22							
	23							
	24							

Timetable for the application of handling-only aspects of chronic mild stress. The shaded areas represent times at which the animal would have received other aspects of the CMS procedure, but instead received no treatment. Handling occurred for 30 seconds with each animal at the beginning and at the end of the period allocated to handling. Animals were weighed and received a clean cage once per week.

APPENDIX 6

	hour	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
	1							
lights	2							
off	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10		cage change		body weight			
lights	11							
on	12							
	13							
	14							
	15							
	16							
	17							
	18							
lights	19							
off	20							
	21							
	22							
	23							
	24							

Timetable for control animals in Experiment CMS6. The shaded areas represent times at which the animal would have received other aspects of the CMS procedure, but instead received no treatment. Control animals were weighed and received a clean cage once per week. These animals were not exposed to food/water deprivation, sucrose preference testing, strobe-lighting, handling or paired housing.