THE ROLE OF CHOLINERGIC NEUROTRANSMISSION IN THE FUNCTIONING OF THE SCN.

By

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Then God commanded, 'Let there be light' - and light appeared. God was pleased with what He saw. Then He separated the light from the darkness, and He named the light 'Day' and the darkness 'Night'. Evening passed and morning came – that was the first day.
Genesis 1: 3-5. Good News Bible.

SUMMARY

Circadian physiology research has applications in many scientific and medical disciplines. There is a circadian basis to many sleep disorders and several psychiatric states have circadian dysfunction as prominent symptoms. The efficacy of pharmacological agents can vary depending on the time of day they are administered and many medical disciplines such as oncology are applying chronopharmacological theories to therapies. Adjustments in lifestyle required by shiftworkers could ultimately be made easier with increased understanding of circadian function, in addition to quicker adaptation to new time zones by trans-meridian travellers. Thus, an increased understanding of the complex neural anatomy involved in the generation and maintenance of circadian rhythms and the mediation of timing by light, may ultimately have benefits across many areas.

This project focussed specifically on the role of acetylcholine in the circadian timing system of mammals, using the rat as an animal model. Acetylcholine is not widely considered to have a dominant role in the system, however the neurotransmitter and it's receptors are found in the suprachiasmatic nucleus (SCN), the site of the biological pacemaker in mammals. Cholinergic agonists have been shown to produce phase shifts in various species, however the pathway mediating these cholinergic effects has not been characterised.

The first part of the project showed that the nicotinic agonist nicotine, caused dose-dependent and time-gated phase delays in the timing of melatonin excretion onset in normal randomly bred (RB) rats, in an identical pattern to light pulses. Further, the agonist caused the expression of c-FOS-like immunoreactivity in the cells of the ventrolateral SCN, again in a dose- and time-dependent fashion. The number of c-FOS-immunopositive cells in the SCN after nicotine administration was reduced by 65% with pre-treatment with the nicotinic antagonist, mecamylamine. The muscarinic agonist, oxotremorine, caused the expression of a very low number of c-FOS-immunopositive cells in the SCN only at the highest dose, however, it failed to induce phase delays in the melatonin rhythm. These data suggest that in rats, the cholinergic effects on the SCN are most likely mediated by a nicotinic receptor subtype and that the cholinergic projection from the basal forebrain to the SCN may be the regulator of these effects.

The second part of the project involved the development of a line of rat with increased sensitivity to cholinergic stimulation. A selective breeding program based on the thermic response to the cholinergic agonist oxotremorine produced the Sensitive to OXotremorine (SOX) rat line and the co-developed Resistant to OXotremorine (ROX) rat line. Circadian analysis of these animals showed inherent differences in the functioning of the SCN between the lines under entrained and constant dark conditions.

The melatonin excretion rate rhythm was used to monitor the timing of SCN output in all generations up to G9 and studies showed that from generation 3 the SOX animals had a significantly earlier onset time than ROX animals under entrained conditions. Further, under entrained conditions the SOX animals also showed a decreased phase angle difference of the temperature offset compared to ROX rats. Thus, under a cycling photoperiod the SCN of the SOX animals appeared to function quite differently. Long-term constant dark studies showed that the SOX animals exhibited significantly shorter free-running periods of the temperature rhythms than both ROX and RB animals. Thus, the increased cholinergic sensitivity of these animals was correlated with a decreased phase angle of melatonin and temperature rhythms under entrained conditions and a shorter free-running period under constant conditions. The altered SCN timing may be a result of an increased level of cholinergic stimulus to the SCN, possibly mediated by the minor afferents from the basal forebrain.

The project has shown that cholinergic effects on the SCN of rats may be mediated through a nicotinic receptor and action of a minor cholinergic projection from the basal forebrain region. Further, the circadian analyses of the SOX line of rat have shown that the endogenous period of circadian rhythms may at least in part be determined by cholinergic tone.

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Errata

Changes in the text are annotated (*) in the left hand column at the lines indicated below.

Chapter 1

pg 18, para 4, line 3 - **before** 'Rusak et al, 1992' **insert** 'Rea, 1989'

pg 25, para 1, line 3 - **replace** 'Consequently' **with** 'Subsequently'

pg 25, para 1, line 12 - **replace** 'rat' **with** 'rate'

pg 34, para 3, line 10 - **replace** 'as light' **with** 'to light'

Chapter 2

pg 52, para 5, line 1 - **after** 'ANOVA' **insert** '(alpha = 0.05)'

pg 55, para 4, line 4 - **after** (30 minutes) **add** 'Differences in onsets between groups were analysed using a student's t-test (alpha = 0.05).

- **after** 'package' **add** 'and differences in period length between the groups analysed by student's t-test (alpha = 0.05).

Chapter 4

pg 95, para 1, line 9 - **replace** 'Across the entire.....' **with** 't-test indicated that across the entire breeding program, SOX parents produced 11.1 pups/litter, a significantly ($p < 0.05$)....'

pg 95, para 2 , line 9 - **add to end of sentence** '($p < 0.01$, Bonferroni correction)'

Chapter 5

Figure 5.2 legend - **replace** 'lights of at 1900h (ZT12)' **with** 'lights remaining off from 1900 - 0700h (ZT12 - 24).

Figure 5.3 - black line should extend from ZT12 - ZT24

Chapter 6

Table 6.1 legend - **after** 'significant difference' **add** ' ($p < 0.05$).

Figure 6.1 and 6.5 - **add** 'x-axis represents double plotted 48 hour period, y-xis represents consecutive days.

Chapter 7

Table 7.1 legend - **after** 'the delays' **add** 'in hours'

RHT	retino-hypothalamic tract
RIA	radioimmunoassay
ROX	Resistant to oxotremorine
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SNAT	serotonin N-acetyltransferase
SOX	Sensitive to oxotremorine
VIP	vasoactive intestinal peptide
vLGN	ventral lateral geniculate nucleus
ZT	zeitgeber time

Chapter 1

Literature review

1.1 RHYTHMS IN LIFE

The proper physiological functioning of many organisms requires a delicate balance between the internal homeostasis and the external environment. Changes in environmental variables such as temperature and lighting can directly affect the behavioural and physiological states of the organism. At the same time, an ability to synchronise behaviour and physiology with these environmental factors allows an organism to anticipate changes in temperature, lighting, food availability and predator activity. The predictable, rhythmic fluctuation in the light/dark cycle is perhaps the most important factor involved in maintaining life in it's delicate balance.

The relevance of the 24 hour light/dark cycle to the physiology of living organisms was first demonstrated by De Mairan who noted that the opening and closing of plant leaves corresponded directly with the fluctuation in light. Various experiments with these plants under constant conditions (24 hour dark or light), led to the proposal of an endogenous pacemaker or clock which continued to oscillate at about 24 hours in the absence of external light cues (DeMairan, 1729). This oscillation was named a circadian (circa-about, diem-day) rhythm as the period of the internal oscillator was approximately one day length.

Higher organisms maintained under conditions devoid of daily light cues demonstrate an endogenous rhythm in many physiological variables with a period slightly less than or greater than 24 hours. For example, rhythms of sleep and body temperature oscillate with a period slightly greater than 24 hours in humans kept in bunkers without external time cues (Winget *et al,* 1975). Similarly, hamsters, mice and rats maintain running activity rhythms under constant conditions which are often different from 24 hours (for review see Hoffman, 1965)). If the biological clock does not receive information about the external light/dark cycle it reverts to it's internal period and the animal is said to be 'free-running'. The only proven link in mammals between the circadian timing system and the external light cycle is the eyes.

1.2 LIGHT AND RHYTHMS

Photic information is received by the retinal ganglion cells and transferred centrally via the optic nerves. The demonstration of a direct tract from the retina which terminated in the

anterior portion of the hypothalamus had a major impact on the field of circadian rhythm research. Cholera toxin and horseradish peroxidase which are easily detectable in sections were injected into the retina and shown to be transported to the paired Suprachiasmatic Nuclei (SCN), with the heaviest label in the ventral portion. From this, the SCN in the anterior hypothalamus were shown to be the major target of the retino-hypothalamic tract (RHT) in the rat (Moore and Eichler, 1972). The hypothesis that such a projection existed in all mammals was tested using tracing studies and in all species examined, a bilateral projection from the retina to the SCN (caudal and ventral region) was demonstrated (Moore, 1973; Millhouse, 1977). In addition, retrograde transport with horseradish peroxidase from the SCN caused bilateral labelling of retinal ganglion cells (Pickard, 1982). The importance of this neural connection in the circadian timing system was shown by experiments in which interruption of the RHT-SCN complex in entrained hamsters (animals in a cycling light/dark photoperiod) produced free-running activity rhythms under the same lighting conditions (Johnson *et al,* 1988). It was concluded that this tract was essential in the entrainment and control of SCN rhythmicity.

Evidence for the SCN as a self-sustaining clock came from a wide range of experiments directly manipulating the nuclei. One of the most important of these studies involved the use of hypothalamic islands, one which contained the SCN and a second used as a control area. Enucleated animals underwent surgery to isolate the areas and electrodes measuring spontaneous neural activity recorded a continuous circadian rhythm of firing only in the island that contained the SCN (Inouye and Kawamura, 1979). This demonstration clearly showed that the SCN maintained rhythmicity in the absence of retinal inputs and independently of neural afferents from other brain regions, and was the only centre in the brain to do so.

Lesion studies demonstrated that bilateral destruction of the SCN resulted in the loss of maintenance of circadian rhythms including the sleep/wake cycle, temperature, food-intake and pineal melatonin (Rusak, 1979). Electrical stimulation of the SCN altered the phase of circadian rhythms in activity and feeding (Rusak and Groos, 1982). Ablation of the SCN in rodents resulted in the loss of circadian rhythms in brain neurotransmitter receptor levels (Kafka *et al*, 1985), the rhythm of corticosterone production (Moore and Eichler, 1972), and behaviour and locomotor activity rhythms (Stephan and Zucker, 1972). Further, explants of tissue containing the SCN were reported to maintain a circadian rhythm of electrical activity (Green and Gillette, 1982) and vasopressin release (Earnest and Sladek, 1987). The most

compelling evidence for the role of the SCN as an endogenous pacemaker came from the transplantation of neural tissue into previously SCN lesioned hamsters. Circadian rhythms could be restored in animals with transplants of fetal nervous tissue containing the SCN (Drucker-Colin *et al*, 1984; Sawaki, 1984). Further, when neural tissue from a mutant strain of hamster having an inherently short circadian rhythm of locomotor activity was transplanted into SCN ablated animals, the restored rhythms exhibited the period of the donor animal (Ralph *et al*, 1990). This confirmed that the period of circadian rhythms was directly controlled by the cells of the SCN. Questions still remain however, about the mechanisms through which the SCN receives and processes light information to control the timing of circadian rhythms in mammals.

The retino-hypothalamic tract is involved in the entrainment process and is the only direct neural pathway from the eyes to the SCN, however it is not the sole afferent. A second important SCN afferent from the retina via the intergeniculate leaflet in the lateral geniculate nucleus is called the retino-geniculo-hypothalamic tract (R-GHT). In rats, the dorsal lateral geniculate nucleus (dLGN) receives a retinal projection as does the contralateral ventral lateral geniculate nucleus (vLGN). A separate projection from the retina was also identified to a group of cells between the dLGN and vLGN called the intergeniculate leaflet (IGL) (Hickey and Spear, 1976). A neural connection between the SCN and IGL was conclusively demonstrated via retrograde tracing studies which injected tracer into the SCN. In the same report a neural pathway from the vLGN was also confirmed using similar retrograde tracing techniques (Harrington *et al*, 1987).

Studies involving interruption of the R-GHT by transection of the optic tracts (Daan and Pittendrigh, 1976), lesions of the vLGN (Dunn *et al*, 1977) or removal of all hypothalamic afferents except the RHT (Rusak, 1977), showed that the SCN did not require an intact R-GHT to respond to light input. Bilateral lesions of the LGN caused no change in the ability of the SCN to entrain (Dark and Asdourian, 1975; Zucker *et al*, 1976), however lesioned hamsters re-entrained more slowly to a new cycle after a 12-hour phase shift (Dark and Asdourian, 1975; Zucker *et al*, 1976; Rusak, 1977; Kafka *et al*, 1985,). Also, IGL destruction affected the magnitude of light pulse-induced phase shifts (Harrington and Rusak, 1986; Pickard *et al*, 1987). Thus, the phase of the entrained rhythm and the rate of phase shift following a photoperiod change appear to be modulated by the R-GHT.

A third afferent from the retina to the SCN projects via the raphe nuclei. Evidence for a projection from the retina to the lateral raphe nuclei in the rat was recently reported by Shen and Semba (1994), using retrograde and anterograde transport of tracers injected into the dorsal raphe nuclei which resulted in staining of ganglion cells in the retina. Further, a direct projection was demonstrated from the dorsal raphe nuclei to the SCN in addition to the retina-raphe connections (Kawano *et al*, 1996). This retino-raphe-SCN tract has been described in the rat (Shen and Semba, 1994; Kawano *et al*, 1996) and cat (Foote *et al*, 1978), however, the existence of this tract in the hamster was not verified by cholera-HRP or cholera toxin immunohistochemical studies (Morin, 1994). This suggests that the circadian timing systems of different species may involve different afferent pathways and neurotransmitters in the control of rhythmicity.

Additional afferents have been reported but very little information is available about their role in SCN rhythmicity. Afferents project from regions such as the septal nuclei and ventral subiculum, anterior hypothalamic area, midbrain periqueductal gray, retrochiasmatic area, paraventricular thalamic nucleus and paraventricular nucleus (Pickard, 1982) as well as forebrain and brainstem regions (Bina *et al*, 1993). Limited research attention has been given to these minor projections, however they should not be discounted completely, as they likely have some influence on circadian rhythmicity.

The RHT, R-GHT and retino-raphe-SCN tracts form the major afferents to the suprachiasmatic nuclei described to date (Figure 1.1). Although transection and lesion studies have supplied the field with invaluable information about the neural connections of the SCN, these processes are highly invasive and can affect other neural tracts. It is important that the dominant neurotransmitters of these neural pathways are characterised so that antagonists may be used in place of the more invasive techniques in the study of the mechanism of circadian rhythmicity. Further, information about the specific neurochemistry underlying SCN function will make it possible to control circadian rhythms, having widespread benefits in the shiftwork and travel industries in addition to improving symptoms involved in circadian based sleep disorders and some psychiatric illnesses.

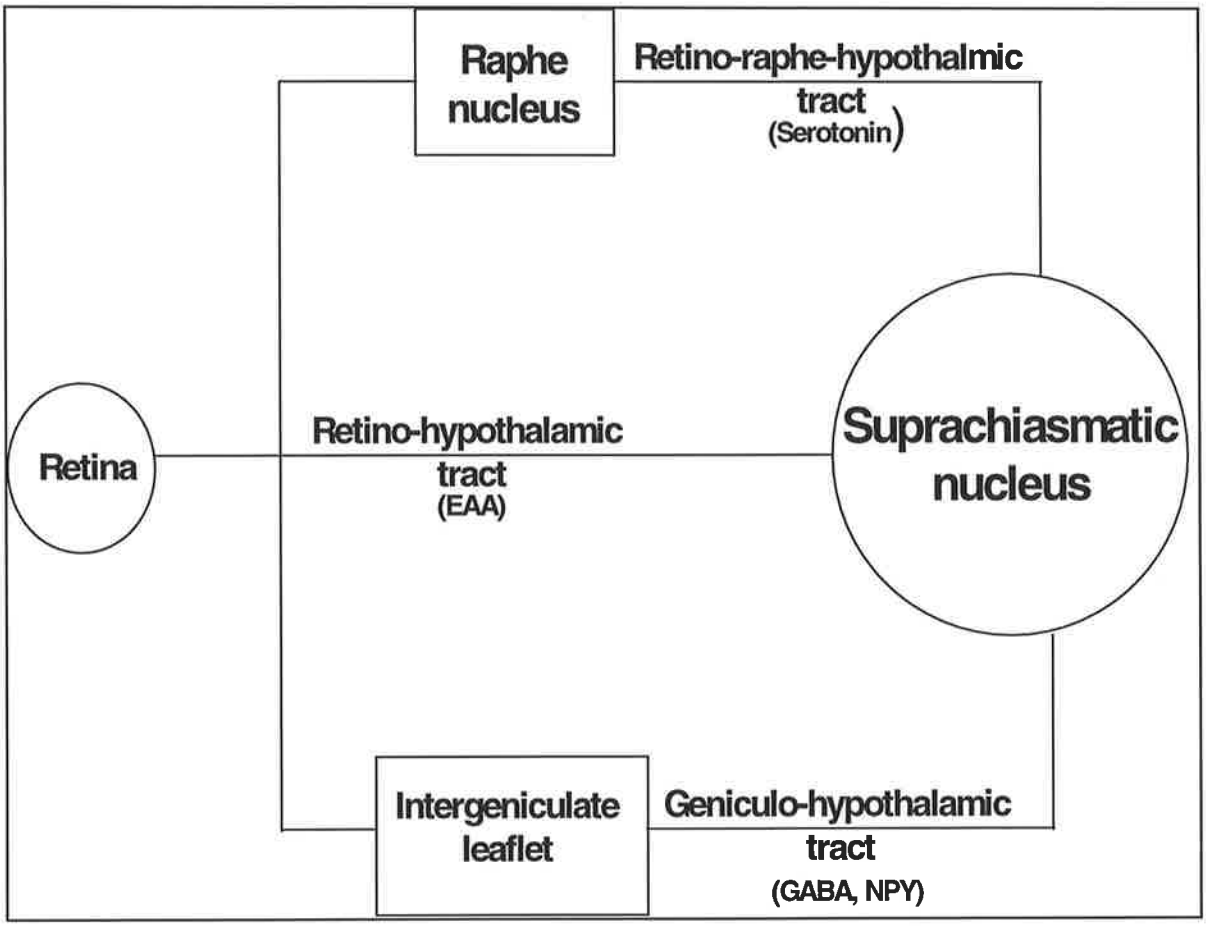

Figure 1.1

Schematic picture of the neural connections of the circadian timing system of mammals. Tracts are thought to project directly from the retina, via the intergeniculate leaflet and via the raphe nucleus. In parentheses under each tract are the major neurotransmitters. EAA - excitatory amino acids, GABA - gamma-aminobutyric acid, NPY - neuropeptide Y.

1.3 CELLULAR BASIS OF CIRCADIAN RHYTHMICITY

The processes by which the SCN maintains an endogenous rhythm and the way light information transferred via SCN afferents in turn entrains that rhythm, are becoming more clearly defined at the cellular level. The induction of immediate-early genes (IEG) occurs as an early, measurable response of cells to discrete stimuli. Such genes code for transcription factors that in turn induce expression of late-response genes ultimately resulting in the response to the initial stimulus. Several IEG are expressed in the SCN as a response to light pulses including *c-fos*, *egr-1* (NGF-1A), and *jun-B* (Aronin and Schwartz, 1991; Kornhauser *et al*, 1992). Expression of IEG in the SCN is induced in mammalian neurons by various stimuli including light (Earnest *et al*, 1990) which makes these genes, particularly *c-fos*, a useful marker for early action in the SCN.

c-fos is a part of all cellular systems and is induced through reception of extracellular stimuli and second messenger systems (Aronin and Schwartz, 1991). *c-fos* mRNA is translated into c-FOS protein which is translocated into the nucleus to form a heterodimer with a member of the *jun* family of oncogenes via the leucine zipper. This dimer then binds to the Activator-Protein-1 (AP-1) site on the promoter of a late response gene triggering the next event in the cascade. AP-1 sites vary in composition and this variation may represent differing target genes, the identity of which remain largely unknown. It is thought that light stimuli may replace or cause alterations in factors already bound at the AP-1 site (Aronin and Schwartz, 1991) resulting in changes in phase and/or period of biological rhythms.

Light stimulation has been shown to cause increased expression of *c-fos* in both the retina and the ventrolateral region of the SCN (Rea, 1989; Earnest *et al*, 1990; Kornhauser *et al*, 1990). In addition to *c-fos*, levels of other immediate early genes including NGFI-A, NGFI-B, *c-jun*, *junB* and *junD* were increased in the SCN in response to light (Rusak *et al*, 1992). Expression of *c-fos* mRNA has a circadian rhythm in constant light (Earnest *et al*, 1992), but exhibits no discernible rhythmicity under constant darkness conditions (Schwartz *et al*, 1995), suggesting that *c-fos* is most likely a clock-controlled gene.

It is apparent that *c-fos* can be expressed only when the SCN is responsive to light, as light exposure that caused phase shifts in free-running activity also resulted in significant *c-fos* expression in the SCN (Rusak *et al*, 1990; Rea, 1992; Rusak *et al*, 1992). This suggests that

the endogenous oscillator was shifted by the light pulses and that *c-fos* induction is one event in the cascade of events involved in light-induced phase shifts, although it may not be an integral part. A non-photic stimulus which caused a phase shift in the circadian rhythm of locomotor activity and in the SCN itself (manifested in other rhythms) did not cause expression of *c-fos* (Mead *et al*, 1992). Rea *et al* (1993b) also reported that *c-fos* expression was neither necessary nor sufficient to induce phase changes in SCN timing. Thus, the induction of the gene can occur without a concurrent phase shift and vice versa, indicating that *c-fos* is not an integral part of the entrainment and phase-shifting mechanisms. *c-fos* therefore provides an excellent marker of neural activity in the SCN and will assist greatly in the search for the mechanisms of light mediated effects on circadian rhythmicity.

1.4 MOLECULAR BASIS OF CIRCADIAN RHYTHMICITY

1.4.1 Drosophila and *per*

Circadian rhythms are generated at the cellular level and research into the mechanisms underlying rhythmicity has predominantly utilised *Drosophila melanogaster*. The ease with which mutants can be created and the extensive knowledge of the genetic makeup of this species have made it a useful tool for analysing the cellular basis of timekeeping. The analysis began with the irradiation of a large batch of flies to determine whether subsequent offspring exhibited altered circadian rhythms (Konopka and Benzer, 1971). One of the simplest rhythms to monitor in the flies is that of eclosion (the emergence of adult flies from the pupa) and within a population it has a very consistent period of about 24 hours. Three 'period' mutants were isolated from this screen, with offspring having either no apparent eclosion rhythm (per^O), a long period of around 29h (per^L), or a short emergence period of around 19h (per^S) (Konopka and Benzer, 1971). Analysis of eclosion, which occurs once in the lifetime of a fly was not by itself sufficient to demonstrate that these lines were rhythm mutants so locomotor activity in constant darkness was also examined. The circadian rhythm of locomotor activity of individual flies from each mutant line had a period almost identical to that of the eclosion rhythm (Konopka and Benzer, 1971). Mapping of the mutations by recombination experiments indicated that all three mutants represented alterations in the same gene. Standard recombination and cytogenic techniques established the location of the period gene or *'per'* on

the *Drosophila* X chromosome and fate-mapping of the site of action of the clock mutation indicated the focus was in the *Drosophila* brain (Konopka *et al*, 1983).

The search began then for the specific transcripts mapping within the *per* locus region, which were altered by chromosomal alterations that disrupted circadian behaviour. Chromosomal rearrangements that altered *per* locus function were localised using cloned DNA and each affected a 7.1kb interval encoding a 4.5kb poly(A)$^+$RNA (Bargiello and Young, 1984). Functional evidence for the fragment as the *per* transcript came after this sequence was transformed into the genome of an arrhythmic per^O fly and the circadian rhythms of locomotor activity and eclosion were restored (Bargiello *et al*, 1984).

This 4.5-kb transcript was studied further through the development of seven lines of *Drosophila* all displaying different periodicities. Molecular and behavioural studies demonstrated an inverse correlation between period length and abundance of PER protein. The per^S and per^l mutations each led to single amino acid substitutions and per^O produced a truncated, apparently functionless protein (Baylies *et al*, 1987). The authors suggested that changes in *per* transcript (abundance or activity) were responsible for changes in period length. per^l mutants produce a hypoactive PER protein and per^S mutants produce a hyperactive protein (Bargiello *et al*, 1984; Baylies *et al*, 1987). The importance of the role of PER protein levels in period length was examined further through experiments showing that mutations around the original per^S mutation also conferred short period phenotypes. Those flies were shown to have the same levels of mRNA as wild types suggesting again that period changes were linked specifically to protein levels (Baylies *et al*, 1992). The precise mechanisms by which this gene and it's protein product controlled circadian rhythms however were still to be elucidated. Early hypotheses had *per* labelled as (1) a proteoglycan, based on a unique threonine-glycine repeat sequence (Yu *et al*, 1987) (subsequently shown to be unnecessary for circadian function), and (2) a modulator of gap junction communication between cells (Bargiello *et al*, 1987). These ideas have been surpassed with work showing fluctuation in PER levels over 24 hours.

Immunoreactive PER protein was found to oscillate in the adult visual system of the fly with intense staining at night, and this fluctuation persisted in constant darkness. RNA assayed from fly heads indicated that both the long and short period mutants had fluctuations in RNA

levels in LD and only the long mutant in DD and that *per*RNA levels were high during the subjective day. The authors suggested the existence of a negative feedback loop, with PER protein regulating its own mRNA levels (Hardin *et al*, 1990).

The feedback loop is reported to act in the following way: *per* mRNA rises during the late portion of the light phase, peaking after dark between ZT13-15 (where ZT0 is lights on). *per* mRNA transcribes PER protein with a lag in accumulation so that the protein peak occurs between ZT21-24. PER protein in turn affects *per* transcription resulting in low levels at the end of the dark period. PER protein breaks down allowing mRNA to build up again at the end of the light phase (Hardin *et al*, 1992). Due to the fact that there is no detectable lag due to post-transcriptional processing it is thought that circadian fluctuation in *per* mRNA levels is transcriptionally controlled. In addition, the PER protein has similar properties to other known transcription factors (Hardin *et al,* 1992). Thus, mutations may affect the ability of the gene to properly respond to PER protein levels, resulting in period, amplitude and phase changes of the circadian oscillation.

The role of *per* in clock function was defined further by the demonstration of a phase shift in locomotor activity in transgenic flies having a heat-inducible copy of PER. When subjected to temperature pulses the phase of the locomotor activity rhythm of these flies was permanently shifted (Edery *et al,* 1994). Thus, a change in the level of PER protein was solely responsible for the altered rhythmicity.

1.4.2 Drosophila and *tim*

For further elucidation of the precise mechanism of *per* in controlling circadian rhythms, screening experiments for other clock mutants were conducted. A second mutant in the fly was found and was called *timeless* (*tim*) due to its apparent total lack of rhythmicity. Flies having alterations in this gene displayed constant activity as well as an arrhythmic eclosion pattern in both LD and DD. The *tim* gene was isolated by positional cloning and chromosome walks to chromosome 2 and is the only reported *Drosophila* clock mutation to map to this interval (Sehgal *et al,* 1994). *per* mRNA levels were examined in *tim* flies and showed a decreased level and no obvious rhythm in fluctuation, circadian or non-circadian (Sehgal *et al,* 1994). This was the first indication that these genes interact within the system. PER protein levels were also studied in *tim* flies and it was shown that nuclear staining for the protein was not present in *tim* flies at any time point (Vosshall *et al,* 1994). This was apparently due to a

blockade by the *tim* mutation of PER nuclear localisation, perhaps as a result of PER containing sequences that somehow inhibited PER nuclear localisation in the absence of *tim*. *tim* RNA oscillated with a circadian rhythm (Sehgal *et al,* 1995) with a similar amplitude and phase to *per* (Sehgal *et al,* 1994). In wild-type and per^S flies, *tim* RNA and *per* RNA oscillated with almost identical phase and period (Sehgal *et al,* 1995), suggesting that PER protein had some role in the cycling of *tim* RNA.

tim RNA and TIM protein cycle in an autoregulatory feedback loop similar to the *per* system (Sehgal *et al,* 1995). Further, it is apparent that they are each responsible for the regulation of the other. It has been shown that a segment of TIM binds directly to a section of the PER dimerisation domain, PAS, and that per^l protein exhibits a defect in binding to TIM (Gekakis *et al,* 1995). As it has been suggested that post-transcriptional mechanisms contribute to the regulation of the PER protein (Zeng *et al,* 1984) it follows that these mechanisms may be disrupted in the *tim* flies.

To date no evidence exists to indicate that the *per* system is affected by light, however recently it was shown that TIM protein is degraded by light stimuli. TIM is not detectable in the nuclei at ZT1 whereas PER remains elevated for several hours after lights on. In addition, a light pulse at ZT19 (when TIM levels are at their peak) caused a total reduction of TIM protein levels by 70 minutes after the pulse, an effect not seen on PER levels (Hunter-Ensor *et al,* 1996). The levels of TIM and PER mRNA however, were not affected by light at this time indicating an alteration to the protein at a post-transcriptional level. This information supplies more pieces for the puzzle of exactly how PER and TIM interact to produce the circadian signal to the animal. It is proposed that at CT12 RNA levels are high and TIM levels begin to rise allowing accumulation of PER. Nuclear entry of the heterodimers of PER and TIM occurs around CT18 and they remain in the nucleus for the next 6 hours. By CT0 the majority of TIM has disappeared but PER is still evident. A light pulse in the early night will cause a reduction in TIM levels and delay the entry of the heterodimer into the nucleus and subsequently the circadian rhythm. A pulse in the second half of the night will reduce TIM levels to those seen at CT0 and advance the phase of the molecular and behavioural rhythms (Hunter-Ensor *et al,* 1996). This exciting work supplies evidence at the molecular level for the mechanisms underlying phase shifts in circadian rhythms.

1.4.3 Hamster and *tau*.

An important mammalian rhythm mutant was discovered by chance in a shipment of hamsters to the Menaker laboratory. The '*tau*' mutant hamster line was established from a single male with a short locomotor activity period (22 hours compared with the normal 24.1 hours) (Ralph and Menaker, 1988). After breeding with 3 normal females, the F1 progeny were examined to see if the trait was inheritable. Fifty percent of the offspring exhibited a short period and 50% a normal period of activity. The authors suggested that the animals with short period (Ts) were heterozygous for the trait and normals (Tn) were wild type. Offspring from subsequent Ts x Ts crosses produced 3 distinct groups of animal. Tn, exhibiting 24h rhythms, Ts, exhibiting 22h rhythms and Tss, exhibiting a 20h period of locomotor activity. Further crosses led to the assumption that a mutation had occurred in a single autosomal locus called *tau* (Ralph and Menaker, 1988). Using Tss animals with locomotor rhythms close to 20h, Davies and Mason (1994) showed that the SCN neurons of these animals also expressed a 20h firing rate rhythm *in vitro*.

Protein differences between wild type and *tau* hamsters were studied using gel electrophoresis on brain sections containing the SCN. Two sets of proteins were found that differed between the lines. P33*tau* was found in all gels from wild type and heterozygous animals but not in homozygous hamsters. P32*tau* was also markedly different between the wild and homozygote animals (Joy *et al,* 1992). These proteins may lead to the site of the *tau* mutation in the hamster, and provide an indication of the action of *tau*, if any in the maintenance of circadian rhythms.

The *tau* mutant hamster has a similar phase response curve to light pulses as wild type animals with a non-responsive zone during subjective day, delays in early evening and advances in late night. The average delays were similar between the two groups, however the *tau* mutants advanced significantly further than the wild types. The authors suggested that the relative duration of the light-responsive portion of the PRC is the same for each group with respect to circadian hours, but that the *tau* mutants experienced 24 circadian hours of 50 minutes duration each rather than a deletion of 4h of subjective day (Grosse *et al,* 1995). Induction of the *c-fos* gene in the SCN by light pulses occurred at times similar to those which elicited phase shifts, although offset of the *c-fos* response occurred at the time of activity offset for each group (Grosse *et al,* 1995). Further study of the characteristics of these 'clock mutants' will undoubtedly help unravel the mechanisms of circadian rhythmicity in mammals.

1.4.4 Mouse and *clk*

Currently the only other mammalian rhythm mutant is the clock mutant mouse. A mutagen (N-ethyl-N-nitrosouren, ENU) was applied to a group of male mice and after recovery of fertility breeding with normal females one of the subsequent 304 offspring was found to have an altered circadian period of locomotor activity (Vitaterna *et al,* 1994). The G1-25 animal produced a line of mouse with a lengthened period of free-running activity. Interestingly, in the homozygote animal, although the long period rhythm was maintained in constant dark for between 5 and 15 cycles, the animals became arrhythmic after this time. This is unlike both *tau* hamsters and *per* mutant *Drosophila*, which both maintain their altered periods. The gene at which this mutation appears to have its effect is named Clock (*clk*) and segregates as a single gene, mapping to chromosome 5 (Vitaterna *et al,* 1994). The clock mutation lengthens the circadian period by 1h in heterozygous animals and 4-5h in homozygous animals, although after 5-15 cycles in DD, homozygous mice become arrhythmic (Vitaterna *et al*, 1994). This gene is homologous to an area on the human chromosome 4. As far as *clk* has been studied, it appears to only affect the circadian system, as the line has no abnormal traits apart from the homozygotes having lower fertility (Vitaterna *et al,* 1994), and in this way it is quite similar to per^{l} in *Drosophila*. The *clock* gene in mice was cloned by the group which identified it (Antoch *et al,* 1997; King *et al*, 1997) and some hypothesise that this gene may be the equivalent in mammals of either the *timeless* gene in *Drosophila* or a third, as yet unidentified clock gene in *Drosophila* that regulates both PER and TIM transcription (Reppert and Weaver, 1997). Sequencing of the *clock* gene has identified further similarities between it and the *Drosophila* genes *per* and *tim*. The *clock* gene encodes a novel member of the basic-helix-loop-helix-PAS family of transcription factors (King *et al,* 1997). Recent research indicated that PER can interact with bHLH-PAS proteins and alter their ability to activate transcription (Huang *et al,* 1993). The cloning of the *per* gene in mammals has brought the classification of the specific function of the *clk* mutation closer.

1.4.5 Mammals and *per*

In groundbreaking and exciting research, Tei *et al* (1997) have identified human and mouse genes that contain sections highly homologous to the period gene of *Drosophila* containing the PAS domain. This report suggested that the mammalian *per* homologues may dimerize with other molecules (as in the *Drosophila* system) which may include the Clock or Tau proteins. The characterisation of the specific molecules that constitute the circadian machinery in mammals should now be imminent.

1.4.6 Rhythmicity in SCN neurons

Demonstration *in vivo* of a persistent circadian rhythm of multiple unit activity from an isolated island containing the SCN provided conclusive evidence that this brain centre maintained spontaneous rhythmic firing (Inouye and Kawamura, 1979). Consequently, *in vitro* studies using brief single-unit recordings showed that spontaneous neuronal firing rate rhythms were maintained in SCN neurons (Green and Gillette, 1982; Groos and Hendriks, 1982). Further, dissociated individual SCN cells were shown to maintain a circadian oscillation of vasopressin release for between 5-30 cycles (Murakami *et al,* 1991; Watanabe *et al,* 1993). Welsh *et al* (1995) cultured SCN cells on multielectrode plates containing an array of microelectrodes and recorded spontaneous action potentials from 50 individual cells. Circadian rhythms in firing rate were consistently observed for up to 7 weeks after dissociation of neurons from the SCN but not the hippocampus. Thus, individual cells of the SCN are each mini-biological clocks, maintaining a circadian rhythm of firing rat. The study also showed that the phases of circadian firing rhythm differed widely between cells and long-term recordings indicated the circadian periods also varied. Most interesting however, was the demonstration that after blockade of action potentials for two and a half days, firing rate re-emerged at the pre-treatment projection times. This suggested that neuronal firing was not an essential part of the clock mechanism of each SCN cell, as individual cells appeared to maintain rhythmicity under blockade.

The desynchronization of individual cells described in the Welsh report (Welsh *et al,* 1995) in terms of phase and period is in contrast with reports of synchronised vasopressin rhythms (Murakami *et al,* 1991; Watanabe *et al,* 1993). Differing culture conditions and/or experimental protocol may account for this discrepancy. Individual rhythms may be synchronised by melatonin feedback (Reppert and Schwartz, 1984; McArthur *et al,* 1991) or an as yet undefined factor produced by SCN cells (Welsh *et al,* 1995). The circadian rhythm established via cycling of clock genes and their transcripts is evident in each individual SCN cell, however it remains to be determined why cells from the same SCN are not synchronised in culture, as well as the mechanism of synchronisation of neuronal firing rate which produces the consistent circadian rhythm of output from the whole SCN.

1.5 NEUROTRANSMITTERS OF THE CIRCADIAN TIMING SYSTEM

The neurochemistry of the circadian timing system became increasingly detailed as further neuronal connections were identified between the brain centres involved. To date, up to 30 neuroactive substances have either been located in the SCN or have been shown to affect SCN rhythmicity (van den Pol and Tsujimoto, 1985). Accurate description of the exact role of each neurotransmitter and its interaction with other systems will ultimately lead to an understanding of the manner in which light information is transferred to the SCN and subsequently processed to modulate mammalian circadian rhythmicity. Investigations of the various neurotransmitter systems have been carried out using *in vitro* and *in vivo* studies in several species. In general terms the SCN is manipulated by the administration of light pulses or agonists/antagonists of the neurotransmitter under investigation, at different time points in the cycle. These agents are applied either peripherally, into the ventricles, directly into brain nuclei, or into culture systems containing the SCN and administration can be chronic or acute depending on the route and model of investigation. Changes in pacemaker output are then recorded via monitoring phase shifts in the rhythms under SCN control. The response of the nuclei to an external intervention such as a light pulse or drug administration depends on the time in the circadian cycle it is applied. For example, light exposure in the early evening may delay behavioural and hormonal rhythms, while the same treatment in the early morning advances the rhythm. By monitoring SCN function after intervention at different time points throughout the 24 hour period, a phase response curve (PRC) is generated. It is expected that dominant transmitters of light information in the circadian system will produce similar responses from the SCN as do light pulses, so the PRC for agonist administration will be similar to the PRC for light administration. In addition, acute and chronic administration of those agents should produce phase shifts in SCN output, evident in shifts in the phase of recordable rhythms such as temperature, locomotor activity, pineal melatonin, vasopressin release and neuronal firing, as do brief and sustained pulses of light. Many of the major neurotransmitters of the body have been investigated to examine their role in the circadian rhythm system and it is perhaps appropriate to begin a discussion of the neurochemistry of the system with the RHT.

1.5.1 Excitatory amino acids (EAA)

It is generally accepted that the excitatory amino acids are the major transmitters in the retino-hypothalamic tract and therefore the major mediators of light-induced effects on SCN function

across all species. *In vitro* and *in vivo* studies in various species have contributed to this theory.

Ultrastructural evidence that glutamate is a neurotransmitter of the RHT came from studies using rats and mice. Immunocytochemical techniques with polyclonal antibodies localised glutamate to retinal terminals of the RHT in the rat (De Vries *et al,* 1993). In another study, retinal terminals were identified with cholera toxin-horseradish peroxidase transported anterogradely from the retina. Sections were stained with anti-glutamate and the results indicated that labelling was densest on retinal terminals of the SCN of mice (Castel *et al,* 1993). Together with pharmacological and electrophysiological data this suggested that glutamate is a major transmitter of the RHT.

Early evidence from *in vitro* experiments using SCN slices showed that stimulation of the optic nerve caused a release of [^{3}H]glutamate and [^{3}H]aspartate in the SCN. Stimulation of the SCN itself also resulted in release of those two excitatory amino acids (EAA) as well as [^{3}H]GABA (Liou *et al,* 1986). Application of glutamate to *in vitro* cultured rat SCN cells stimulated 60% of the spontaneously firing neurons (Bos and Mirmiran, 1993) and in hypothalamic slices prepared from hamsters, 1mM glutamate caused an increase in discharge in most cells but not all (Meijer *et al,* 1993). Metabolic activity of cultured golden hamster SCN was stimulated by glutamate only at night (Tominaga *et al,* 1992b). In contrast, in rat hypothalamic slices, glutamate elicited an excitatory response dose-dependently with no day/night differences (Shirakawa and Moore, 1994). These results suggest that the EAA are the predominant transmitter of the RHT however their role in SCN function may vary between species.

N-methyl-D-aspartate (NMDA) receptors, one subclass of EAA receptors, were identified in the SCN of male Long Evans rats and Syrian hamsters via autoradiographs of sections incubated with the agonist MK-801 (Hartgraves and Fuchs, 1994). Further, *in situ* hybridisation histochemistry detected NMDA receptor mRNA in female and male Wistar rat SCN (Mikkelsen *et al,* 1993; Ishida *et al,* 1994). The evidence for a circadian rhythm in expression of these receptors is not conclusive. One group found no day/night differences in glutamate receptor (NMDA, AMPA and metabotropic glutamate) mRNA levels using *in situ* hybridisation in the Sprague-Dawley rat SCN (Gannon and Rea, 1994). Measuring two time points in 24 hours, Ishida *et al* (1994) found levels of the NMDA receptor subunit mRNA epsilom3 and gamma1 were higher during the light phase than the dark phase in Wistar rat

SCN. *In vitro* autoradiography of MK-801 binding sites in Long Evans rat and Syrian hamster SCN, failed to detect differences in levels as a result of photoperiod or time of day (Hartgraves and Fuchs, 1994). This discrepancy in results may be due to the use of different techniques or different animal models. Further clarification of day/night differences in EAA receptor densities is therefore required. Definition of the mechanisms of regulation of neurotransmitter components by light and/or the circadian clock would have important implications in the identification of the important signalling pathways in the circadian timing system.

Work with the immediate early gene, *c-fos,* has also provided information about the role of EAA in SCN function. Light pulses caused an increase in Fos-like immunoreactivity and *c-fos* mRNA in the SCN at night in the rat (Aronin *et al,* 1990) and hamster (Rea *et al,* 1991). Administration of NMDA was also shown to induce the expression of c-FOS protein in the SCN of hamsters at night (Ebling *et al,* 1991). EAA receptor antagonists have been shown to inhibit the photic induction of c-FOS in the hamster rostral SCN and ventrolateral portion of the caudal SCN, but failed to do so in the dorsolateral SCN (Abe *et al,* 1991). Another study showed a similar distribution of inhibition of light-induced Fos-lir by the NMDA antagonists CPP (3(2-carboxypiperazin-4-yl)-propyl-phosphoric acid) and ketamine and the non-NMDA antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione). These antagonists prevented the appearance of Fos-lir in the rostral SCN and ventrolateral area but not dorsolateral area of the caudal SCN (Abe *et al,* 1992). MK801 (3mg/kg) injections partially suppressed the light-induced rise in *c-fos* expression in the rat retina (Gudehithlu *et al,* 1993) and application of MK801 and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an EAA antagonist, to the SCN via stereotaxically guided cannulae prevented light-induced *c-fos* expression in hamster SCN (Rea *et al,* 1993a). Taken together these results suggest a major role for the EAA in the processing of light information at the level of the SCN of hamsters and perhaps to a lesser degree in the rat as neither the agonist and antagonist actions are of the same magnitude in that species.

The role of the SCN in the control of reproductive function through photoperiod changes is well known (Rusak and Morin, 1976; Turek and Campbell, 1979; Eskes, 1984). MK801 (10mg/kg) administered before a weekly light pulse to hamsters in short days, blocked the stimulatory effect of the light pulses on gonadal function (Colwell *et al,* 1991b). One of the few studies involving direct application of NMDA showed that hamsters in short days were stimulated in a manner similar to light pulses (Urbanski and Ojeda, 1990). This may be an

indirect result of NMDA-induced release of luteinising hormone which would have a similar effect to light pulses on gonadal function.

The role of EAA in pineal melatonin output is another important area of this research. MK801 (3mg/kg) blocked the effect of a light pulse on melatonin receptor density in the SCN in previously pinealectomised rats (Gauer *et al,* 1994). Similarly, injection of MK801 (0.01-10mg/kg) to hamsters blocked the light-induced suppression of pineal melatonin in a dose-dependent manner. A 10mg/kg dose resulted in levels similar to those seen in animals maintained in darkness (Colwell *et al,* 1991a), however a large amount of variation is evident in this data, suggesting the effect was not uniform. Rowe and Kennaway (1996) reported that MK801 was unable to block the effect of a light pulse on the rhythm of melatonin metabolite excretion at doses of 0.1-3mg/kg in rats. They also showed no effect of NMDA on 6-sulphatoxymelatonin levels, acutely or on the following nights, even at a dose of 30mg/kg (Rowe and Kennaway, 1996). In the same paradigm Kennaway (1997) reported that the non-NMDA EAA antagonist DNQX failed to block the effects of a light pulse. These results raise doubts that EAA are of singular importance in the control of pineal rhythmicity in the rat and therefore question their dominant role in SCN function in this species.

Administration of MK801 (0.1-1.5mg/kg) to free-running mice reduced the magnitude of light-induced phase advances and delays of the circadian rhythm of locomotor activity in a dose-dependent manner. MK801 also reduced light-induced delays in retinally degenerate mice (Colwell *et al,* 1991a) and the same results were found with hamsters injected with MK801 (1.2-6mg/kg) (Colwell *et al,* 1990). At doses in the range of 0.1-0.2mg/kg locomotor activity is generally elevated in rats (Maj *et al,* 1991), but the high doses used in these experiments would be sufficient to cause an acute reduction in activity and these animals appear to have stopped running completely for up to 24 hours. Restraint or immobilisation can act as a weak zeitgeber and has been shown to block accelerated re-entrainment induced by benzodiazepines such as triazolam (Van Reeth *et al,* 1991). In addition, MK801 was not injected alone (without the light pulse) which would seem the appropriate control in such an experiment to determine any effect the drug itself had on the phase of the circadian rhythm. Injection of glutamate and aspartate into the SCN of hamsters caused phase shifts in the circadian rhythm of running activity similar to those caused by dark pulses in the light (Meijer *et al,* 1988b; De Vries and Meijer, 1991), an opposite PRC to that expected from a major neurotransmitter of the circadian timing system.

Electrical stimulation of the optic nerves and anterior optic chiasm at CT12-16 caused similar phase-dependent shifts in the hamster circadian rhythm of locomotor activity as those induced by light pulses (De Vries *et al,* 1994). Such stimulation is thought to cause a release of neurotransmitter at the RHT terminals in the SCN and as the electrically-induced phase shifts were blocked by MK-801 (6mg/kg), the authors suggested that the EAA mediate the effects of light on the circadian pacemaker (De Vries *et al,* 1994). Intraperitoneal administration of the antagonist does not however supply any information about the specific site of action and it is also difficult to say that stimulation of the optic nerves results in phase changes mediated solely through the RHT. Rowe and Kennaway (1996) have shown that in rats, MK801 (3 mg/kg) failed to block the phase delay in running onset following a 15 minute light pulse. They report a 60% decrease in wheel running in the 8 hours following injection and reduced running on the subsequent 2 nights. It was suggested therefore that drug-induced immobility does not impinge on light effects in rats as it appears to in hamsters and mice. In addition, the failure of the antagonist to block light effects on aMT.6S and the failure of the agonist NMDA to mimic light effects on melatonin production suggest that EAA are less important in light effects in this species than previously thought.

Thus, while the EAA are major transmitters of the retino-hypothalamic tract, the relative importance of this tract and therefore its dominant transmitter appears to vary according to species. The circadian timing system of the rat and hamster may have evolved separately to utilise alternative pathways for light information transfer and control of circadian rhythms. With this in mind it becomes obvious that results taken from one species cannot automatically be extrapolated to another.

1.5.2. Serotonin (5-HT)

The midbrain raphe nuclei project to the SCN, resulting in dense serotonergic innervation and contributing the serotonin found in the SCN (Van der Kar and Lorens, 1979). When this input was destroyed by infusion of the selective neurotoxin 5,7-dihydroxytryptamine (5,7-DHT), into each lateral ventricle, the circadian rhythm of corticosterone in rats was lost or severely dampened (Banky *et al,* 1986). The neurotoxin also advanced the phase of the locomotor activity rhythm in hamsters, an effect maintained for up to 60 days post treatment (Smale *et al,* 1990). However, there was no difference in the period of locomotor activity between the control and treated animals placed in constant dark, suggesting that the system destroyed by the toxin may not have been a vital part of the clock mechanism in the hamster (Morin and

Blanchard, 1991). The destruction of the serotonergic projection may have a modulatory effect rather than producing a fundamental change in pacemaker mechanism in hamsters.

Serotonin receptor subtypes have been identified in the SCN using various experimental methods. Prosser *et al* (1993) reported abundant 5-HT_{1a} and 5-HT_{1b}, very few 5-HT_{2a} and 5-HT_{2c} receptors and no 5-HT_3 receptors in the SCN of rats. In contrast, mRNA probes for 5-HT_{1a} receptor identified no hybridisation in the SCN (Pompeiano *et al,* 1992; Roca *et al*, 1993) and an antibody against the 5-HT_{1a} receptor similarly failed to identify labelling in the SCN (Kia *et al,* 1996). Further, reports indicated very few 5-HT_{1b} receptors (Roca *et al,* 1993) or 5-HT_{2a} receptors (Wright *et al,* 1995) were localised in the SCN. The Prosser study utilised ligand binding to identify the presence of receptors, whereas the studies outlined above used mRNA localisation, a more specific and reliable technique (Prosser *et al,* 1993). Recently, a new serotonin subclass was described in the SCN, the 5-HT_7 receptor (Lovenberg *et al,* 1993), however this isolated report has not been followed with *in situ* localisation of mRNA for the receptor in the SCN. Indeed, a subsequent report suggested that no 5-HT_7 mRNA was located in the SCN (Gustafson *et al,* 1996).

Although Prosser *et al* (1993) identified very few 5-HT_{2a} receptors in the SCN, several other reports present conflicting data. *In situ* hybridisation of 5-HT_{2c} mRNA located moderate levels of receptor in the SCN (Hoffman and Mezey, 1989) and Roca *et al* (1993) demonstrated intense hybridisation signal for the 5-HT_{2c} receptor mRNA. In addition, *in situ* hybridisation signal for the 5-HT_{2c} receptor mRNA was identified in the SCN by Pompeiano *et al* (1994) and Wright *et al* (1995). Our group has also recently identified 5-HT_{2c}-like immunoreactivity in the rat SCN (Moyer and Kennaway, unpublished). It is apparent then that the 5-HT_{2c} subtype may indeed be a dominant mediator of serotonergic effects in the SCN.

In vitro studies have contributed greatly to the characterisation of the role of serotonin in SCN function. Neuronal firing of rat SCN cells exhibits a diurnal cycle in response to iontophoresis of serotonin, and a circadian rhythm of recovery from suppression of firing by 5-HT (Mason, 1986). Further, microiontophoretic application of 5-HT to rat SCN cells caused 40% of cells to be suppressed dose-dependently. No visual SCN cells were 5-HT-sensitive but both visual and non-visual vLGN cells were (Meijer and Groos, 1988). This supplies further evidence that the afferent SCN input from the raphe nuclei and vLGN has some control of pacemaker function. It is still necessary to illustrate a circadian effect of serotonin and its agonists

inasmuch as the transmitter has a direct effect on clock function in a manner similar to light, if it is to be said to have a major role in the processing of light information. In addition, the specific receptor subtype(s) involved must be characterised.

Monitoring of SCN rhythmicity via locomotor activity has shown that 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin, a specific 5-HT_{1a} receptor agonist) caused phase advances in the circadian rhythm of locomotor activity of hamsters in constant light at CT8 (where CT12 was defined as onset of activity) (Tominaga *et al,* 1992a; Cutrera *et al,* 1994). Further, 8-OH-DPAT was reported to inhibit the phase-shifting effects of light on hamster free-running locomotor rhythms (Rea *et al,* 1994). The serotonergic antagonist NAN-190 was reported to potentiate the phase shifts induced by light pulses in hamsters (Rea *et al,* 1995). Edgar *et al* (1993) demonstrated dose-dependent phase advances in the circadian rhythm of locomotor activity of rats when quipazine was injected at CT6 and no significant shifts at CT18. By contrast, Kennaway *et al* (1996) showed significant and permanent delays in running activity onset of rats after quipazine administration at CT18 and no change at CT6. This discrepancy in studies both using the rat remains unexplained although the models do differ in relation to the circadian model being used ie. 2 weeks continuous darkness versus acute continuous dark. Thus in general, it appears that serotonergic agonists cause phase changes in rats and block light-induced shifts in hamsters. However, the large variation in study protocol makes comparison very difficult. In addition, those studies reporting phase-shifting effects after agonist administration around CT6 suggest that 5-HT is not mimicking the effects of light in a phase-dependent manner. Light pulses at that phase of the circadian cycle do not result in altered SCN rhythmicity. This remains unresolved, however, it is important to recognise that the serotonergic system of the hamster and rat may have very different roles in the control and timing of circadian rhythmicity.

Hypothalamic slice perifusion has been used widely as an *in vitro* method of studying 5-HT effects on SCN function. Prosser *et al* (1990) used this technique to demonstrate an altered phase of rat SCN output after treatment with the non-selective 5-HT agonist quipazine. The sensitivity was dose-dependent and it was suggested that the phase of the clock could be affected by the stimulation of 5-HT receptors in the SCN. A further study by this group reported that coapplication of tetrodotoxin and quipazine *in vitro* still produced phase-shifts suggesting that the effect was not at SCN efferents but 5-HT receptors on the clock itself (Prosser *et al,* 1992). Application of serotonin itself to rat hypothalamic slices containing the

SCN caused phase advances at CT7 but no phase changes during the subjective night (Medanic and Gillette, 1992). It is important to give results from such *in vitro* phase shifting studies the appropriate emphasis. The phase changes reported in such experiments are often of much greater magnitude than those reported using slave rhythms in whole animals. This fact alone suggests that the SCN isolated from the rest of the circadian timing machinery responds differently to manipulation. Another important functional difference between results gleaned from *in vitro* and *in vivo* studies is the direction of the phase changes and the phase-dependent nature of the changes. In the case of serotonin, phase shifts were reported after CT7 administration (Medanic and Gillette, 1992), a time when a light pulse given to an intact rat would have little or no phase-shifting effect on a slave rhythm. Further, work examining the firing rate rhythm of individual SCN cells has shown that the phase and periods of cells from the same animal can vary widely (Welsh *et al,* 1995), and thus definitive conclusions cannot be drawn with regard to the degree of phase change seen in these studies. However, important information can still be obtained from such techniques. Serotonin and its agonists do induce a response from the SCN implying a role for the transmitter in circadian rhythmicity.

Studies with the specific 5-HT_{1a} receptor agonist 8-OH-DPAT, showed an inhibitory effect on SCN neurons. Perfused coronal hypothalamic slices were exposed to 1 hour treatments with 8-OH-DPAT which reset the phase of the endogenous oscillator when administered during the subjective day (Shibata *et al,* 1992). Further, 8-OH-DPAT and to a lesser extent RV24969 (5-$HT_{1a/1b}$ selective agonist) mimicked the effect of quipazine *in vitro* during the subjective day and NAN-190 blocked quipazine-induced effects. NAN-190 acts at the 5-HT_{1a} receptor suggesting that this receptor subtype is the mediator of 5-HT effects on the clock (Prosser *et al,* 1993). Microiontophoretic application of 8-OH-DPAT and 5-HT to IGL and vLGN cells demonstrated a dose-dependent suppression of spontaneous activity in these cells and photic responses via a 5-HT_{1a}-like receptor (Ying *et al,* 1993). Initial work pointed towards serotonergic neurotransmission being modulated by the 5-HT_{1a} receptor but the evidence, though strong, was not conclusive. The discovery of a completely separate 5-HT receptor very similar to the 5-HT_{1a} subtype gave investigators further answers and questions.

Work with 5-HT agonists having varied specificity for the receptor subtypes has shed much light on the mediation of serotonergic effects on SCN function. Ritanserin which has high affinity for the 5-HT_7 subtype was shown to block 8-OH-DPAT induced phase changes, whereas the 5-HT_{1a} antagonist pindolol was less effective (Lovenberg *et al,* 1993). These

authors surmised that the 5-HT_7 receptor was mediating the phase shifts. As some doubt remains as to the existence of the 5-HT_7 receptor in the SCN, it is possible that the 5-HT_{2c} subtype was mediating the effects of ritanserin at the level of the SCN, as ritanserin has a high specificity for the 5-HT_{2c} receptor. Subsequent studies supported this view.

A significant phase delay of the 6-sulphatoxymelatonin rhythm occurred following quipazine (non-selective serotonergic agonist) administration at CT16 in the rat. This phase delay seen with doses of 1, 3 and 10mg/kg, persisted on the following 2 subjective nights indicating an effect on the circadian phase. The selective 5-HT_{1a} agonist 8-OH-DPAT also caused small but significant phase delays in the 6-sulphatoxymelatonin rhythm as did buspirone (Kennaway *et al,* 1996). Quipazine was also shown to be effective at inducing *c-fos* in the SCN of rats at ZT18 but not at ZT6, a pattern similar to light (Moyer *et al,* 1997). This effect was partially blocked with the serotonergic antagonist ritanserin (with higher affinity at the 5-HT_{2c} than the 5-HT_{2a} receptor) (Kennaway and Moyer in press). It was further demonstrated that significant phase delays of both 6-sulphatoxymelatonin and core body temperature rhythms occurred after DOI (0.5mg/kg s.c.) administration (a serotonergic agonist with high affinity for the 5-$HT_{2a/2c}$ receptor subtype) (Kennaway and Moyer in press). This agent produced phase delays of a similar magnitude to light pulses and also induced *c-fos* in the SCN to a comparable level. These investigators suggested that the serotonergic input to the SCN, most likely via the raphe nuclei, is important in light effects on the rat melatonin rhythm and that this is mediated by the 5-HT_{2c} receptor.

It is undisputed that the projection from the raphe nuclei to the SCN is predominantly serotonergic and serotonergic projections from the dorsal raphe nucleus extend to the IGL and vLGN and then continue through to the ventral SCN (Morin, 1994). Thus, a role for this transmitter within the system is expected. Clarification of the manner in which 5-HT interacts with other neuroactive substances both in the raphe and geniculate body and also in the SCN will elucidate further it's exact role in the system. As much of the early research into circadian rhythmicity was done using hamsters, the relative importance of 5-HT in the circadian timing system of all species may have been underplayed. The serotonergic projection from the retina to the SCN, present in rats but not in hamsters is the likely route of 5-HT-induced phase shifts in the rat. As serotonin can have opposing effects in the hamster as light, its specific role in that species remains to be elucidated.

1.5.3 Gamma-aminobutyric acid (GABA)

GABA is the main inhibitory amino acid in the brain, having widespread effects throughout the body. For this reason it is not plausible to do *in vivo* physiological studies to the same extent as with other neurotransmitters. Thus, investigations are limited mainly to *in vitro* histological and culture studies as well as *in vivo* microinjection.

In situ hybridisation and immunocytochemical techniques have demonstrated that the GABA synthetic enzyme, glutamate decarboxylase (GAD) mRNA and GABA are present in the SCN (Okamura *et al,* 1989), with a circadian rhythm of GABA levels (Aguilar-Roblero *et al,* 1993). GABA nerve endings present in the rat SCN are reputedly colocalized with serotonin (Bosler, 1989), and with vasoactive intestinal peptide (VIP) neurons (Francois-Bellan and Bosler, 1992). Other reports also stated that VIP neurons receive both 5-HT and GABA afferents (Bosler, 1989; Francois-Bellan and Bosler, 1992). Moore and Speh (1993) suggested that nearly all neurons in the IGL and SCN of rats are GABA-producing. However, an earlier study suggested GABA immunoreactive terminals accounted for half of the total synaptic input to the SCN (Decavel and van den Pol, 1990). Single-labelled immunocytochemistry demonstrated distribution of GABA containing cells in the SCN of sheep but a more dense distribution of cells containing GAD. In addition, VIP distribution was similar to that of GAD (Jansen *et al,* 1994). This was suggestive of a modulatory role for GABA neurons of VIP neuronal activity. In rat hypothalamic slices, stimulation of the optic tract failed to cause a release of GABA in the suprachiasmatic nucleus (Liou *et al,* 1986) which suggested a limited role for GABA in the direct transfer of light information to the SCN.

Binding sites for GABA and benzodiazepines are functionally coupled as part of the GABA/BZ receptor complex. GABA/BZ-like immunoreactivity was found in the raphe nuclei but not the SCN or IGL using immunocytochemistry and autoradiographic demonstration of benzodiazepine binding. The dorsal LGN and lateral area of the vLGN also contain GABA/BZ receptor staining (Michels *et al,* 1990). An apparent absence of receptors in the SCN may indicate a minor role for GABA in the functioning of the SCN.

GABA produced inhibitory responses in 65% of SCN cells in a rat hypothalamic slice, as did the benzodiazepines diazepam, flurazepam and zopiclone, muscimol (a $GABA_a$ agonist) and baclofen (a $GABA_b$ agonist). Bicuculline, a selective $GABA_a$ antagonist blocked the effects of muscimol suggesting predominance of the $GABA_a$ receptor (Liou *et al,* 1990). In hamster

brain slices GABA inhibited only 55% of SCN neurons with no day-night differences in the response (Liou *et al,* 1990). In post-natal rat SCN neurons both GABA and muscimol induced a large current response, blocked by bicuculline. However, diazepam elicited no response (Kawahara *et al,* 1993). Only the $GABA_b$ antagonist has been shown to inhibit light-induced Fos-like immunoreactivity in the SCN of hamsters (Colwell *et al,* 1993a). Ralph and Menaker (1989) suggested that the $GABA_a$/BZ and/or $GABA_b$ receptor subtypes were important in the SCN. It is evident from this information that conflicting views exist as to the receptor subtype mediating the role of GABA in SCN function.

Muscimol applied to neural cultures has given the most information about the role of the $GABA_a$ receptor. Under organ culture conditions, muscimol applied in the early to mid subjective day produced a large phase advance in the neural firing rhythm (Tominaga *et al,* 1994). This follows the pattern for changes caused by dark pulses in constant light, producing a PRC opposite to that for light pulses, not what is expected for a major neurotransmitter of light effects on SCN function, although similar to glutamate and aspartate treatment in the whole animal.

Little work has been done in the area of behavioural rhythms with GABAergic drugs with most studies concentrating on hamsters. Triazolam, a benzodiazepine, administered to hamsters produced phase changes again similar to those following dark pulses in constant light (Turek *et al,* 1986). Results from these animals apparently free-running at about the same period, suggest the effects of triazolam on the circadian clock do not occur via a direct route to the SCN from the retina. Smith *et al* (1990) showed that phase changes in the circadian rhythm of locomotor activity induced by muscimol and baclofen could be blocked by the antagonists bicuculline and picrotoxin. There appear to be some problems with the use of hamsters as models for the study of the circadian rhythm system via locomotor activity. Firstly there is a lot of variation in the free-running period between animals. The genetic differences between individual hamsters causing one to free-run with a period about 25 hours and another about 23 hours, make it difficult to compare their individual clock response to artificial manipulation. Secondly, these animals often exhibit bi-phasic rhythms which indicate again the vast genetic diversity within strains, a diversity so obvious that animals are being selected and bred according to their specific free-running periods to study clock function. Thirdly, as mentioned earlier, hamsters are highly susceptible to a stimulus of immobility or induced exercise, both of

which are often caused by injected agents. Each of these factors is evident in the representative animals in this particular paper and raises questions about the interpretation of these results.

As GABA is the transmitter found in the highest concentration in the SCN, it would not be prudent to discount it as a major player in the system. Along with serotonin it may be important in the R-GHT but as with the other transmitters the interaction with other systems involved in SCN function is still being debated.

1.5.4 Other transmitters

Several neuropeptides appear to have important actions in the circadian timing system along with the classical neurotransmitters. The distribution of vasoactive intestinal peptide (VIP)-immunoreactive neurons overlaps the retino-recipient area in the ventrolateral part of the SCN (Okamura *et al,* 1987) and VIP-immunoreactive perikarya are also densely distributed in this region (Card *et al,* 1981; Okamura *et al,* 1986). Also present in the ventrolateral region of the SCN are peptide histidine isoleucine (PHI) and gastrin releasing peptide (GRP) (Okamura *et al,* 1986) and arginine vasopressin immunoreactive cells were located to the mediodorsal region of the SCN (Vandesande *et al,* 1975). Somatostatin exhibits a circadian rhythm in concentration in constant darkness suggesting a fundamental role for this peptide in circadian rhythm control (Reppert *et al,* 1987). Levels of the other aforementioned peptides follow the light/dark cycle and have no sustained rhythm in constant conditions (Reppert *et al,* 1987). Neuropeptide Y is present in the neurons projecting from the IGL to the SCN and also appears to have some role in SCN function (Card and Moore, 1989).

1.6 ACETYLCHOLINE (ACh)

The enzyme that synthesises acetylcholine, choline acetyltransferase is present in the SCN (Brownstein *et al,* 1975), together with its product (van den Pol and Tsujimoto, 1985). Ocular enucleation experiments showed no endogenous circadian rhythm of ACh levels in the SCN (Murakami *et al,* 1984). However, when a light pulse was given to intact animals 2 hours after the dark period began, acetylcholine levels rose 3-fold in the SCN 30-60 minutes later (Murakami *et al,* 1984), suggesting a role for ACh in light information transfer to the SCN. More than 80% of SCN neurons *in vivo* were excited by iontophoretic application of ACh and

65% of neurons that responded to stimulation of the optic tract also responded to cholinergic stimulation (Nishino and Koizumi, 1977). Photically responsive cells of the SCN were also shown to be responsive to nicotinic stimulation and mecamylamine, a nicotinic antagonist, blocked this response. Mecamylamine also prevented any photic response from the active cells (Miller *et al,* 1987). Atropine, a muscarinic antagonist, also inhibited ACh-induced activity in SCN neurons (Morin, 1994). This information suggests that there may be a functional role for acetylcholine in light transfer to the SCN, however the class of receptor mediating the effect is not defined.

Fibres that are immunoreactive for choline acetyltransferase (ChAT), the biosynthetic enzyme for ACh are present in the SCN (Ichikawa and Hirata, 1986). However, as no cholinergic somata are found in the nuclei, the neurons that contribute these fibres must be located elsewhere in the brain. Kiss and Halasz (1996) demonstrated the existence of separate contacts between choline-acetyltransferase-immunopositive elements and SCN neurons, and retrograde tracing from the SCN has located cholinergic neurons in the basal forebrain (basal nuclear complex) and brainstem (mesopontine tegmentum). Stimulation of these cells caused the release of ACh in the SCN (Bina *et al,* 1993), indicating a possible route for cholinergic action at the level of the SCN. Further, it was shown that the basal forebrain area receives retinal terminals in hamsters and rats (Pickard, 1982) indicating ACh activity in the SCN may be photically controlled.

Studies have localised α-bungarotoxin (α-BTX) (nicotinic antagonist) binding sites in the brain of rodents. α-BTX binds specifically and non-reversibly to the nicotinic cholinergic receptor. Stereotaxic infusion of [^{3}H]α-BTX into the third ventricle of rats resulted in label dispersion throughout the brain, being most intense in the arcuate nucleus, the tuber cinereum, the ventral aspect of the lateral hypothalamic area dorsal to the optic tract, supraoptic nucleus, central amygdaloid nucleus, preoptic suprachiasmatic and suprachiasmatic nuclei (Silver and Billiar, 1976). Autoradiographic localisation of [^{125}I]α-BTX binding sites indicated high levels of nicotinic receptor in the rat hippocampus, supraoptic, suprachiasmatic and periventricular nuclei, ventral lateral geniculate and the mesencephalic dorsal tegmental nucleus (Segal *et al,* 1978). Binding assays of homogenised rat hypothalamic nuclei indicated high α-BTX binding sites in the suprachiasmatic nucleus, dorsomedial and preoptic suprachiasmatic nuclei (Block and Billiar, 1981). Further, autoradiographic localisation of [^{3}H]ACh and [^{3}H]nicotine

demonstrated intense labelling in the interpenduncular nucleus, thalamic nuclei, superior colliculus, and medial habenula. [^{3}H]α-BTX binding was highest in the rat cerebral cortex, hippocampus, inferior colliculus and hypothalamic nuclei including the suprachiasmatic, supraoptic, paraventricular and posterior nuclei (Clarke *et al,* 1985). Muscarinic receptors were also localised to the SCN using monoclonal antibodies to purified muscarinic and nicotinic cholinergic receptor proteins. A near total co-localisation of the two receptor classes was found in cholinoceptive neurons in the rat SCN (van der Zee *et al,* 1991).

As mentioned previously, expression of *c-fos* mRNA and c-FOS protein is stimulated by light in the SCN of rodents. Mecamylamine administration prior to a 5 minute light pulse reduced the Fos-like immunoreactivity in the SCN of hamsters, most pronounced in the dorso-medial region (Zhang *et al,* 1993). Mecamylamine appears to interact with the EAA system (O'Dell and Christensen, 1988) and the mechanism for the reported antagonism may be via blockade of EAA receptors in the RHT, specifically in the hamster. However, EAA antagonists do not prevent light-induced Fos-lir from appearing in the dorsomedial section of the SCN (Abe *et al,* 1991; Abe *et al,* 1992). Abe *et al* (1991) suggested that Fos-lir in that portion of the SCN was 'induced by a mechanism which is not antagonised by MK-801', nor indeed by the non-NMDA receptor antagonist DNQX (Abe *et al,* 1992). As the dorsomedial region does receive retinal inputs, albeit to a lesser extent than the ventrolateral region, it is possible that the cholinergic and EAA inputs to the SCN are spatially segregated in the hamster. This work has not been followed up in any other animal model. A single study examining the effect of cholinergic agonists on c-FOS expression in rats showed that mRNA expression was stimulated in the fetal SCN but not in the adult SCN (Clegg *et al,* 1995). The authors indicate however, that the treatment was applied around CT6-7, which is a time that the SCN is unresponsive to photic stimuli, and therefore if the agonist was mimicking the effects of light on c-FOS induction one would not expect Fos-lir at this time.

The role of cholinergic pathways in pineal gland rhythmicity has also been examined. The cholinergic agonist carbachol has been widely used along with the antagonists α-bungarotoxin and mecamylamine. In the pineal gland the activity of the enzyme serotonin N-acetyltransferase (SNAT) is higher during the dark period than during the day. Injection of carbachol near the SCN at CT15-16 (Zatz and Brownstein, 1979) or into the lateral ventricles at CT18 (Miller and Billiar, 1986) mimicked the effect of light on pineal SNAT by causing an immediate reduction in SNAT levels. This has not been reported to occur with any of the other

neurotransmitter agents. Further to this, the same group demonstrated that α-BTX blocked the effect of carbachol and of light itself on pineal SNAT when injected near the SCN (Zatz and Brownstein, 1981). However, Miller and Billiar (1986), were unable to replicate this blocking effect of α-BTX suggesting at the same time the possibility of differences in the bungarotoxin preparations.

Reproduction is a physiological function mediated by pineal gland function which is in turn controlled by the SCN (Reiter, 1980)). Hamsters in constant dark or short days undergo testicular regression which can be prevented with regular exposure to short light pulses near the middle of the dark period (Elliott, 1976). Carbachol administered intraventricularly, every 23.33 hours to hamsters in constant dark mimicked light and maintained gonad function, a similar stimulatory effect on the reproductive system as light (Earnest and Turek, 1985). These studies further suggest that acetylcholine has a role in the control of light/SCN interactions. Further direct physiological evidence comes from locomotor activity experiments.

The free-running rhythm of locomotor activity in rodents is readily monitored and provides an excellent marker for SCN function. The light-dark cycle mediates the timing of the rhythm in a manner similar to regular brief pulses of light (Pittendrigh, 1981). Applying cholinergic drugs to free-running animals to determine phase changes can test the hypothesis that ACh has a role in the mediation of light effects on SCN function. Intraventricular administration of carbachol to hamsters in the early subjective night caused a phase delay of running activity and a phase advance when administered in the late subjective night (Earnest and Turek, 1985). Carbachol administered intraventricularly produced dose-dependent phase-shifts in the circadian rhythm of locomotor activity of hamsters, higher doses producing larger phase shifts in both directions (Meijer *et al,* 1988a; Wee *et al,* 1992). In hamsters, mecamylamine, a nicotinic antagonist, administered 10 minutes before a 5 minute light pulse, blocked or reduced both the phase advances and phase delays caused by the pulse (Keefe *et al,* 1987). This information suggests that acetylcholine is involved in mediating effects of light on the SCN but as previously stated, results from hamster work can be confounded by the manner in which their SCN responds to non-photic stimuli such as immobilisation and induced running.

The rat and mouse while not having locomotor activity rhythms as defined as the hamster, are still viable models for such studies. In mice, intraventricular injection of carbachol mimicked

the effect of light on the circadian rhythm of wheel running (Zatz and Herkenham, 1981). Carbachol implants near the SCN caused shortening of the free-running rhythm of drinking in rats suggesting that a constant supply of carbachol acts in the same way as constant light (achieved by removal of eyelids) (Murakami *et al,* 1986). Results in rats and mice are similar to those found in hamsters in that they both indicate a role for acetylcholine in the mediation of light effects on rhythms controlled by the SCN. No other agent has been reported to mimic light at all times of the circadian cycle, as has carbachol. Carbachol has a phase response curve identical to light in the early and late subjective night (Colwell *et al,* 1993b), while many other neurotransmitters produce PRC similar to dark pulses in constant light. This fact alone suggests that acetylcholine is involved in SCN function at some level and deserves further clarification.

Suprachiasmatic nucleus explants exposed to nicotine exhibited phase advances in the timing of output at all times of the cycle (Trachsel *et al,* 1995). The authors suggested that the phase-independent advances caused by nicotine may be a change in period rather than a true phase-advance however, because they used only a portion of one circadian cycle post-treatment they could not make any definitive conclusions about period vs. phase changes. As outlined earlier results from *in vitro* studies such as this need to be examined prudently. Removed from the rest of the circadian timing system machinery and slowly dying (the preparations are supplied only with salts and glucose), the SCN must function quite differently and thus the degree and direction of phase shifts cannot be directly compared to those reported *in vivo*. It can be said however that nicotine did induce changes in the output of SCN neurons of the rat. Similarly, SCN neuronal activity was used as a marker to assess phase-shifting effects of other cholinergic agonists. Carbachol, acetylcholine and two specific M1 agonists caused large phase shifts when applied in the mid-subjective night and nicotine elicited smaller responses (Liu and Gillette, 1996). These studies supply evidence for action of cholinergic agents at the level of the SCN, an action suggested by the previous identification of muscarinic and nicotinic receptors in the SCN. However, as mentioned the significance of the phase shifts (magnitude and direction) is not fully defined.

Thus, while acetylcholine is not a transmitter of major SCN afferents a large body of evidence exists suggesting it plays an important role in the control of circadian rhythmicity. The mechanism of action of acetylcholine within the circadian timing system is yet to be explained.

1.7 TEMPERATURE REGULATION

The maintenance of internal body temperature despite challenge from the internal milieu (such as occurs from pyrogens) or the external environment is vital to the survival of the organism. The process by which this occurs, referred to as thermoregulation, is said to be an integrated and coordinated mobilisation of physiological processes used to actively defend the set point temperature of the body (Myers, 1984). The centre for the integration of temperature information and subsequent initiation of regulatory processes is thought to be in the medial preoptic area of the anterior hypothalamus (POAH) (Nakayama, 1985). This site receives input from peripheral receptors, both warm and cold and integrates this information with the temperature of the blood circulating through the hypothalamus. In this way a negative feedback loop is established incorporating peripheral receptors, neural networks, receptors in the POAH and effector organs to maintain the body's temperature.

The POAH contains thermosensitive neurons that alter their steady state discharge in response to local temperature changes. Cold-sensitive neurons decrease their firing rate when local temperature decreases and trigger pathways for heat production. Warm-sensitive neurons increase their firing rate in high temperatures and activate pathways for heat loss (Boulant, 1994). Even under synaptic blockade these neurons exhibit an inherent thermosensitivity (Nakayama, 1985). Stimulation of the POAH either electrically or thermally caused substantial disorganisation of thermoregulation with the amplitude of the circadian oscillation of body temperature exaggerated and selection of preferred ambient temperature being disrupted (Briese, 1989). Local warming and cooling of the POAH produced heat loss (panting, cutaneous vasodilatation), and heat production (shivering) processes respectively (Hori, 1991).

At the turn of the century it was reported that epinephrine, when infused into the cerebellum of the rabbit, could cause a transient and intense hyperthermia (Myers, 1974). What followed was the discovery that many other chemical substances could also alter the set point temperature of the body. In the 1930's, excess calcium was shown to produce a fall in body temperature when injected directly into the hypothalamus (Myers, 1984). In the subsequent period, theories have been put forward combining various neurotransmitters to explain the mechanism of thermoregulation. Serotonin, norepinephrine, dopamine and acetylcholine have all been shown to play important roles in the maintenance of thermal homeostasis. A brief summary of the effect of each neurotransmitter on body temperature and their interaction with

each other will endeavour to describe in part the mechanisms underlying control of body temperature.

Serotonin: Nerve terminals containing 5-HT present in the POAH were stimulated to release endogenous 5-HT when the animal was peripherally cooled (Myers, 1984). After exposure to 45°C, samples taken from the POAH of sheep showed a decrease in the levels of the 5-HT metabolite 5-HIAA, implying reduced levels of serotonin in the area (Alam and Mallick, 1991). Microinjection of 5-HT into the POAH produced hypothermia in a dose-dependent manner, and 5-HT antagonists applied to the POAH prevented temperature responses (Myers, 1984). Electrical stimulation of midbrain raphe nuclei resulting in elevated levels of 5-HT in the hypothalamus produced a decrease in metabolic heat production and hypothermia (Lin, 1984). However, different agonists of this transmitter elicited opposite changes in core body temperature. For example, 8-OH-DPAT (5-HT_{1a} agonist) caused hypothermia while DOI (5-HT_{2c} agonist) caused hyperthermia (Gordon, 1990; Linden *et al,* 1991). Therefore activation of 5-HT receptors in the hypothalamus can produce different effects dependent on the receptor subtype being activated and the anatomical origin of the cell bodies.

Norepinephrine: Adrenergic agonists injected directly into the hypothalamus caused increased metabolism and hyperthermia. Using a norepinephrine nerve depletor, lesion of noradrenergic neurons depressed metabolic heat production (Lin, 1984). Stimulation of adrenergic neurons has the reverse effect to serotonergic activation, ie-hypothermic effects.

Dopamine: Direct administration of dopamine to the hypothalamus produced hyperthermia in rats (Lin, 1984), suggesting that dopaminergic activation in the temperature regulation centres promotes decreased metabolic heat production.

Acetylcholine: It has been reported that both hyper- and hypothermic responses are mediated by cholinergic mechanisms (Lipton and Clark, 1986) and it is proposed that ACh plays a major linking role in the neurochemistry of thermoregulation. ACh is released in the mid- and caudal regions of the hypothalamus in response to monoamine application to the POAH. ACh in the hypothalamus is released at higher levels when an animal is exposed to cold air as opposed to warm (Myers, 1984). Core body temperature has been shown to fall after oxotremorine, pilocarpine, nicotine and acetylcholine (acetylcholine agonists) (Lipton and Clark, 1986), while cholinergic blocking agents led to a rise in body temperature (Lomax *et al,* 1969). Carbachol

also produced significant rises in body temperature, in a dose-dependent manner (Avery, 1970). Cholinergic pathways are also activated in response to serotonin or norepinephrine release due to activation of cold-sensitive or warm-sensitive neurons in the hypothalamus, to activate heat exchange processes. Thus, the cholinergic system plays an important role in the regulation of body temperature.

Temperature-sensitive neurons have recently been reported in the SCN (Derambure and Boulant, 1994). The circadian pacemaker situated in this nuclei controls various thermoregulatory events and it is also well known that temperature can affect certain circadian rhythms in lower vertebrates (Underwood and Calaban, 1987; Firth *et al,* 1991). SCN neurons show a circadian rhythm in their firing rates with highest firing occurring during the day (Derambure and Boulant, 1994). At this time however, very few temperature-sensitive neurons can be recorded in the SCN, the majority of these being reported active at night. The thermosensitive neurons in the SCN are thought to play a role in the circadian regulation of core body temperature and the SCN is considered by some as an integral part of the thermoregulatory system (Derambure and Boulant, 1994).

1.8 ESTABLISHING A UNIQUE ANIMAL MODEL

The study of the neurophysiology of circadian rhythms and their control by the SCN has generally been done by administering drugs to randomly bred animals. This type of experiment has provided invaluable information about the role of these neurotransmitters in the control of circadian rhythms but it has also encountered many problems. Routes of administration vary widely and despite all precautions being undertaken to confine the site of substance administration there is always a risk of the drug acting at areas other than those specified. In addition to this, different species of animal have been used in almost every area of circadian rhythm research, which makes comparison of experiments very difficult. The challenge remains to establish a model for rhythm research that escapes these limitations, and in particular for this project a model for the study of the cholinergic system in the control of circadian rhythms.

The reported link between SCN function, the cholinergic system and temperature regulation provides a basis for the establishment of this model. An animal that is sensitive to cholinergic stimulation will exhibit this physiologically through an exaggerated drop in core body temperature as a response to cholinergic treatment. The core body temperature response of a rat to a cholinergic agonist administered centrally varies from 0-3 degrees (Lipton and Clark, 1986). Those animals more sensitive to this stimulation will have a larger drop in temperature and this can be seen after a single injection. If this animal is "cholinergically upregulated" and this is manifested in a different temperature response it may also be that this upregulation is manifested in other systems under cholinergic control.

The Overstreet group applied this approach with much success in the 1980's. The project established a line of cholinergically upregulated animals as a model for the study of genetic sensitivity to the anticholinesterase di-isopropyl fluorophosphate (DFP) (Overstreet *et al,* 1979). Animals were challenged initially with DFP and those exhibiting the most extreme responses in a series of selection criteria were selected as the basis for the Flinders Sensitive Line (FSL) and those showing the least response for the Flinders Resistant Line (FRL). Each new generation was screened for the response to DFP and breeders were selected from this phenotyping program. The lines had diverged significantly in their temperature response (as one of the selection criteria) to this drug by generation 8. As the response to DFP treatment could be quite extreme in some cases (dose = LD40), phenotyping was later carried out with the muscarinic agonist oxotremorine which produced a safer but equally effective change in core body temperature (Daws *et al,* 1991b) and as such temperature change was used as the sole measure of sensitivity.

The FSL animals had a cholinergically upregulated system which was maintained between generations (Overstreet, 1991). The sensitive animals have higher levels of muscarinic receptors in the brain (Overstreet *et al,* 1984) in addition to having increased sensitivity to various cholinergic agents (Daws *et al,* 1991b). The circadian timing system of the FSL animals was studied through the temperature and drinking rhythms both of which were advanced compared to the FRL animals (Shiromani *et al,* 1991; Shiromani and Overstreet, 1994). Period differences of these rhythms between the lines suggested inherent differences in the functioning of the SCN. The FSL rat having a genetically altered cholinergic sensitivity therefore provided a unique opportunity to study the role of the cholinergic system in the functioning of the SCN. The line is no longer maintained in Australia and was therefore not

available for use in the current program. Further, as our emphasis was on circadian parameters the monitoring of rhythmicity was required from the outset of the breeding program under controlled lighting conditions.

1.9 SUMMARY

The suprachiasmatic nuclei of the anterior hypothalamus control the circadian fluctuation in behavioural and physiological parameters via a constant rhythm of neural output from the cells of the nuclei. Impinging upon this inherent rhythm are various environmental factors, the most important for mammals being light. Photic information is received at the retina and transferred directly and indirectly (through various brain centres), to the SCN to affect the phase and period of the inherent rhythm and in turn the physiological rhythms under clock-control. This process of information transfer involves several neurotransmitter systems that interact in an as yet undefined manner. The puzzle is slowly being filled in and appears to involve many of the major transmitters such as EAA, serotonin, GABA and acetylcholine. It was the aim of this project to further examine by various models of investigation, the role of the cholinergic system in the functioning of the SCN.

1.10 HYPOTHESIS

Cholinergic neurotransmission plays a major role in the processing of light information at the level of the suprachiasmatic nucleus.

This general hypothesis can be separated into two more specific hypotheses.

1) Cholinergic agents, applied peripherally will cause phase changes in melatonin rhythmicity in a similar time-gated manner as light. Further, agonists of ACh will elicit the induction of c-FOS-positive cells in the SCN of rats.

2) A selectively bred line of rat, having inherited cholinergic sensitivity will exhibit altered circadian rhythmicity under entrained and free-running conditions.

The specific aims of the project are:

1) To monitor the effects of 2 cholinergic agonists on the melatonin rhythm and the induction of the immediate early gene *c-fos* in the SCN of randomly bred rats.

2) To develop two lines of rat through a selective breeding program that exhibit inherited increased and decreased cholinergic sensitivity.

3) To monitor the development of the lines with respect to physiological parameters, breeding parameters and circadian parameters.

4) To determine any correlation between altered cholinergic sensitivity and SCN rhythmicity.

Chapter 2

General Methods

2.1 4-DAY TEST FOR MELATONIN RHYTHMICITY

Melatonin rhythmicity was monitored via assessment of the excretion of the major urinary metabolite of melatonin in the rat, 6-sulphatoxymelatonin (aMT.6S). The system used for urine collection is shown in Figure 2.1 and has been employed extensively in previous studies in our laboratory (Kennaway, 1993; Kennaway, 1994; Kennaway and Rowe, 1994; Kennaway *et al,* 1996; Rowe and Kennaway, 1996). Animals were placed in the top section of a Techniplast metabolic cage four days before urine collection commenced. The animals were provided with a liquid diet of Osmolite HN (Ross Laboratories, Columbous, Ohio) and water *ad lib*. This diet promotes high urine flow and provides all the nutritional requirements for normal growth. After the initial acclimatisation period during which the animals were maintained in a 12L:12D photoperiod (lights off 1900h) identical to that which they had previously been exposed, the experiment began. Samples were collected for four consecutive nights over the experimental period.

Urine was collected in a conical tube set inside the normal collecting vial. The urine was drawn off continuously from this tube by a multi-channel peristaltic pump (Technicon Pump II - flow rate 3ml/min) via polyvinyl tubing (2mm internal diameter). The tubing was connected to a fraction collector (LKB 2211 SuperRac, LKB Produckter, Bromma, Sweden), which enabled hourly fractions to be collected from up to 10 rats automatically. The animals urinated frequently when provided with the liquid diet and the urine was drawn off instantaneously and continuously and deposited in the urine collecting vials. No overlap occurred and samples were not contaminated by previous samples. The urine collecting vials contained 10mg boric acid crystals and a random sample of 20 were pre-weighed for each collection period. Weights ranged from 1.980g - 2.010g, with tubes from the same batch being used in the same collection.

A standard pattern of urine collection and photoperiod designated the **'4-day test'** was used throughout this project. The lighting protocol followed that of Aschoff's Type II method for assessing phase shifts (Aschoff, 1965a).

Experimental protocol.

Night 1 (control night) - on the control night the lights went off at the normal time of 1900h (as with the previous photoperiod) and they remained off for the entire experiment.

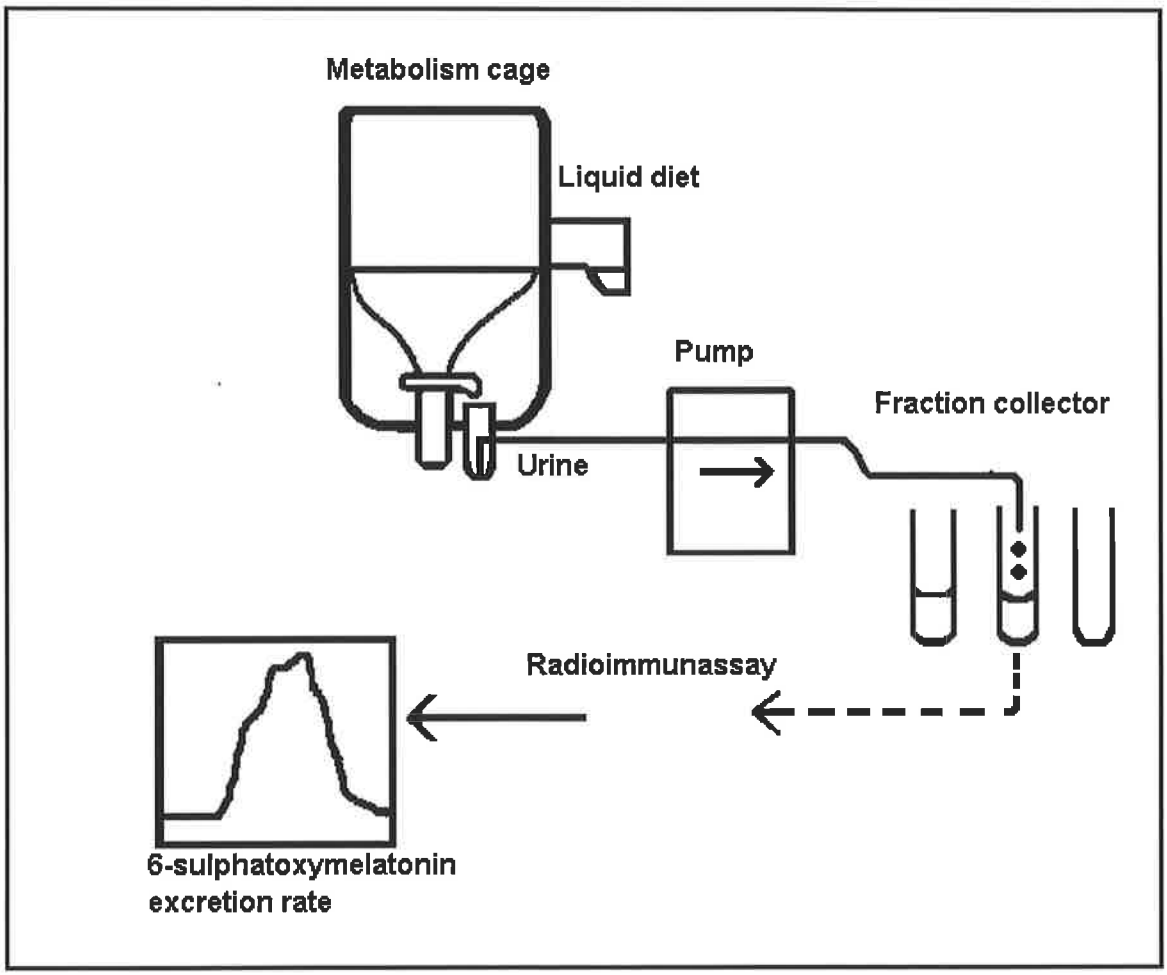

Figure 2.1
Schematic representation of the urine collection system used in the 4-day test.

Night 2 (treatment night) - animals were exposed to a light or drug stimulus at the circadian time specified in each experiment.
Night 3 (post-treatment night) - animals were left untreated in constant darkness.
Night 4 (post-treatment night) - animals were left untreated in constant darkness.

The post-treatment nights served to detect changes in the timing of the onset of the melatonin excretion rhythm as assessed via radioimmunoassay (section 2.2) from the control night.

Vials of urine were individually weighed and the volume estimated by subtracting the average weight of the vials before the collection from the total weight. Vials were capped and stored at -20°C for assay.

2.2 6-SULPHATOXYMELATONIN RADIOIMMUNOASSAY

Urine samples were assayed for 6-sulphatoxymelatonin content by radioimmunoassay (Aldhous and Arendt, 1988) using reagents purchased from Stockgrand Ltd., Surrey U.K. Urine samples were diluted 50-fold in tricine buffer (17.9g tricine, 9.0g sodium chloride, 1.0g gelatin, 0.2mg sodium azide, 1.0L distilled water) by adding 20µl sample to 980µl buffer.

50µl of the diluted sample, standard and quality controls were aliquoted into 550µl tricine buffer using a Hamilton Digital Diluter (Reno, Nevada, U.S.A.). 6-sulphatoxymelatonin antibody (100µl of a 1:360,000 dilution) was added to all tubes except totals and NSB together with 100µl of [^{125}I]aMT.6S (approximately 4000cpm/tube). Tubes were multi-vortexed for 20 seconds and incubated overnight at 4°C.

To separate bound and free aMT.6S, 100µl of a charcoal solution (1.0g charcoal, 50ml tricine buffer, prepared the previous day and stirred overnight at 4°C) was added and the tubes vortexed two at a time. Samples were immediately centrifuged at 4°C and 4000rpm for 15 minutes. The supernatant was decanted and the tubes were blotted dry on tissue paper. The precipitated charcoal pellet was counted for 60 seconds in a 1261 Multigamma Gamma Counter (Wallac, Oy., Finland).

Samples from individual animals were assayed together and control and experimental animals alternately within each assay.

2.2.1 Assay particulars

The concentration of aMT.6S in the sample was interpolated from a standard curve generated from standard solutions of 6, 12, 25, 51, 102, 205 and 410 fmol/100µl. The sensitivity of the assay was 3 fmol/tube. A total of 391 assays were carried out across the project and exhibited 20%, 50% and 80% displacement of the radioligand at doses of 9, 48 and 219 fmol/tube. Interassay variation for a sample in each assay was less than 10% at 20 fmol/tube. Intra-assay variation was less than 10%, as replicates with greater variation or replicates having levels above 200 pmol/tube were re-assayed.

Average 6-sulphatoxymelatonin excretion profiles were plotted for each treatment group (generally 5-10 animals). Figure 2.2 shows a schematic representation of a 4-day test with a light pulse administered at 6 hours after subjective dark onset (CT18).

2.2.2 Assessment of aMT.6S excretion rate onset

The time of onset of the 6-sulphatoxymelatonin excretion was used as the primary phase marker of the melatonin excretion rhythm. Onset time was determined for individual animals for each night of the experiment, and was set as the time at which the excretion rate rose above 20 pmol/h. This threshold has been used very successfully in previous studies and exhibits minimal variability on the control nights. For each animal the hours around the time of onset of aMT.6S excretion rate were individually plotted and the time at which the levels rose above 20 pmol/h was estimated to one decimal point. Figure 2.3 shows a representative plot of the time of onset of aMT.6S excretion rate for a normal animal on the control night of an experiment. Hourly samples are the most reliable as half- or quarter-hourly samples do not provide regular urine volumes for assay. All time lines in figures depict circadian time (CT) where CT0 is the time of subjective lights on and CT12 is subjective lights off.

2.2.3 Statistical analysis of onset times

- Statistical analysis of phase changes was done using a repeated measures ANOVA. The 4-day test protocol allows us to use individual animals as their own control. Thus, instead of comparing the degree of change in a control group to the degree of change in a treatment group we are able to analyse the change in onset time of each animal in the group.

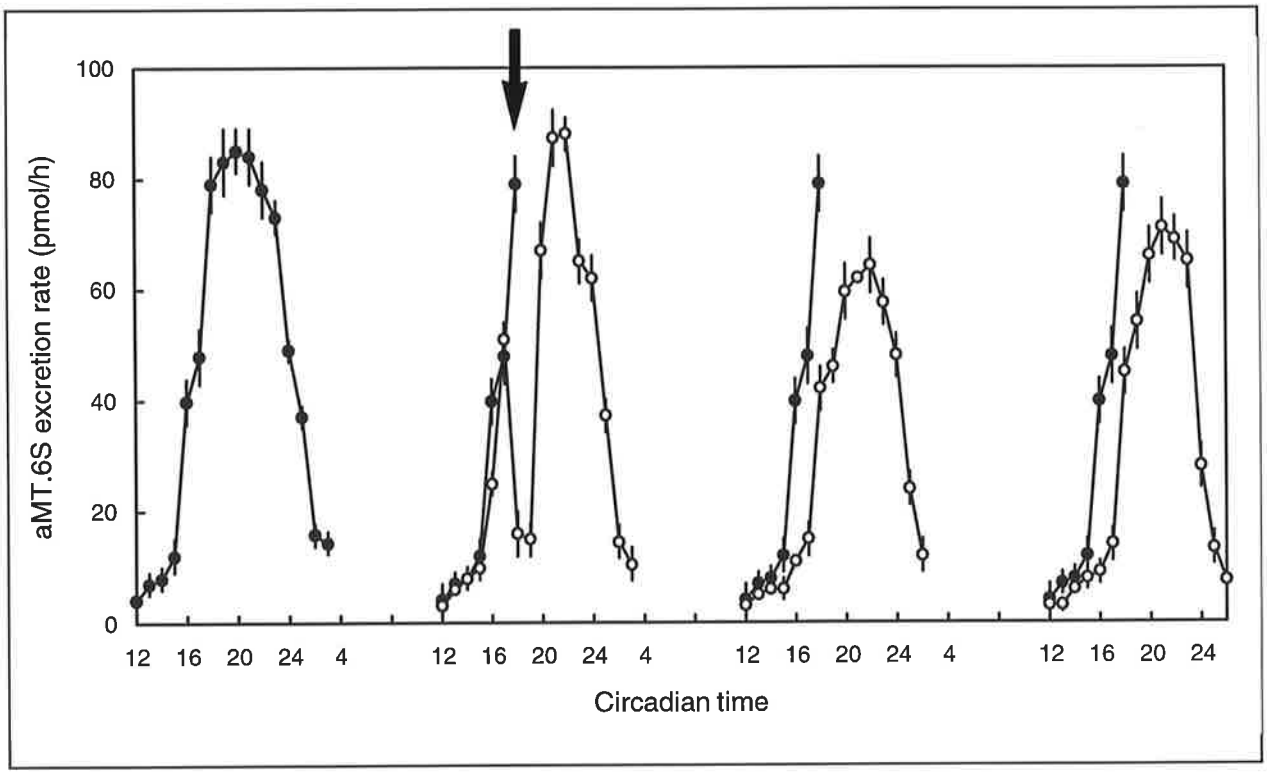

Figure 2.2
Schematic representation of the melatonin profiles produced using the 4-day test protocol for animals treated with a light pulse at CT18 on night 2 (6 hours after subjective lights off). Four consecutive nights are shown and lights remain off from CT12 on night 1. Data from the control night (night 1) represented by solid symbols, overlays the treatment night and two subsequent post-treatment nights, represented by open symbols (nights 2, 3 and 4). Arrow indicates the time of treatment on night 2.

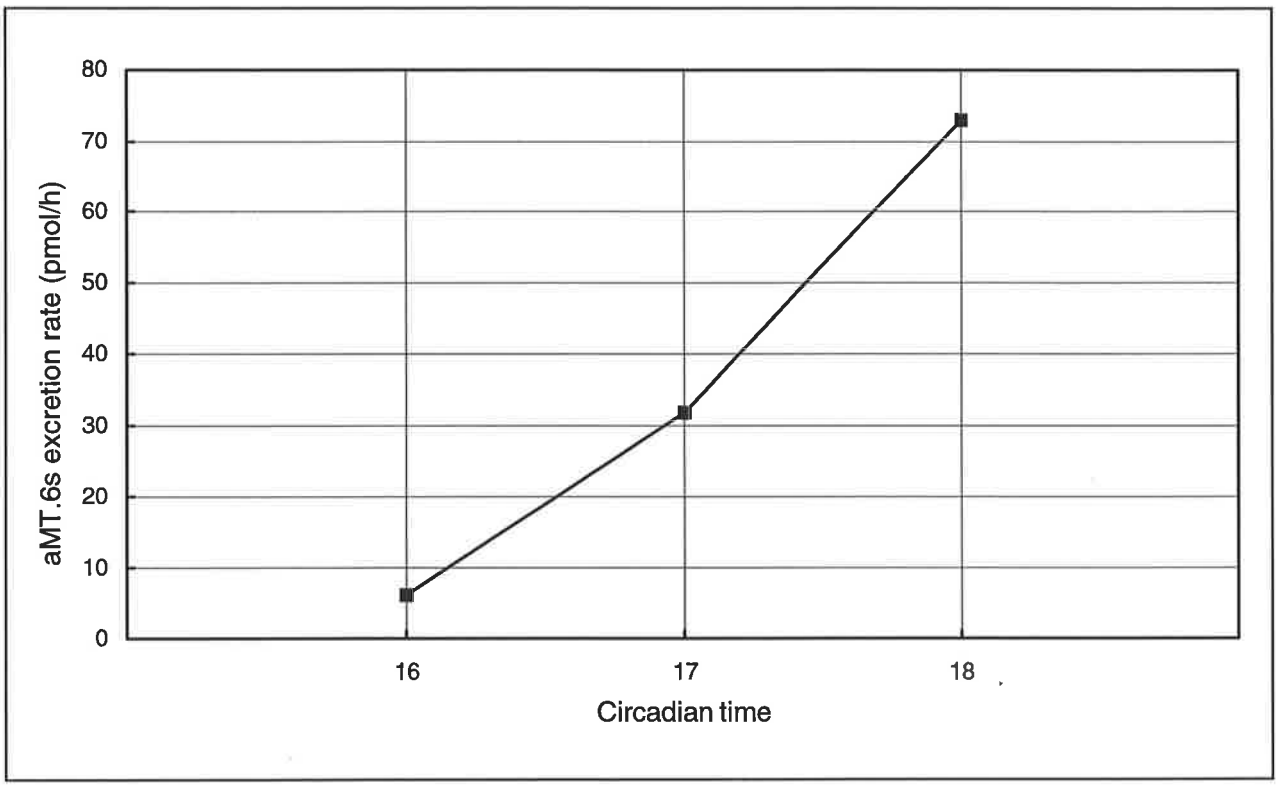

Figure 2.3
A representative plot of the 6-sulphatoxymelatonin excretion rate for a normal randomly bred animal in the hours around the time of onset. Onset time is recorded as the time at which the excretion rate goes above 20pmol/h (ie CT16.5).

The onset times of all normal randomly bred (RB) animals used in this project were pooled to gain an estimate of the variability between experiments, and to provide an indication of the normal phase shifts of untreated animals maintained in constant darkness for four days. Figure 2.4 shows the onset times for all normal animals that were administered a saline injection or left untreated versus animals that were exposed to a light pulse (1 minute/2 lux) at CT16 on the treatment night. This figure provides an estimate of the variability of the onset times over four nights and indicates that a small phase shift occurs from night 1 to night 3 as a result of a free-run in untreated animals.

The average onset of aMT.6S excretion in RB animals on the control night is CT16.1 ± 0.5h (mean ± SD), and this has delayed non-significantly to CT16.4 ± 0.5 on night 3, indicating a drift of approximately 0.3h. Animals treated with a light pulse had a similar time of onset on the control night (CT16.1 ± 0.4h) and on night 3 this was delayed to CT18.3 ± 0.7h.

2.3 CONTINUOUS TEMPERATURE/ GENERAL ACTIVITY MONITORING

Continuous monitoring of core body temperature and general activity was carried out via radiotelemetric recording. Under 3% halothane/oxygen anaesthesia animals were implanted with temperature transmitters (model TA10TA-F20, DataSciences Inc.) in the peritoneal cavity. Approximately 2 hours after the surgery the temperature had recovered to normal baseline and no ill effects of the surgery were observed in any of the experiments. Animals were kept in individual cages in a photoperiod designated in the individual experiments. The temperature and activity data was sampled continuously by the Dataquest IV computer system for 10 seconds at 10 minute intervals.

2.3.1 Assessment of temperature offset

The phase marker used to assess shifts in the rhythm of core body temperature was the time of the morning decline. For each animal the time of offset was determined as the time at which the temperature dropped below the mean temperature for that animal and remained below that value for 3 samples (30 minutes). In addition, long-term experiments were analysed using the TAU computer package.

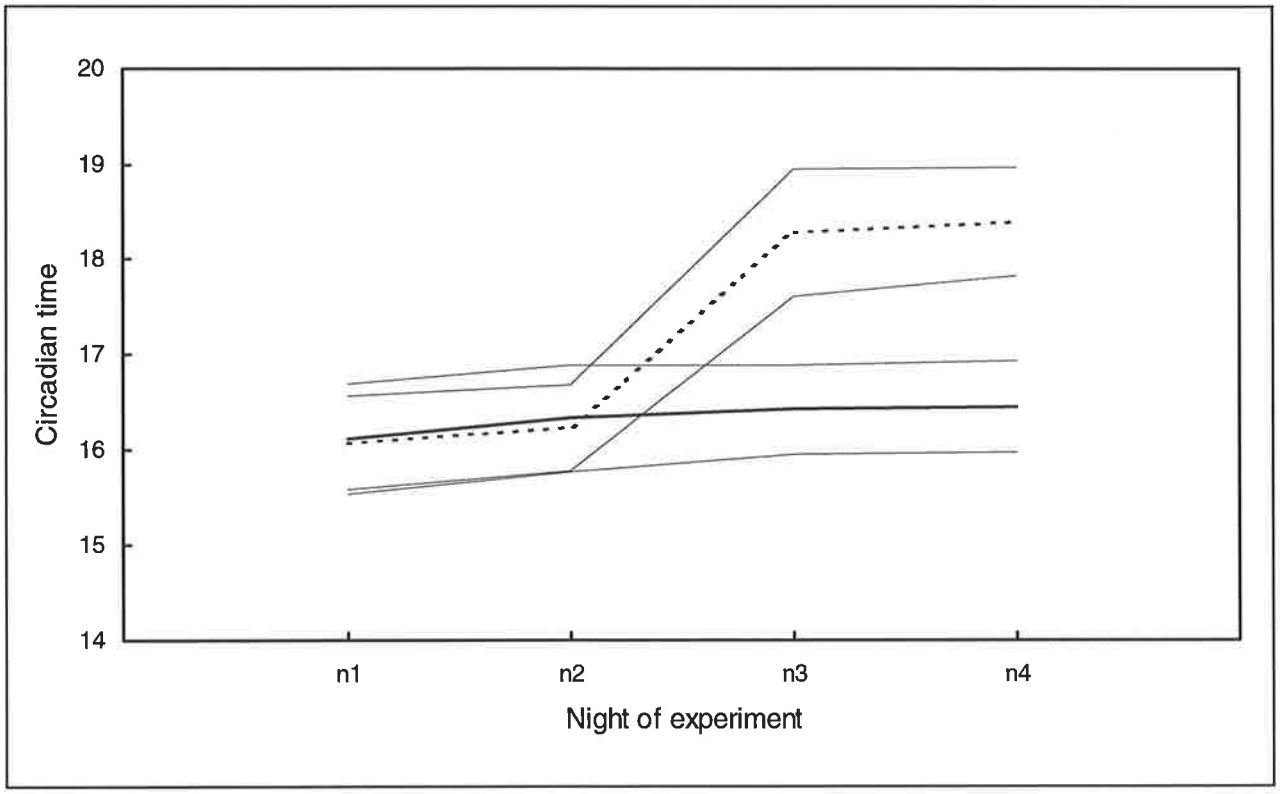

Figure 2.4
Timing of the onset of 6-sulphatoxymelatonin excretion in normal animals treated with a light pulse at CT16 on experimental night 2 or animals used as controls (ie. Saline or untreated animals). The control animals are represented by the heavy unbroken line and the data set is the average onsets for 60 normal randomly bred animals and the lighter lines indicate one standard deviation from the mean. The heavy dashed line represents the average onsets for 25 normal animals treated with a 1 minute/2 lux light pulse at CT16 on night 2 of the experiment and the lighter lines either side indicate one standard deviation from the mean.

2.3.2 Assessment of general activity offset

The phase marker for assessing general activity rhythmicity was the time of activity offset. Under LD conditions the light-dark transition triggers a burst of activity from the majority of animals making the activity onset an inappropriate marker. The time of activity offset was defined as the time at which the activity measure dropped below the average for that animal and remained below for 3 samples (ie 30 minutes). In addition, long-term experiments were analysed using the TAU computer package.

2.4 RUNNING ACTIVITY MONITORING

Activity in individual animals was recorded via custom-built running wheel assemblies (Kennaway, 1994). Animals were placed in home cages connected via an open mesh tunnel to running wheels 7cm wide and 25cm in diameter, fitted with an external graphite axle. Each running wheel was fitted with a magnet and a magnetic microswitch, with the microswitch closures monitored by a Squirrel 1207 data logger (Grant Instruments Limited, Cambridge, England). The logger was set to monitor 10 wheels in the following manner: on each rotation of the wheel the microswitch closed, charging a capacitor with 1.5 volts. When the switch opened again the capacitor slowly discharged. In the absence of the capacitor, the voltage surge upon microswitch closure lasted for milliseconds and was not consistently detected by the data logger which was set to scan each channel every 2 s. The capacitor ensured that there was a detectable signal for up to 10 s after each event and thus, that no rotation of the wheels were missed. On each scan of the channels the recorded voltage was stored in temporary memory and every 10 minutes the average voltage was placed in permanent memory. In addition to the 16 voltage/current channels, the 1200 series Squirrel data logger has two channels operating as simple event counters. Two wheels were therefore fitted with a second microswitch leading to these channels to provide a calibration of wheel rotations vs. stored voltage/10 min. There was an approximate linear relationship between voltage stored and the number of revolutions up to 350 revolutions/10 min. The average voltage was printed out every 10 min on line onto computer paper and every 7 days the stored data was transferred to disk using a program from Grant Instruments Software. For the visual assessment of entrainment following changes of the lighting schedule the data was graphed using the TAU computer package (Mini-Mitter Inc., Sunriver, OR).

2.5 c-FOS IMMUNOCYTOCHEMICAL STUDIES

Animals were maintained in home cages in a 12L:12D photoperiod in groups of four or five. On the day of the experiment animals received the treatment specified in each individual experiment at the specified time. Two hours after treatment animals were decapitated and brains removed within 30 seconds. Brains were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After 30 hours in fixative brains were rinsed in phosphate buffered saline (PBS) and four coronal sections encompassing the SCN were cut from each brain on a Vibroslice® microtome. Free-floating sections were stored overnight in Triton-X100 in PBS at 4°C.

Endogenous peroxidase was inhibited for 3 minutes with a 10% solution of hydrogen peroxide. After repeated washing with PBS, sections were incubated for one hour in 0.5% solution of bovine serum albumin and 1% horse serum in 0.05M phosphate buffer and incubated for a further 60h at 4°C with a 1:20,000 dilution of a rabbit anti-Fos antibody (Arnel Products Co., New York) (Moyer *et al,* 1997). An anti-rabbit ABC Vectastain® kit was used in combination with a modified nickel enhanced technique to localise the primary antibody. Quantitation of immunopositive cells was achieved using a computerised VidoePro 32® image analysis system (Leading Edge Pty. Ltd., Australia). The rectangular counting area was 480 x 331 μm with the long side tangential to the ventral indenture of the SCN into the optic chiasm and the shorter side of the frame in line with the third ventricle. Results are expressed as the number of immunopositive cells per animal (calculated by averaging the number of positive cells in the left and right SCN). Data was analysed by Kruskal Wallis non-parametric ANOVA and Mann-Whitney U test post hoc.

2.6 RECEPTOR BINDING STUDIES

Animals were decapitated between CT7-9 and brains were rapidly removed. Using a brain slicer (Jacobowitz rat brain slicer, Zivi-Miller Lab. Inc., PA) with 1mm gradations, gross sections were made of the areas under investigation. Initially, separation of the rhombencephalon by a transverse section from the rest of the brain produced 3 sections. A second transverse section was then made at the optic chiasma, separating the cerebellum into

two parts. These sections were frozen in isopentane and liquid nitrogen and stored at -20°C. From decapitation to freezing of individual sections the process took no longer than 2 minutes.

At the time of homogenising individual brain regions were dissected from the gross sections. The hypothalamus was dissected taking the anterior commissure as a horizontal reference and the line between the posterior hypothalamus and mamillary bodies as the caudal limit. The striatum was dissected using the external walls of the lateral ventricles as internal limits and the corpus callosum as external limits. The hippocampus was then separated from the midbrain and cortex. The cortex was dissected from the anterior and middle sections and combined (for review see (Glowinski and Iversen, 1966).

Sections from individual animals were homogenised in 10 vols of Tris buffer, 50mM, pH 7.4 (6.61g Tris.HCl, 0.97g Tris.base, 1 litre distilled water) and homogenates were stored frozen at -20°C until assay.

2.6.1 Nicotinic receptor (nAChR) studies

In the nicotinic studies the methods were adapted from (Bennett, 1978; Wonnacott *et al,* 1992). [^{125}I]Tyr^{54}-α-bungarotoxin preparation was purchased from DuPont New England Nuclear Research Products, Boston, MA. A 16μCi/μg solution was used in all studies. For assessment of non-specific binding a 0.1M solution of nicotine was used. Nicotine di-d-tartrate (3.072g nicotine di-d-tartrate = 1.0g nicotine) was purchased from Research Biochemicals International.

Nicotinic studies were only conducted in the hypothalamus and preliminary studies were carried out on hypothalami of RB animals to optimise assay conditions. The homogenates (containing approximately 5mg tissue per tube) were incubated in the presence of [^{125}I]α-BTX in varying concentrations for 60 minutes at 25°C in a final volume of 1ml of Tris buffer (0.12M, pH 7.4) in glass tubes. Non-specific binding was assessed by incubating the homogenates with [^{125}I]α-BTX in the presence of nicotine (0.1M). Incubations were terminated by the addition of 2ml ice cold Tris buffer containing 1% BSA followed by immediate centrifugation for 15 minutes at 4°C and 4000rpm (Wonnacott *et al,* 1992). The supernatant was then tipped off and the pellet was resuspended with another 2 ml ice cold wash

buffer and re-centrifuged. The pellet was counted on a 1261 Multigamma Gamma counter (Wallac, Oy., Finland) for 1 minute.

The binding curve for nicotinic receptors in the hypothalamus is represented in Figure 2.5. The non-specific binding was relatively high using the above assay conditions and separation techniques, however the [^{125}I]α-BTX complex was found to adhere to the filters when vacuum filtration separation was attempted even after treatment of filters with 0.1% polyethyleneimine. Thus, the centrifugation step was employed and was found to produce the highest level of specific binding. The scatchard plot derived from this binding data is shown in Figure 2.6. The KD for nAChR in the hypothalamus was 0.33nM.

2.6.2 Muscarinic receptor (mAChR) studies

The methods for the muscarinic binding assays were adapted from (Bennett, 1978). Quinuclidinyl Benzilate,L-[Benzilic-4,4'-^{3}H] (QNB) was purchased from DuPont New England Nuclear Research Products, Boston, MA. A 49Ci/mmol solution was used in all studies. For assessment of non-specific binding a 1μM solution of atropine was used. Atropine was purchased from Sigma Chemicals Pty Ltd.

Analysis of muscarinic receptor levels was conducted in the hypothalamus, striatum, hippocampus and cortex. Assay conditions were optimised for each brain region using RB animals. The hypothalamus and striatum homogenates contained approximately 1mg tissue, while cortex and hippocampus contained 0.2mg tissue per tube. Homogenates were incubated in the presence of [^{3}H]QNB in varying concentrations for 90 minutes at 25°C in a final volume of 1ml of Tris buffer (0.12M, pH 7.4). Non-specific binding was assessed by incubating homogenates with [^{3}H]QNB in the presence of atropine (1μM). Incubations were terminated by the addition of 4ml ice-cold Tris buffer containing 0.2% BSA followed by immediate vacuum filtration over Whatmann GF/B glass fibre filters. Filters were washed a further two times with 4ml buffer and radioactivity was counted after a period of 2 hours by scintillation spectrometry (ReadySafe scintillant) using 1 minute counts on a beta counter (Beckman LS 6000LL, Beckman Instruments, Fullerton, CA). Scatchard analyses of the binding data were carried out to determine the affinity of the muscarinic receptors in each brain region.

The binding curve for mAChR in the hypothalamus is shown in Figure 2.7 and the scatchard derived from the data in Figure 2.8. The KD for mAChR in the hypothalamus of RB animals was 75pM.

The binding curve for mAChR in the striatum is shown in Figure 2.9 and the scatchard derived from the data in Figure 2.10. The KD for mAChR in the striatum of RB animals was 160pM.

The binding curve for mAChR in the hippocampus is shown in Figure 2.11 and the scatchard derived from the data in Figure 2.12. The KD for mAChR in the hippocampus of RB animals was 36pM.

The binding curve for mAChR in the cortex is shown in Figure 2.13 and the scatchard derived from the data in Figure 2.14. The KD for mAChR in the cortex of RB animals was 39pM.

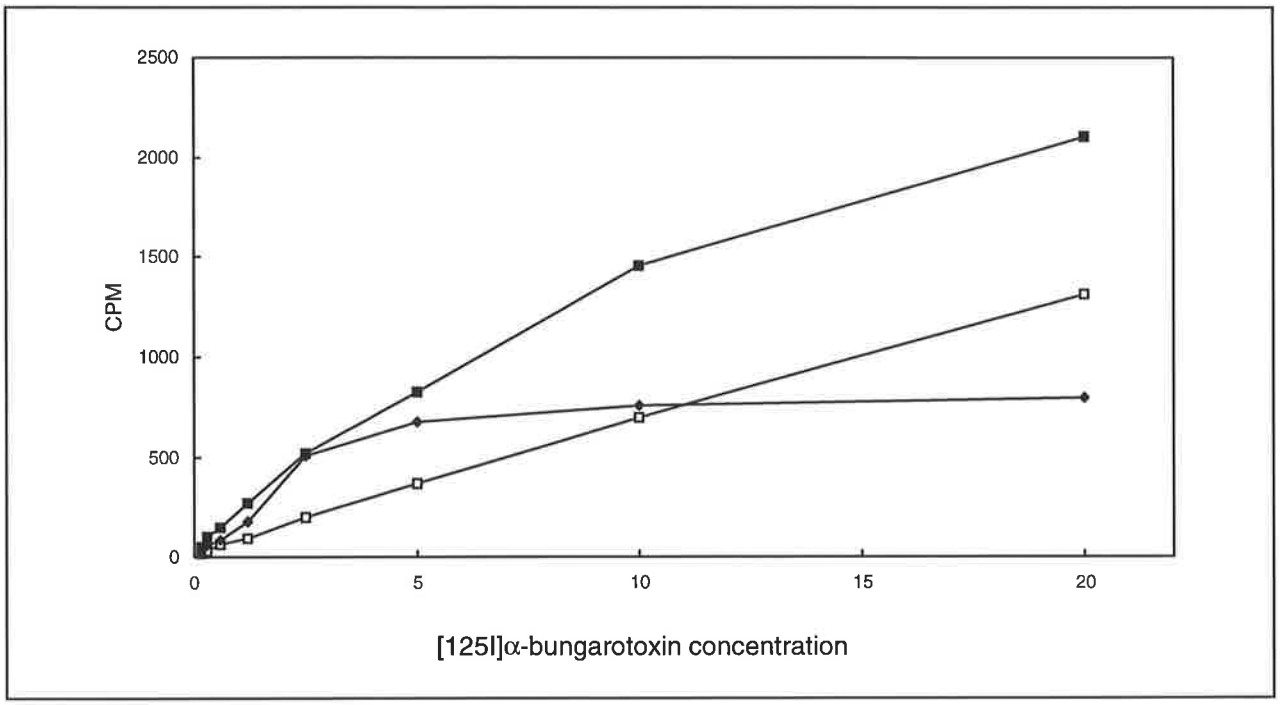

Figure 2.5
Binding curve for nicotinic receptors in the hypothalamus of normal randomly bred rats. Homogenates were incubated with increasing concentrations of [^{125}I]α-BTX with or without nicotine to assess non-specific binding. Total counts are represented by the closed squares, non-specific binding by the open squares and specific binding (the difference between total and non-specific binding) is represented by closed diamonds.

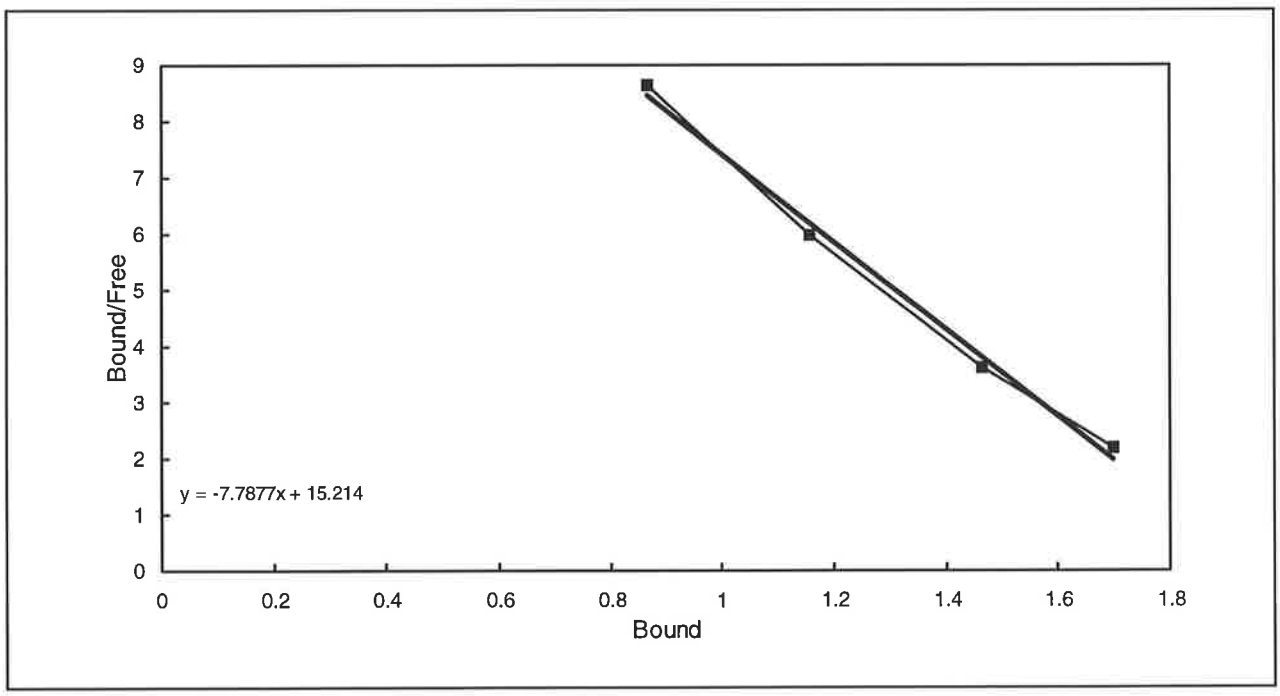

Figure 2.6
Transformation of the data contained in the binding curve for nicotinic receptors in the hypothalamus of normal RB rats according to the method of Scatchard. The experimental data is represented by the solid squares and the heavy line represents the trendline for the data. The equation for the trendline is expressed on the graph.

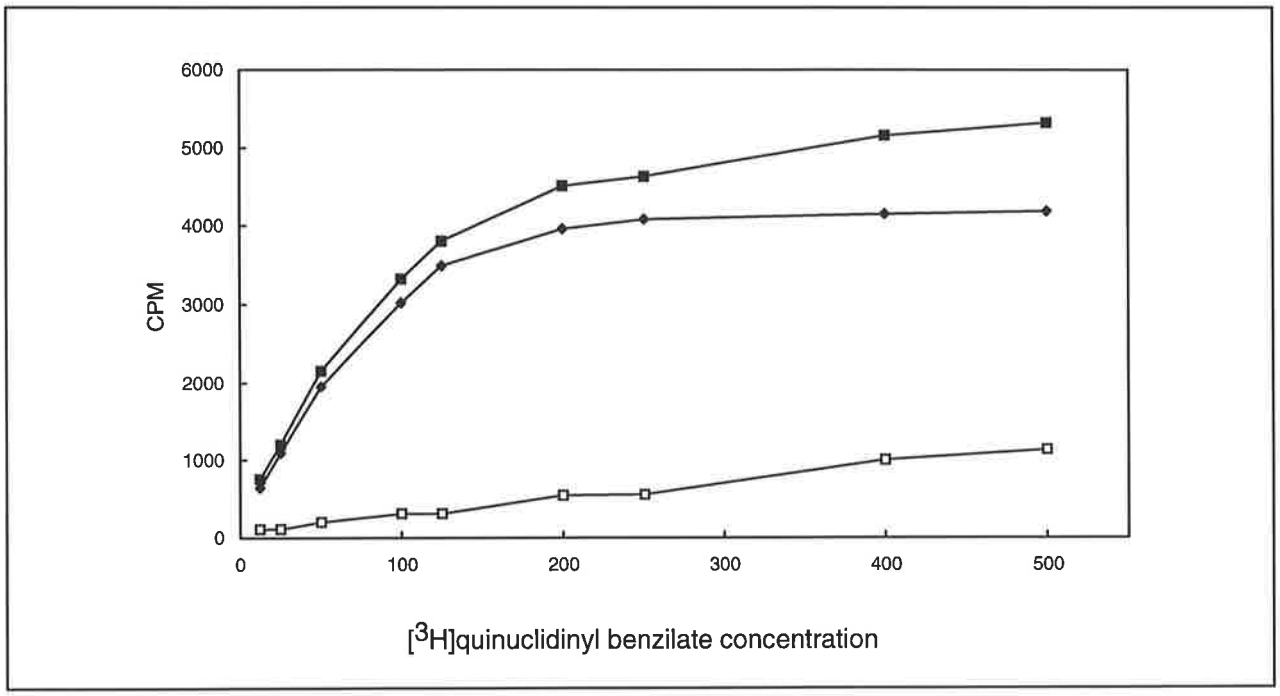

Figure 2.7
Binding curve for muscarinic receptors in the hypothalamus of normal randomly bred rats. Homogenates were incubated with increasing concentrations of [^{3}H]QNB with or without atropine to assess non-specific binding. Total counts are represented by the closed squares, non-specific binding by the open squares and specific binding (the difference between total and non-specific binding) is represented by diamonds.

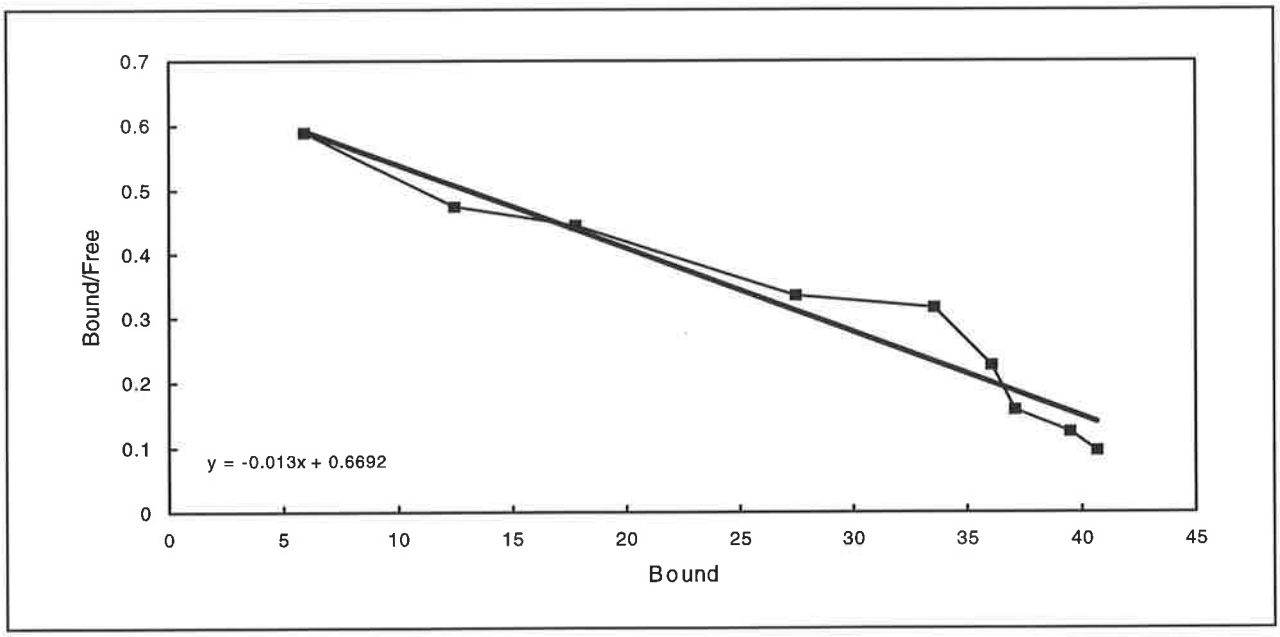

Figure 2.8
Transformation of the data contained in the binding curve for muscarinic receptors in the hypothalamus of normal RB rats according to the method of Scatchard. The experimental data is represented by the solid squares and the heavy line represents the trendline for the data. The equation for the trendline is expressed on the graph.

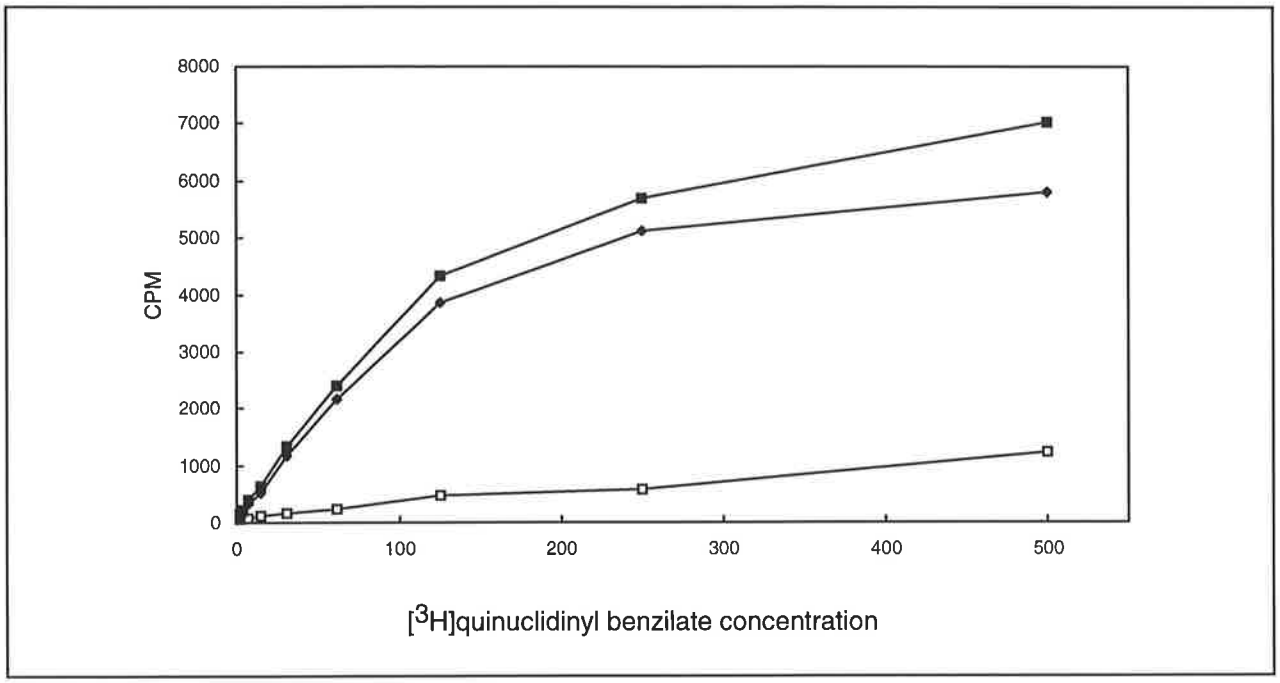

Figure 2.9
Binding curve for muscarinic receptors in the striatum of normal randomly bred rats. Homogenates were incubated with increasing concentrations of [^{3}H]QNB with or without atropine to assess non-specific binding. Total counts are represented by the closed squares, non-specific binding by the open squares and specific binding (the difference between total and non-specific binding) is represented by diamonds.

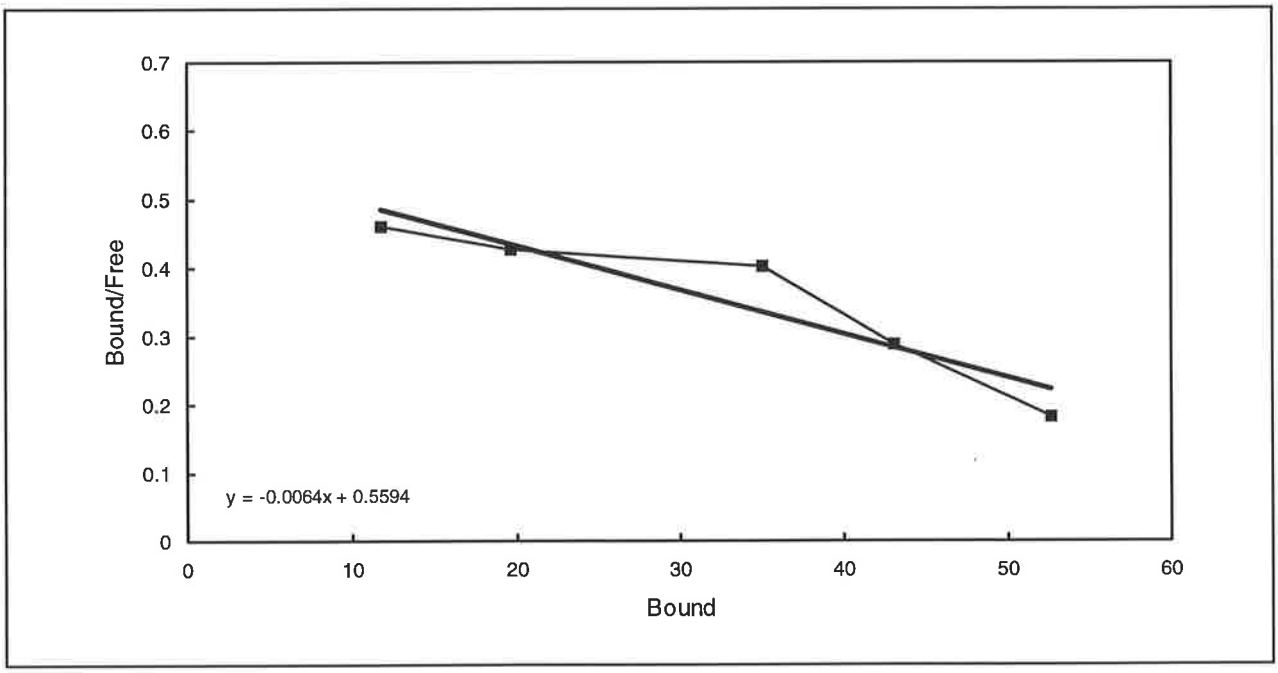

Figure 2.10
Transformation of the data contained in the binding curve for muscarinic receptors in the striatum of normal RB rats according to the method of Scatchard. The experimental data is represented by the solid squares and the heavy line represents the trendline for the data. The equation for the trendline is expressed on the graph.

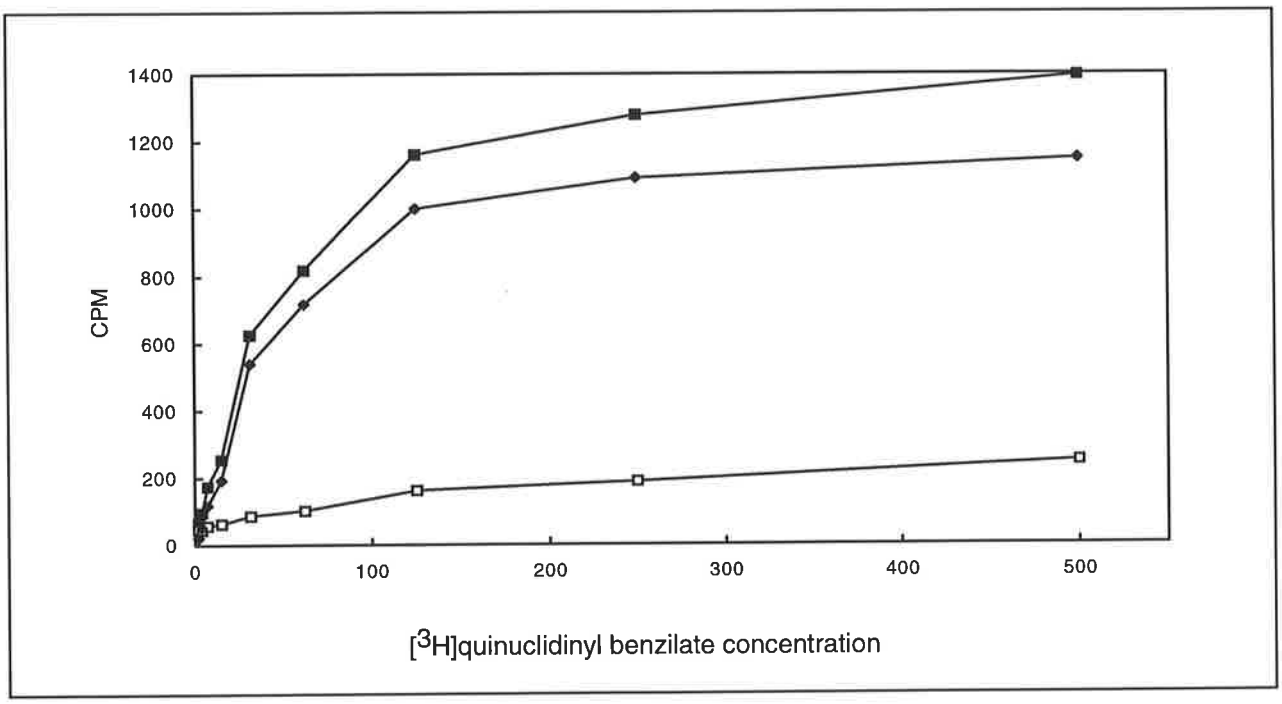

Figure 2.11
Binding curve for muscarinic receptors in the hippocampus of normal randomly bred rats. Homogenates were incubated with increasing concentrations of [^{3}H]QNB with or without atropine to assess non-specific binding. Total counts are represented by the closed squares, non-specific binding by the open squares and specific binding (the difference between total and non-specific binding) is represented by diamonds.

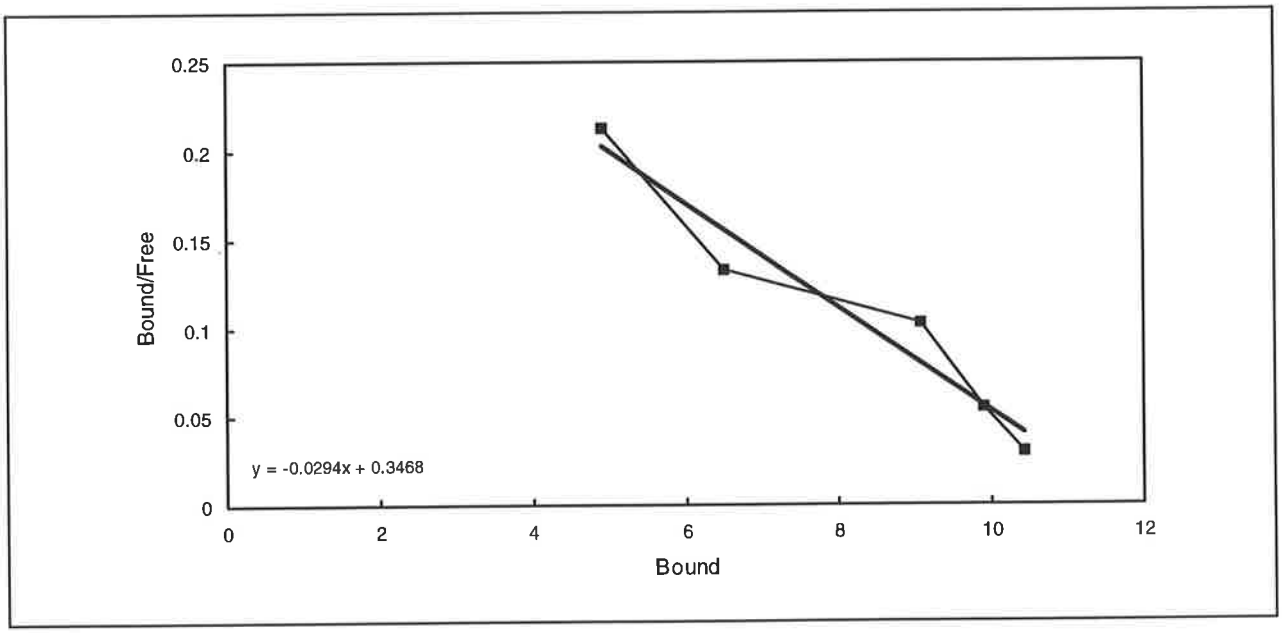

Figure 2.12
Transformation of the data contained in the binding curve for muscarinic receptors in the hippocampus of normal RB rats according to the method of Scatchard. The experimental data is represented by the solid squares and the heavy line represents the trendline for the data. The equation for the trendline is expressed on the graph.

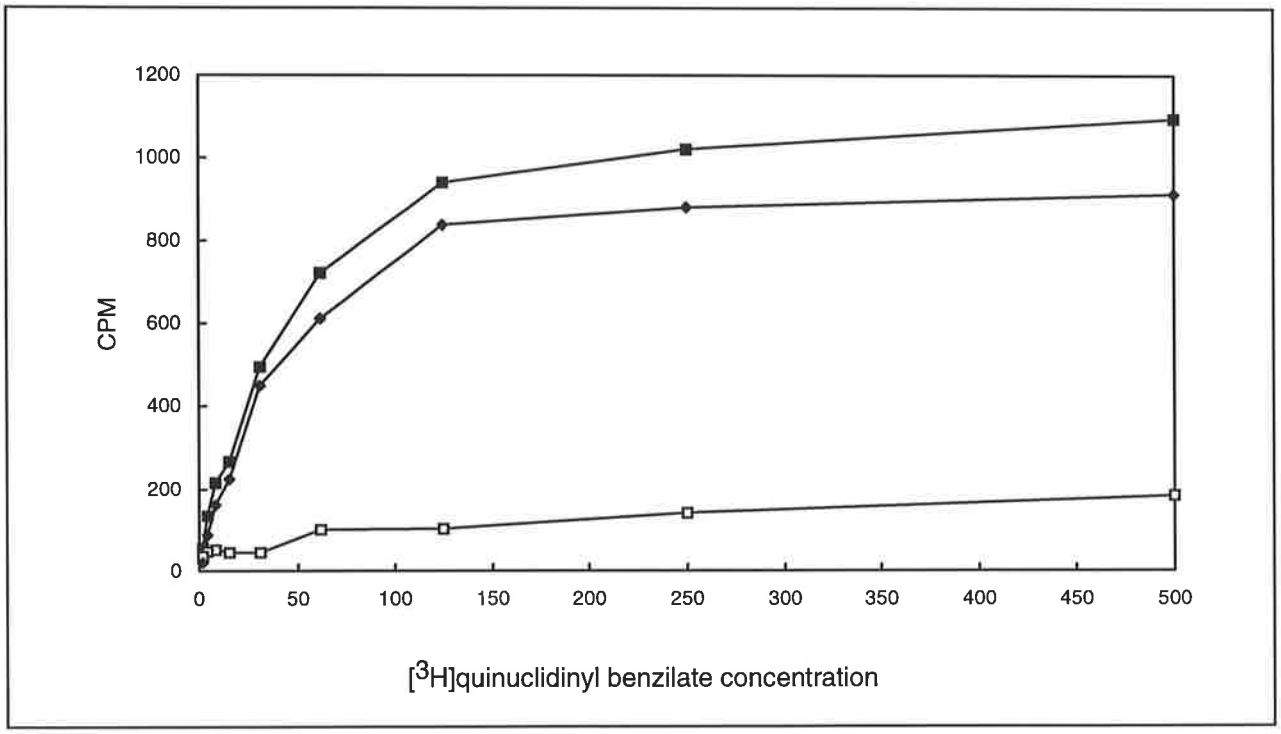

Figure 2.13
Binding curve for muscarinic receptors in the cortex of normal randomly bred rats. Homogenates were incubated with increasing concentrations of [^{3}H]QNB with or without atropine to assess non-specific binding. Total counts are represented by the closed squares, non-specific binding by the open squares and specific binding (the difference between total and non-specific binding) is represented by diamonds.

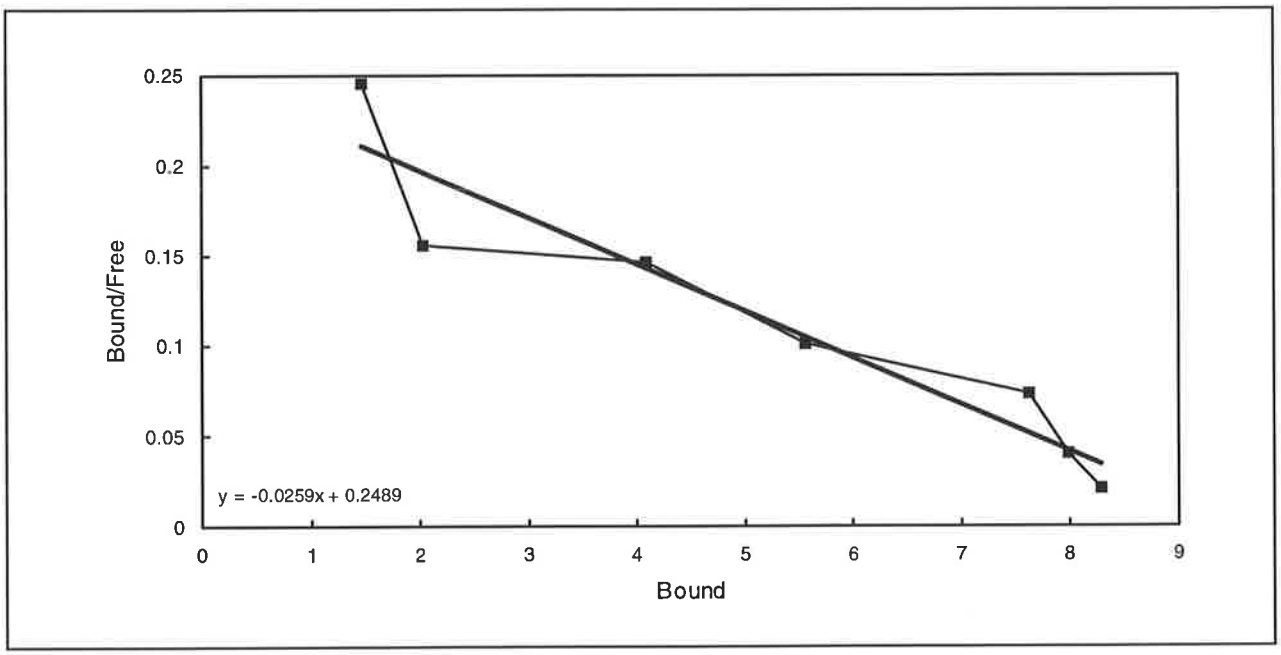

Figure 2.14
Transformation of the data contained in the binding curve for muscarinic receptors in the cortex of normal RB rats according to the method of Scatchard. The experimental data is represented by the solid squares and the heavy line represents the trendline for the data. The equation for the trendline is expressed on the graph.

2.7 FORCED SWIM TEST

The protocol used for the forced swim test was adapted from that of Porsolt *et al* (1978) and Overstreet *et al* (1986). A cylindrical perspex container (height 60cm, diameter 25cm) was used in all experiments. The container was filled with tap water to a level of 35 cm and was maintained at 37°C throughout the experiment rather than 24°C as in the original report. Animals were placed in the container for a five minute period during which they were monitored closely. At the end of the testing period the animal was removed from the container, towel dried and placed under a heat lamp until completely dry, and then returned to the home cage. Fecal pellets were removed after each animal and water was changed after every five animals.

2.7.1 Assessment of immobility

All testing was videotaped for assessment of time spent immobile by two independent assessors. Both experimenter and assessors were blind to the strain or treatment group of animals. Using a stopwatch, the time spent immobile was recorded for each animal's five minute testing period. When an animal was moving only one paw in an attempt to keep the nose above the water it was defined as being immobile.

Chapter 3

Circadian rhythms and acetylcholine

3.1 INTRODUCTION

The importance of acetylcholine as a neurotransmitter in the circadian timing system is not well understood. Although ACh is not a transmitter in any of the three major SCN afferents, it has been identified in the SCN (van den Pol and Tsujimoto, 1985), together with choline acetyltransferase (Brownstein *et al,* 1975) and muscarinic and nicotinic cholinergic receptors (Block and Billiar, 1981 ; Swanson *et al,* 1987; van der Zee *et al,* 1991). Early work with the non-specific ACh agonist carbachol suggested that it could cause changes in the timing of the SCN of rats (Zatz and Brownstein, 1979), hamsters (Wee *et al,* 1992) and mice (Zatz and Herkenham, 1981). More recently, nicotine was reported to cause phase shifts in the neuronal firing rate rhythm in SCN explants (Trachsel *et al,* 1995), as were carbachol and atropine (Liu and Gillette, 1996). An important study implicating a role for acetylcholine in the mediation of light effects at the level of the SCN showed that a light pulse 3 hours after dark onset caused the levels of ACh in the SCN to increase three-fold (Murakami *et al,* 1984). The pathways mediating such effects within the circadian timing system are yet to be elucidated.

There are no cholinergic somata in the SCN and thus acetylcholine found in the nuclei arises from other brain regions. Cholinergic projections have been identified that originate from the brainstem and forebrain areas (Bina *et al,* 1993), basal forebrain and mesopontine tegmentum (Kiss and Halasz, 1996) and several other hypothalamic areas (Rao *et al,* 1987; Tago *et al,* 1987). Further, the brainstem and forebrain areas also receive retinal projections (Bina *et al,* 1993) and although the cells on which the retinal projections terminate have not been clearly implicated as minor SCN afferents, the possibility remains that this may be the pathway of action of ACh in the circadian timing system. A more recent study has provided morphological evidence that cholinergic afferents to the SCN may act directly on neurons in the nucleus. Kiss and Halasz (1996) demonstrated the existence of separate contacts between choline acetyltransferase-immunopositive elements and SCN neurons. Thus, while there is compelling evidence for a fundamental role for acetylcholine in SCN function, two questions remain. The first is which pathways are mediating the actions of the neurotransmitter and the second is which receptor subtype mediates the reported effects.

This part of the project examined the effect of two peripherally administered cholinergic agonists, nicotine and oxotremorine, on the timing of the aMT.6S rhythm and the induction of

c-fos in the SCN. This was done in normal randomly bred animals to assess the phase-shifting abilities of these cholinergic agents and to attempt to differentiate between their actions.

3.2 METHODS

In each experiment randomly bred (RB) male Wistar albino rats weighing 80-100g at the beginning of the experiment were used. Previous to the experiments the animals were maintained in home cages in groups of 5 under 12L:12D conditions, with lights off at 1900h. All manipulations during the experimental period were conducted in complete darkness using infrared viewers and torches.

3.2.1 Melatonin studies

Animals were transferred to metabolism cages in light-controlled (12L:12D, lights off 1900h) environment chambers, fed a liquid diet and allowed to acclimatise for four days. Protocol for these experiments followed that of the 4-day test as outlined in section 2.1, however in some of the reported studies only one post-treatment night was used. Urine was collected hourly over each subjective night and urine vials were weighed and stored frozen for RIA (see section 2.2). Animals were assigned to one of the following protocols.

Part 1.

A dose-response curve was established across three doses for both nicotine and oxotremorine. On the treatment night (night 2 of the experiment), animals received either CT16 administration of nicotine (0.3mg/kg, 1mg/kg of 3mg/kg s.c.), oxotremorine (0.8mg/kg, 2mg/kg or 4mg/kg s.c.), a light pulse (1 minute/2 lux) or saline. NB. The light pulse used in the reported studies (1 minute/2 lux) was chosen on the basis of previous reports illustrating significant acute and phase delaying effects on the aMT.6S excretion rhythm and a significant number of c-FOS immunopositive cells in the SCN (Rowe and Kennaway, 1996, Moyer *et al,* 1997).

Part 2.

A partial phase-response curve was also defined for nicotine administration across a section of the PRC that light pulses have previously been shown to be effective at eliciting phase shifts

(Kennaway and Rowe, 1994). On the treatment night animals received either nicotine (1mg/kg s.c.) or saline at CT14, CT16 or CT18 and underwent a 4-day protocol. There was a minimum of five animals in each treatment group and each animal received only one treatment.

Part 3.

The effects of the cholinergic nicotinic antagonist mecamylamine on the phase shifts induced by nicotine were assessed to further clarify the receptor mediating these effects. On the treatment night animals received either mecamylamine (20mg/kg) prior to nicotine (1mg/kg) or mecamylamine (20mg/kg) prior to saline. Pre-treatments occurred at CT15.5 and post-treatments at CT16. Five animals were assigned to each treatment group.

3.2.2 c-FOS immunocytochemical studies

A similar set of experiments was carried out using c-FOS as a marker of SCN response. Animals were maintained in home cages (5 to a cage) in a 12L:12D photoperiod, lights off at 1900h. On the experimental night the lights went off at the normal time (1900h or ZT12). For subjective day treatments (CT6), the lights did not come on at the time of normal dawn.

Part 1.

A dose-response curve was again established for nicotine treatment, however only the highest dose of oxotremorine was used as the drug was found to have no effect on aMT.6S excretion levels. On the experimental night animals received ZT16 administration of nicotine (0.3mg/kg, 1mg/kg or 3mg/kg s.c.), oxotremorine (4mg/kg s.c.), a light pulse (1 minute/2 lux) or saline.

Part 2.

To ensure the responses recorded after treatment during subjective night were time-gated as light effects are, both light and nicotine were administered during subjective day. Nicotine (1mg/kg s.c.), saline or a light pulse (1 minute/2 lux) were administered at CT6 (after 18 hours darkness and 6 hours after subjective dawn).

Part 3.

To assess the specific receptor subtype mediating the nicotine-induced responses animals injected with nicotine were pre-treated with the cholinergic nicotinic antagonist,

mecamylamine. Animals were treated with nicotine (1mg/kg s.c.) at ZT16 (4 hours after lights off) after pre-treatment with mecamylamine (0.3mg/kg s.c.) or saline 30 minutes prior.

A minimum of 9 animals was assigned to each group in each part of this experiment. Two hours after treatment animals were decapitated and brains rapidly removed and fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for processing (see section 2.5).

3.3 RESULTS

3.3.1 Melatonin studies

Part 1.

A light pulse at CT16 (1 minute/2 lux) caused an acute suppression of the aMT.6S excretion rate at the time of treatment and a subsequent phase delay in the timing of onset of the rhythm on the following night (Figure 3.1). Saline administration at this time had no effect either acutely or on the following nights (Figure 3.1).

Nicotine at each dose (0.3mg/kg, 1mg/kg and 3mg/kg) administered at CT16 caused a small transient decrease in melatonin excretion rate at the time of treatment (Figure 3.2), however this was significantly less than that seen after a light pulse. A significant phase delay in the timing of aMT.6S excretion onset was seen on night 3 after treatment with a dose of 3mg/kg (2.2 ± 0.5h) and 1mg/kg (1.7 ± 0.3h) however, at 0.3mg/kg no significant delay resulted (0.1 ± 0.3h) (Figure 3.2) (Table 3.1). Oxotremorine administered at CT16 had no effect either acutely or on the following night at any dose used (Table 3.1).

Part 2.

The timing of 6-sulphatoxymelatonin excretion onset was phase delayed in animals treated with nicotine (1mg/kg) at CT16 and CT18 but not at CT14 or in animals treated with saline at any time (CT14 data not shown) compared with the control night (Figure 3.3)(Table 3.1).

Part 3.

Mecamylamine treatment (5mg/kg) at CT15.5 caused complete suppression of the melatonin production from the time of administration (Figure 3.4). When administered prior to a saline injection the antagonist did not cause any phase shifts in the aMT.6S excretion rhythm (Figure 3.4). Further, mecamylamine had no effect on the phase delay caused by nicotine administration, as animals receiving mecamylamine prior to nicotine exhibited phase delays of 1.1 ± 0.2h on night 3 and 1.9 ± 0.3h on night 4 (Figure 3.4).

3.3.2 c-FOS immunocytochemical studies

Part 1.

At each dose of nicotine (0.3mg/kg, 1mg/kg and 3mg/kg) administered at CT16 significantly more c-FOS immunopositive cells were observed in the SCN than followed saline administration (Figure 3.5). While there was a dose-dependent increase in the number of labelled cells, even at the maximal dose (3mg/kg) the response was a third of that observed after treatment with a light pulse (Photo 3.1). In both nicotine and light treated animals the majority of the immunopositive cells were identified in the ventrolateral region of the SCN and a lower proportion in the ventromedial area. Administration of oxotremorine (4mg/kg) also resulted in the appearance of immunopositive cells in the SCN and the number was similar to that provoked by the lowest dose of nicotine (Figure 3.5)(Photo 3.1).

Part 2.

Nicotine (1mg/kg) administered at CT6 did not result in the appearance of c-FOS-positive neurons in the SCN. In addition, both a light pulse (1 minute/2 lux) and a saline injection failed to induce the gene at this time (Figure 3.5).

Part 3.

There were a significant number of c-FOS-positive cells identified in the SCN of animals treated with saline prior to nicotine (1mg/kg) (56.1 ± 11.1 cells/SCN). This number was reduced by 65% in animals pre-treated with the antagonist mecamylamine to 19 ± 2.1 cells/SCN (Photo 3.2).

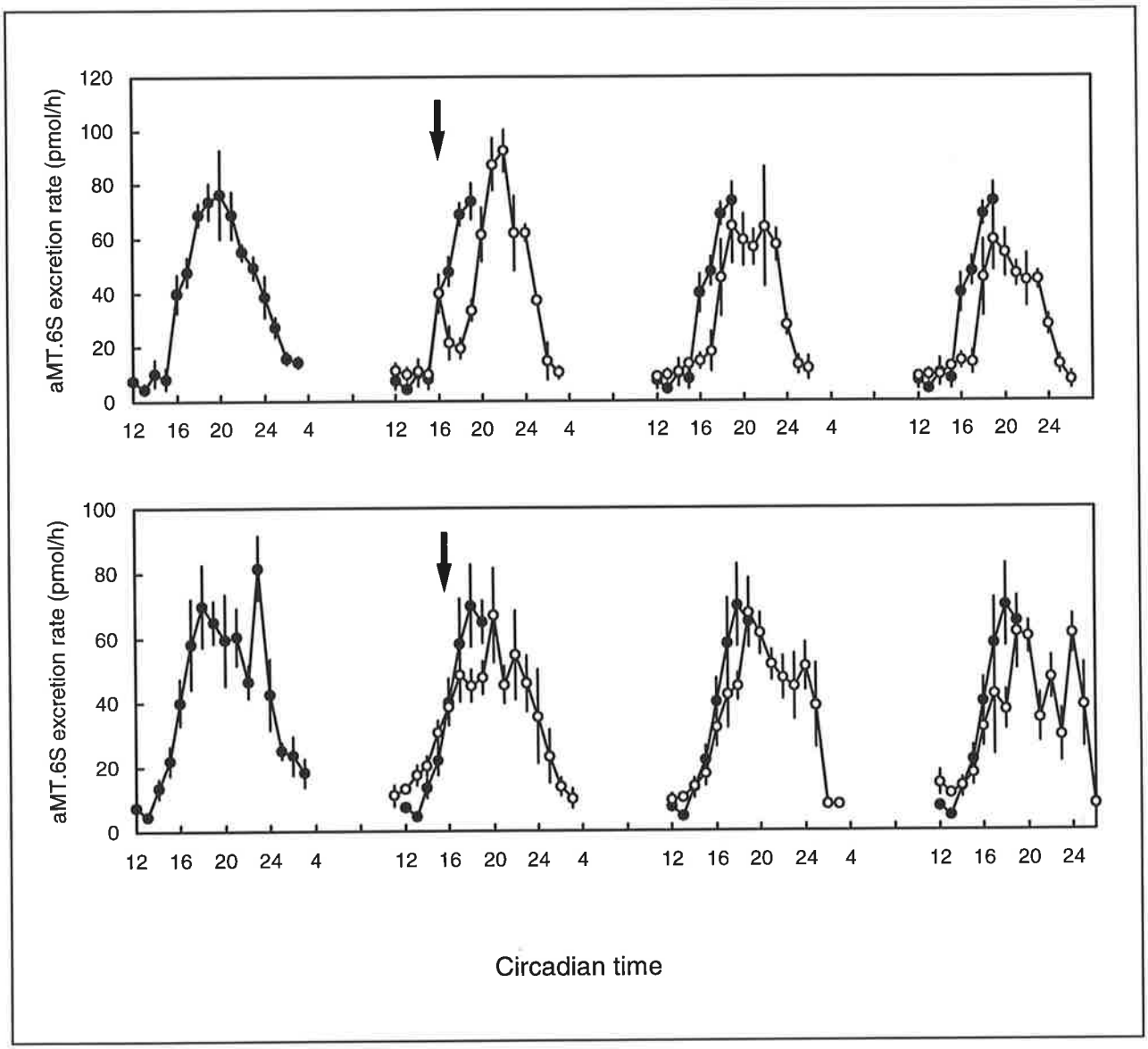

Figure 3.1
6-sulphatoxymelatonin excretion profiles for animals treated with a 1 minute/2 lux light pulse (upper panel) or saline (lower panel) at CT16 (2300h). Four consecutive nights are shown. In each panel data from night 1, represented by solid symbols overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate time of treatment on night 2. Each data point represents the mean ± SEM for five animals.

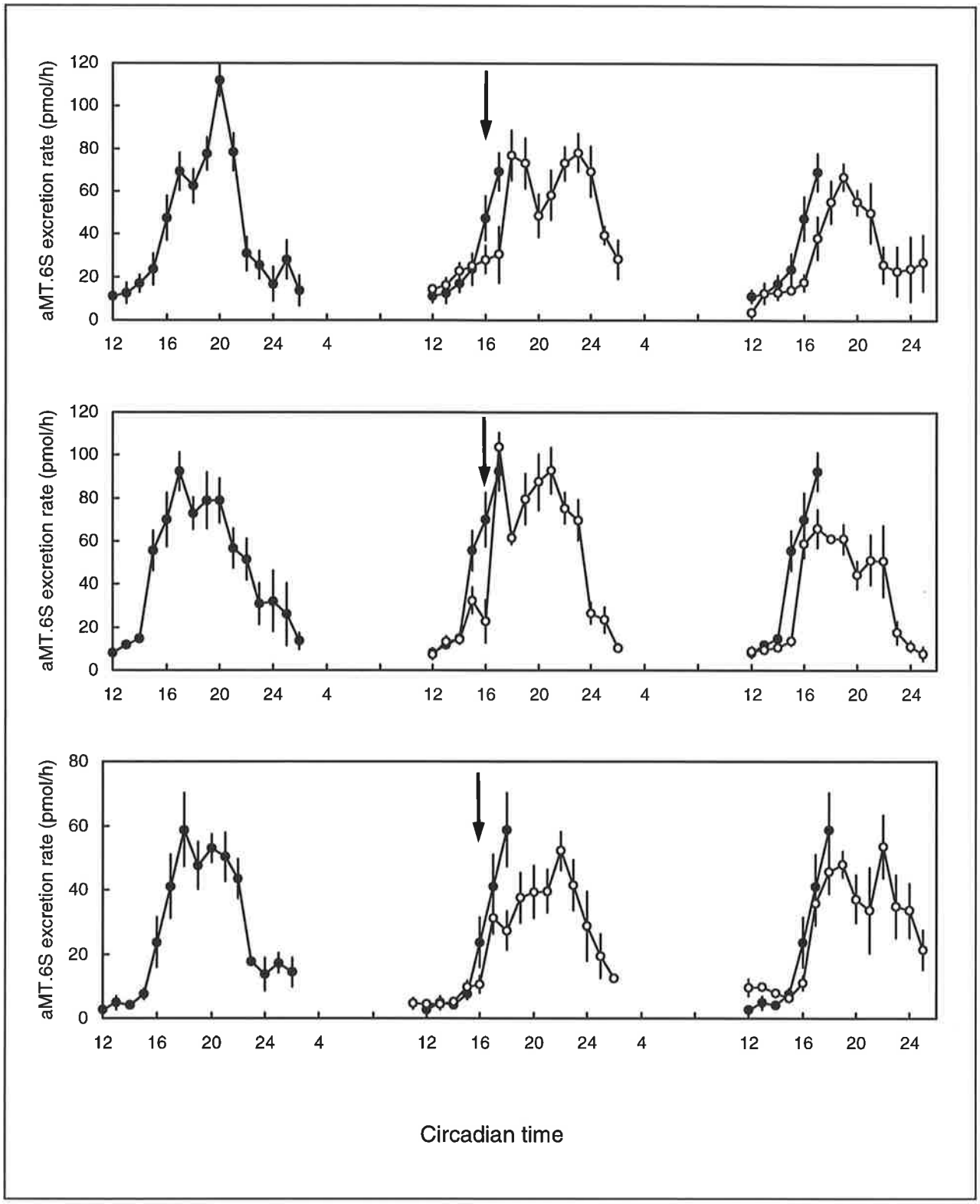

Figure 3.2
6-sulphatoxymelatonin profiles for RB rats treated with nicotine 3mg/kg (upper panel), 1mg/kg (centre panel) or 0.3mg/kg (lower panel) at CT16 (2300 h). Three consecutive nights are shown. In each panel data from night 1 represented by solid symbols overlays data from nights 2 and 3 (open symbols). Arrows indicate time of treatment on night 2. Each data point represents the mean ± SEM for five animals.

Table 3.1.
Effects of cholinergic drugs or light on the timing of the onset of melatonin production in randomly bred rats.

Treatment	Dose	Time	Delay on night 3	Significance
Nicotine	3mg/kg	CT16	2.2 ± 0.5	Y
Nicotine	1mg/kg	CT16	1.7 ± 0.3	Y
Nicotine	0.3mg/kg	CT16	0.1 ± 0.3	N
Nicotine	1mg/kg	CT18	1.7 ± 0.2	Y
Nicotine	1mg/kg	CT14	0.8 ± 0.3	N
Oxotremorine	4mg/kg	CT16	0.1 ± 0.2	N
Oxotremorine	2mg/kg	CT16	0.2 ± 0.1	N
Oxotremorine	0.8mg/kg	CT16	0.2 ± 0.2	N
Light	1 minute/2 lux	CT16	2.3 ± 0.2	Y
Light	1 minute/2 lux	CT18	2.6 ± 0.2	Y
Saline		CT16	0.3 ± 0.1	N
Saline		CT18	0.3 ± 0.1	N

The data show the mean ± SEM (hours, n=5) delay in the onset of aMT.6S excretion on the night following administration of the various treatments.

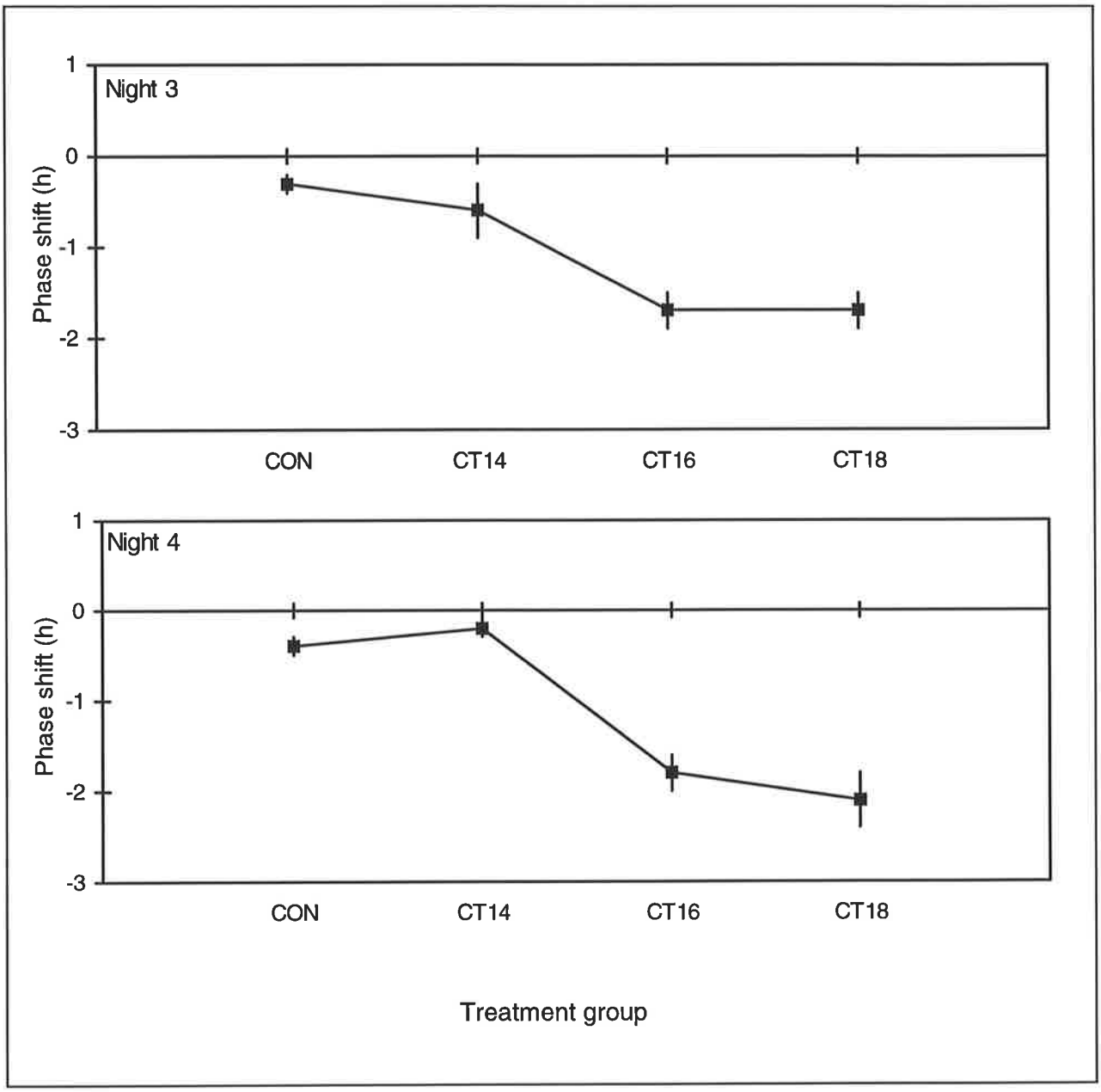

Figure 3.3
Phase shifts caused by nicotine (1mg/kg) administration at various circadian times. Delays from night 1 are represented by negative values. Top panel contains phase shifts on night 3 as compared to the control night and the bottom panel contains night 4 shifts. CON indicates the phase shift of the aMT.6S excretion rate rhythm after treatment with saline at CT16. Each data point represents the mean ± SEM for five animals. Stars indicate a significant delay.

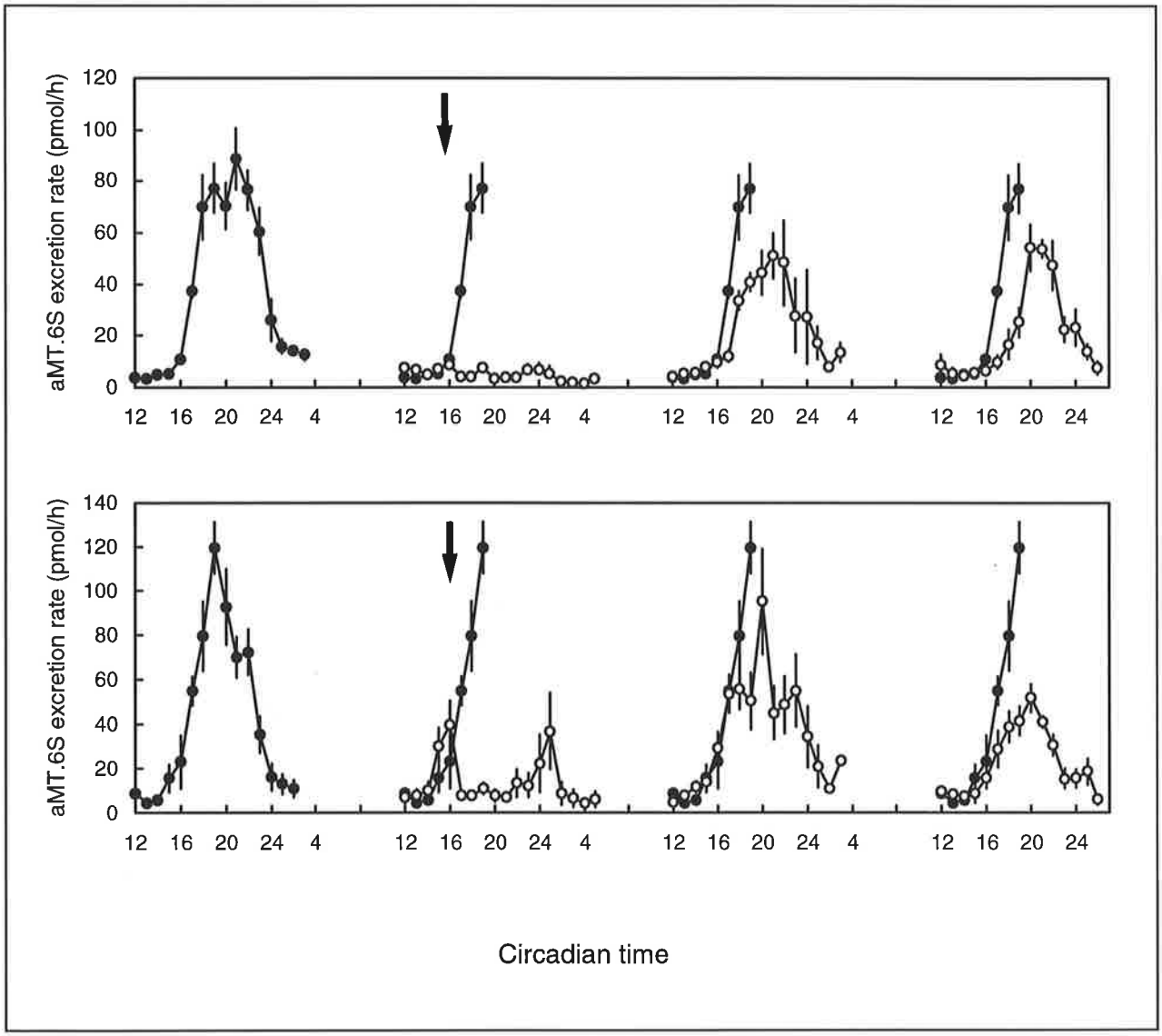

Figure 3.4
6-sulphatoxymelatonin excretion profiles for animals treated with mecamylamine (5mg/kg) at CT15.5 prior to nicotine (1mg/kg) (top panel) or saline (bottom panel) at CT16. Four consecutive nights are shown. Data from night 1 represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate time of treatment on night 2. Each data point represents the mean ± SEM for five animals.

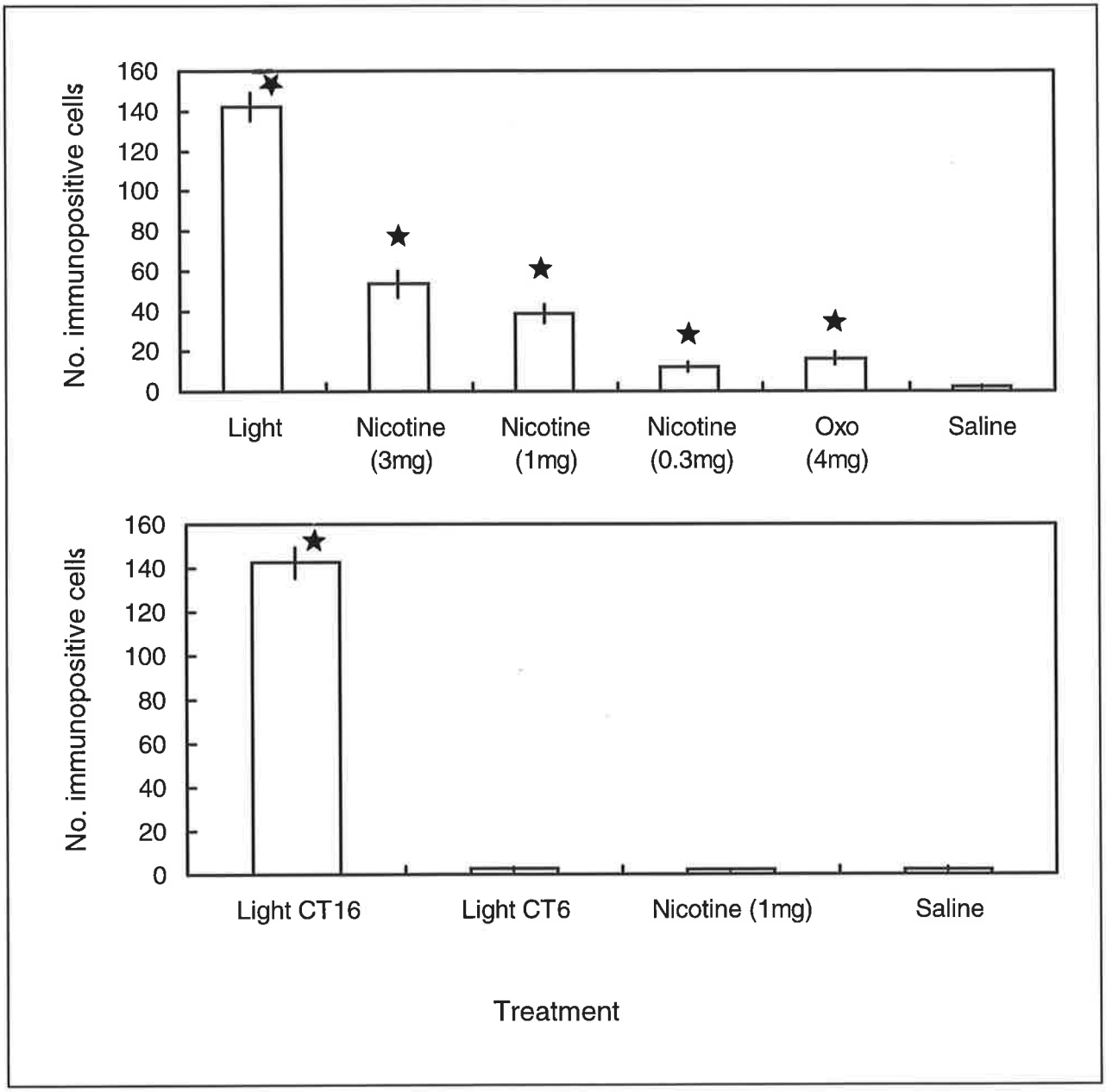

Figure 3.5
The number of immunopositive cells recorded after treatment with cholinergic or light stimuli. The top panel contains data from animals treated with nicotine (3mg/kg, 1mg/kg or 0.3mg/kg sc), oxotremorine (4mg/kg), light (1 minute/2 lux) or saline at ZT16 (4 hours after lights off). The lower panel contains data from animals treated with light (1 minute/2 lux) at ZT16, or light (1 minute/2 lux), nicotine (1mg/kg) or saline at CT6 (after 18 hours of darkness). Each data set represents the mean ± SEM for nine animals. Stars indicate significant difference from saline treated group ($p < 0.05$).

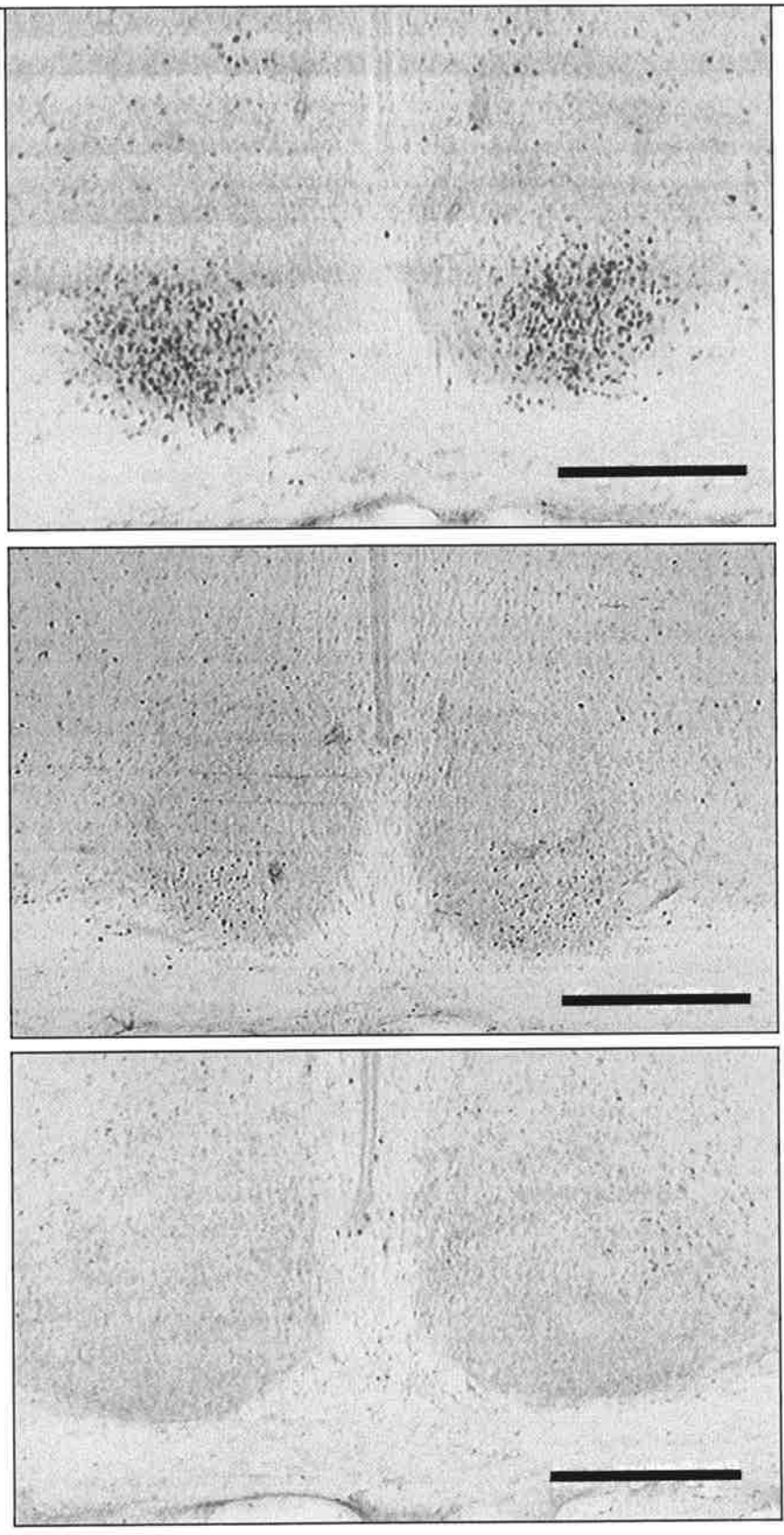

Photo 3.1
Representative micrographs of the SCN region of normal randomly bred rats treated with a 1 minute/2 lux light pulse (top panel), nicotine (1mg/kg) (centre panel) or oxotremorine (4mg/kg) (lower panel) at ZT16. Horizontal bars = 500μm.

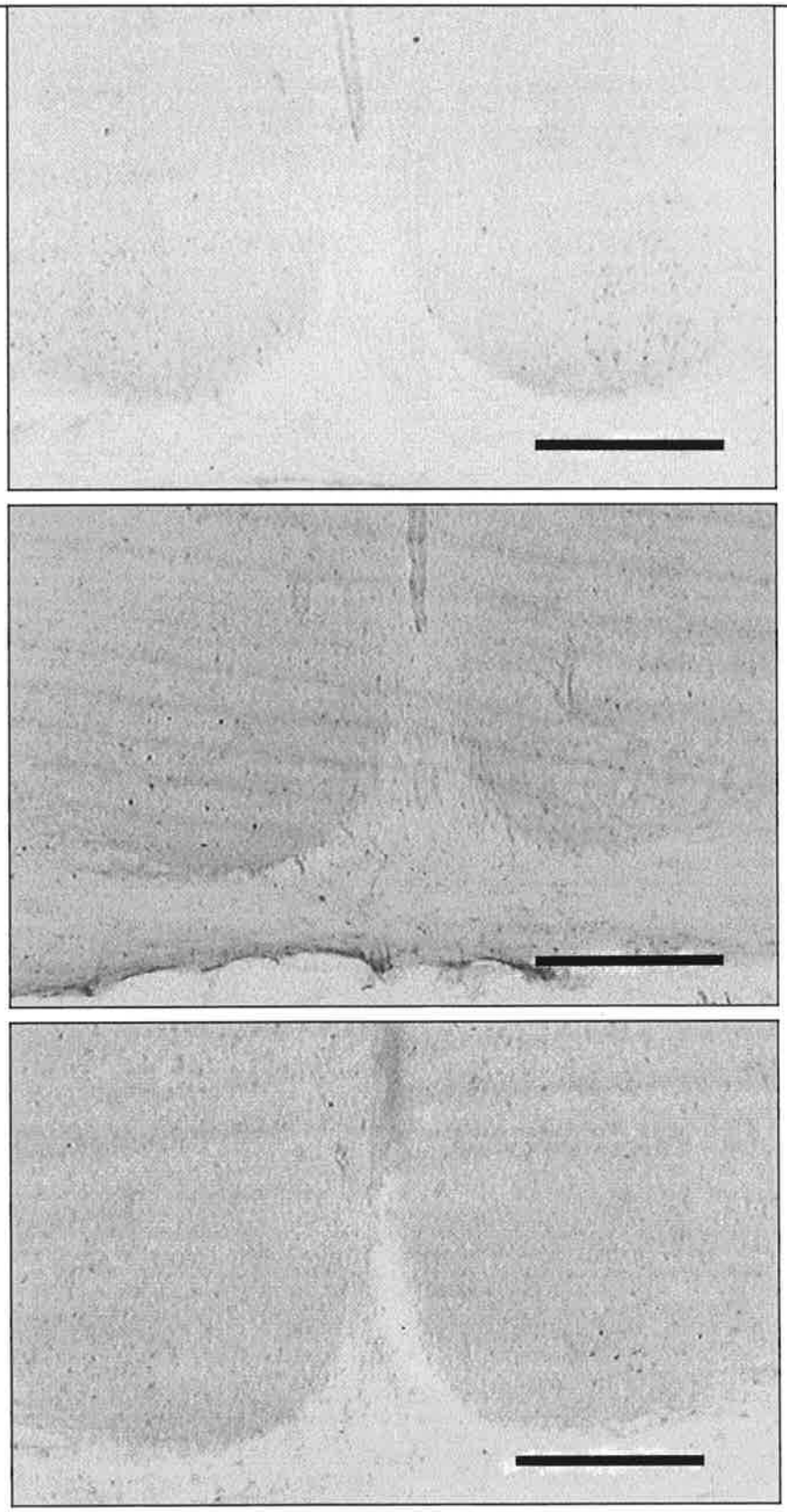

Photo 3.2
Representative micrographs of the SCN region of normal randomly bred rats treated with either saline (top panel), mecamylamine (20mg/kg) (centre panel) or mecamylamine (20mg/kg) 30 minutes prior to nicotine (1mg/kg) (lower panel) at ZT16. Horizontal bars = 500μm.

3.4 DISCUSSION

The cholinergic agonist nicotine delayed the timing of the onset of the 6-sulphatoxymelatonin excretion rhythm in a dose-dependent and time-gated manner. In addition, the drug stimulated induction of the immediate-early gene, *c-fos* in the suprachiasmatic nucleus, a response partially blocked by the nicotinic antagonist, mecamylamine. Oxotremorine (a muscarinic cholinergic agonist) had no effect on the timing of the aMT.6S excretion rhythm but did induce a small number of immunopositive cells when administered at the highest dose. While mecamylamine failed to block the phase shifts of the aMT.6S excretion rhythm it may not be the ideal nicotinic antagonist to use in this system due to the acute effect on melatonin production. Together, this data suggests that acetylcholine, possibly acting through the nicotinic receptor may be important in the mediation of light effects on the timing of melatonin excretion and that the drug is acting (directly or indirectly) on the SCN.

Nicotine at the two higher doses (1 and 3mg/kg) caused significant phase delays in the timing of the aMT.6S rhythm when administered at CT16. The lowest dose used (0.3mg/kg) was not effective and oxotremorine was ineffective at all doses. The highest dose of oxotremorine used in this experiment (4mg/kg) was 5-fold higher than that used to produce a significant decrease in core body temperature during the day (Overstreet, 1991), suggesting that the dose was sufficient to invoke central effects. Using the minimal effective dose of nicotine (1mg/kg), a partial phase response curve was established over three time points in the section of the curve in which light pulses have been shown to elicit phase shifts in the aMT.6S excretion rhythm. A 15 minute light pulse given 4 or 6 hours after dark onset delayed the timing of aMT.6S excretion, however a pulse at 2 hours after dark onset resulted in no delay (Kennaway and Rowe, 1994). The results from this section of the project show that the nicotine-induced delays were phase-dependent in a similar manner to light-induced delays, with the drug effective at CT16 and CT18 but not CT14. Although the protocol used in the previous study applied Aschoff's method IV (phase response in LD) and the present study used method II (acute continuous darkness)(Aschoff, 1965a), the phase delays observed following light treatment 4 hours after (subjective) lights off are comparable (2.4 ± 0.2h; (Kennaway and Rowe, 1994) cf. 2.6 ± 0.2h; present study). Thus, nicotine administered under similar conditions has elicited similar phase delays in the timing of SCN output as measured by aMT.6S excretion, in an identical time-gated manner as light.

The dose-response curve was established using only one post-treatment night to assess the degree of phase shift that occurred after nicotine treatment. Subsequent studies used two post-treatment nights to ensure the accurate interpretation of phase changes. However, the phase delays on night 3 in the dose-response section of this study were analysed in relation to the individual shifts from the control night in addition to the degree of shift seen in normal animals having either no treatment or saline injection (section 2.1.2). Using the system of automated urine collection and RIA in a 4-day protocol, significant delays on night 3 are always followed by similar magnitude delays on night 4. We have not recorded a significant delay using this method on night 3 that was not subsequently maintained on night 4. For these reasons the data gathered from the 3-day tests is considered statistically and physiologically meaningful.

Much of the work examining the role of ACh in the circadian timing system has focussed on the non-specific agonist, carbachol. *In vivo* studies showed that intraventricular administration of carbachol caused phase shifts in the circadian rhythm of locomotor activity of hamsters similar to those seen after a light pulse. Phase advances were demonstrated after treatment during the late subjective night/early subjective morning, and phase delays after treatment during early subjective evening (Meijer *et al,* 1988a). Similarly, hamsters maintained in DD for at least two weeks were treated with intraventricular or intra-SCN carbachol 2 or 10 hours (CT14 or CT22) after activity onset and identical phase changes were reported (Wee *et al,* 1992). This result was repeated in mice also using wheel-running activity as a marker (Zatz and Herkenham, 1981). Carbachol administered intraventricularly to the rat caused phase delays of the N-acetyltransferase rhythm in the pineal gland similar to those seen with light pulses (Zatz and Brownstein, 1979) and continuous infusion of carbachol shortened the free-running period of drinking behaviour of rats (Murakami *et al,* 1986; Furukawa *et al,* 1987). This shortening of the period under continuous carbachol infusion was the same result as seen with constant light administration, thus the cholinergic agonist directly mimicked the effect of light.

More recently, a similar pattern of carbachol-induced phase shifts in hamster locomotor activity was reported and the shifts were blocked with the muscarinic antagonist atropine, but not the nicotinic antagonist mecamylamine (Bina and Rusak, 1996). In a separate study, mecamylamine was shown to block light-induced phase shifts of locomotor activity in the hamster (Keefe *et al,* 1987). This discrepancy between studies using the same species may be explained by a possible interaction between the cholinergic system (specifically mecamylamine)

and the glutaminergic system (O'Dell and Christensen, 1988), as the EAA are a dominant transmitter in the circadian timing system of the hamster. This problem is compounded with evidence that the neural control of SCN function may vary between species. The EAA, primary transmitter in the hamster circadian timing system, appear to be less important in the rat (Rowe and Kennaway, 1996) while serotonin may be more important in mediating effects of light in that system (Kennaway *et al,* 1996; Moyer *et al,* 1997). Much of the early research into the control of circadian rhythms was done using hamsters as a model and it now appears that generalisation of results across species is not valid. It is yet to be established however, if the importance of ACh in SCN function varies between species.

Although the non-specific agonist carbachol has been shown to induce phase shifts in the three main species studied (hamster, rat and mouse), data are not conclusive with regard to the receptor subtype mediating these effects. The use of specific agonists for the cholinergic receptors has been somewhat limited thus far. The early work of Miller *et al* (1987) demonstrated that suprachiasmatic nucleus neurons were responsive to nicotinic stimuli. Both α-bungarotoxin and D-tubocurarine, antagonists of nicotinic receptors, blocked the photic- and carbachol-induced effects on the circadian rhythm of pineal enzyme in rats (Zatz and Brownstein, 1981). A more recent study using the circadian rhythm of neuronal activity in suprachiasmatic nucleus explants has also shown nicotine to be effective at shifting the timing of SCN output in the rat (Trachsel *et al,* 1995). Phase advances were produced by nicotine at all times of the cycle studied. A similar protocol demonstrated carbachol to be effective at causing phase advances while nicotine also 'caused a small phase advance' (Liu and Gillette, 1996). As discussed in the literature review it is important to give results from such studies appropriate emphasis.

The Trachsel study reported phase advances after nicotine treatment at all times of the cycle studied (Trachsel *et al,* 1995), suggesting a lack of phase-dependence of the response of the SCN. This conflicts directly with delays seen after nicotine treatment around CT14-18 in the present study and with the PRC for light pulses (Daan and Pittendrigh, 1976). Together with the large magnitude phase changes (up to 6h) recorded by Liu and Gillette (1996) and commonly reported in *in vitro* studies, it is evident that the SCN responds differently to manipulation *in vivo* and *in vitro* and consequently direct comparison between such studies, particularly with respect to magnitude and direction of phase changes, may not be appropriate. These experiments do indicate that the SCN is responsive to both carbachol and nicotine and

the discrepancy between them may be accounted for by study protocol. The present study however, has shown that nicotine caused phase delays at the same time as a light pulse in the intact animal, while oxotremorine had no effect. Therefore nicotine appears to be mimicking the effect of light on the melatonin rhythm in the rat via a direct or indirect action on the SCN. Mecamylamine was applied in the present study, in an attempt to define the receptor subtype mediating the cholinergic effects. When administered prior to saline the antagonist caused an absolute suppression of melatonin production for almost the entire night but failed to cause any change in the timing of the aMT.6S excretion rate. This suggested action at the level of the input to the pineal gland from the superior cervical ganglion rather than changes in function of the SCN. Mecamylamine administered prior to nicotine also caused absolute suppression but failed to block the nicotine-induced phase delay. As mecamylamine interfered with the actual measurement in this model it is not the most appropriate tool for assessment of receptor action in this system. Further analysis with mecamylamine will need to involve intrahypothalamic administration or the monitoring of alternative rhythms such as temperature or locomotor activity.

The neural mechanisms through which nicotine affects the timing of the circadian rhythm of melatonin excretion were further examined using the induction of the immediate-early gene, *c-fos* in the SCN. Rea (1989) first illustrated the phase-dependent response of *c-fos* induction in the rat SCN, showing that light pulses during the subjective night caused expression of the c-FOS protein whereas pulses during the day did not. Studies in our laboratory have recently shown that a light pulse as short as 1 minute and at an intensity of only 2 lux given 3-6 hours after lights off resulted in a large (though submaximal) number of c-FOS immunopositive cells appearing in the rat SCN and confirmed a lack of *c-fos* induction when light was administered during the subjective day (Moyer *et al,* 1997). The present results show that nicotine caused the induction of the c-FOS protein at the same circadian time as a light pulse (ZT16) and also failed to induce c-FOS at CT6, as did a light pulse in this study. It is important to note the proportion of cells immunopositive for c-FOS protein in the nicotine treated animals was approximately a third of the level seen after a light pulse. The highest dose of nicotine used in this study was 3mg/kg at which the animals experience some hypoxia causing them to gasp intermittently for about two minutes after treatment. An attempt to elicit the same number of c-FOS immunopositive cells with nicotine as followed light using higher doses was not made as the animals would have been unduly stressed. While the treatment (3mg/kg) did cause some degree of physiological stress, this alone could not have caused the induction of *c-fos* in the

SCN as no c-FOS immunopositive cells were identified after treatment with nicotine at CT6. Only one other study has reported the effect of nicotine of *c-fos* induction in the rat and showed that the drug resulted in *c-fos* mRNA production in the fetal SCN, but not the maternal SCN (Clegg *et al,* 1995). The treatment time used by the authors (ZT6-7), is in the 'dead' phase of the adult SCN response curve and a number of studies in addition to the present one, have shown that light does not induce the gene at this time (Rea, 1989; Moyer *et al,* 1997). In the hamster, carbachol was incapable of inducing Fos-like immunoreactivity in the SCN 8 hours after activity onset (Colwell *et al,* 1993a), a result not reported in the rat to date. The animals in that study were processed 60 minutes after a 15 minute light pulse or carbachol treatment at CT20, conditions similar to those reported previously in the rat (Moyer *et al,* 1997). This result differs from that reported in the present study, however the possibility exists that the role of the cholinergic system in light stimulation of the SCN differs between rats and hamsters as it may with respect to the serotonergic and EAA systems and this requires further investigation. The only other c-FOS-related study reported that mecamylamine blocked the CT19 light-induced *c-fos* induction in hamsters (Zhang *et al,* 1993), which as mentioned, may be due to an interaction of mecamylamine with the glutaminergic system. In our hands mecamylamine reduced the number of nicotine-induced c-FOS-positive cells in the SCN by more than 60%, suggesting that nicotine is acting via a nicotinic receptor however, further studies are required using higher doses of the antagonist. The present study is the first to demonstrate cholinergic stimulation of *c-fos* in the SCN of adult rats.

Oxotremorine also induced a small but significant number of immunopositive cells in the SCN, a response similar to that seen with the lowest dose of nicotine (0.3mg/kg). Neither oxotremorine at any dose, nor nicotine at the lowest dose caused delays in the aMT.6S rhythm however, oxotremorine administration at ZT16 did result in *c-fos* induction. It has been suggested that induction of the gene does not necessarily result in subsequent phase shifts or indeed, that phase shifts do not require the induction of the gene (Rea *et al,* 1993b), rather that the two events are separate in a cascade stimulated by light. Alternatively, the number or type of cells positive for c-FOS after treatment may be important in the generation of phase changes. As this data does not supply information about the neurochemistry of the specific cells being activated by the stimuli, it is difficult to make definitive conclusions about the relationship between Fos induction and phase changes in these studies. The possibility remains that the cells stimulated by oxotremorine in this experiment are responding to the thermic effects of the cholinergic agonist. Derambure and Boulant (1994) reported the existence of

temperature-sensitive neurons in the SCN which are thought to be part of the circadian thermoregulatory system. It may be these neurons that have been stimulated by oxotremorine treatment in the normal randomly bred animals. Thus, the two cholinergic agents, nicotine and oxotremorine may be acting via different pathways to induce the *c-fos* gene. Description of the specific cells being activated by these drugs will further define the action of ACh in the circadian timing system.

The mechanisms of action of acetylcholine in the functioning of the SCN are becoming clearer. The presence of the receptors (Swanson *et al,* 1987; van der Zee *et al,* 1991) and the synthetic enzyme (Brownstein *et al,* 1975) for ACh in the SCN, along with the report that ACh levels rise in the nucleus after a light pulse (Murakami *et al,* 1984) supply functional evidence for a role for acetylcholine in the circadian timing system. Excitation of SCN neurons by iontophoresis of ACh (Nishino and Koizumi, 1977) or systemic injection of cholinergic agonists (Miller *et al,* 1987), provide electrophysiological evidence, and the pharmacological studies reporting phase shifts with carbachol and other cholinergic agonists (Earnest and Turek, 1983; Wee *et al,* 1992; Liu and Gillette, 1996) confirm that the cholinergic neurotransmitter system is involved in the regulation of SCN function at some level. A recent study has provided the field with morphological evidence that cholinergic afferents of the SCN may act directly on neurons in the nucleus.

Kiss and Halasz (1996) demonstrated the existence of separate contacts between choline acetyltransferase-immunopositive elements and SCN neurons. There are no cholinergic somata in the SCN (Rao *et al,* 1987; Tago *et al,* 1989) and it is reported that cholinergic neurons whose terminals are located in the SCN may originate from brainstem and forebrain areas (Bina *et al,* 1993), basal forebrain and mesopontine tegmentum (Kiss and Halasz, 1996) and several hypothalamic areas (Rao *et al,* 1987; Tago *et al,* 1989). It may be that the cholinergic pathways from the forebrain/brainstem regions play a modulatory role in light information transfer to the SCN. The lower magnitude phase shifts and smaller number of c-FOS-immunoreactive cells induced by nicotine treatment as compared to a light pulse are possibly due to activation of a different population and/or a smaller number of cells in the SCN. While the population of cells positive for c-FOS after nicotine and light treatment is topographically comparable the specific cells have not been defined. Characterisation of the structure and neurochemistry of the specific cells activated by nicotine and light will further elucidate the precise role of acetylcholine in the circadian timing system of rats.

This section of the project has demonstrated that nicotine administered peripherally can induce time of day-dependent delays in an identical pattern as that seen with light pulses. In addition, the cholinergic agonist had an effect at the level of the SCN as indicated by the induction of the immediate-early gene *c-fos* in the nucleus. This effect was partially blocked with the cholinergic nicotinic antagonist mecamylamine, suggesting the nicotinic receptor is mediating these effects. However, further studies will need to be done by monitoring alternative rhythms to examine the ability of this drug to block phase shifts. This data provides further confirmation that acetylcholine may be acting through a nicotinic receptor to have a modulatory role in the functioning of the SCN of rats.

Chapter 4

The development of the SOX and ROX lines

4.1 INTRODUCTION

For most of this century circadian researchers have been examining the way in which the cycles of physiological parameters are controlled. De Mairan's series of experiments manipulating the light cycles of plants demonstrated the maintenance of rhythmicity under constant conditions and indicated that organisms possess an endogenous pacemaker (DeMairan, 1729). Subsequent work in mammals has defined the location of that pacemaker as the suprachiasmatic nucleus (SCN) in the anterior hypothalamus of the brain (Moore and Eichler, 1972; Moore, 1973). Destruction of this nucleus caused the disruption of a wide range of circadian rhythms (Rusak, 1979; Kafka *et al,* 1985) and transplantation of neural tissue containing the SCN into previously SCN ablated animals resulted in the resumption of rhythmicity (Drucker-Colin *et al,* 1984; Sawaki, 1984). Further, neural pathways from the retina to the SCN, both direct and indirect have also been described, defining in part the manner in which the light/dark cycle entrains the endogenous rhythm of the SCN (Hickey and Spear, 1976; Millhouse, 1977; Pickard, 1982; Shen and Semba, 1994). The complex neuroanatomy and neurochemistry of the circadian timing system is under continued investigation with the aim of elucidating the specific mechanisms by which circadian rhythms are controlled.

Early work on the control of mammalian circadian rhythms was conducted primarily in hamsters. These animals have very strong circadian rhythms in locomotor activity and as such have been used to assess the response of the SCN to many stimuli. From such studies it was determined that the retino-hypothalamic tract (RHT), of which the excitatory amino acids (EAA) are the primary transmitter, is the major pathway involved in circadian rhythm entrainment and light-mediated effects on SCN function (Johnson *et al,* 1988; Meijer *et al,* 1988a). However, this does not appear to be the case for all species utilised in the study of circadian rhythmicity. Rowe and Kennaway (1996) demonstrated that in the rat, EAA probably have a lesser role in SCN function and the same group showed that serotonin acting through the projection from the raphe was important in the rat (Kennaway *et al,* 1996; Moyer *et al,* 1997). Thus, the neurochemistry of the circadian timing system may not be the same in all species and results in one species cannot be regarded as true for all. Further, neurotransmitters such as serotonin, previously thought to play a very minor role in SCN function are now being recognised as vitally important in the mediation of light effects on

rhythmicity in some species. Another neurotransmitter considered to have a relatively minor role, if any in SCN function is acetylcholine.

Acetylcholine appears to play some role in the control of circadian rhythms in most species studied (for review see section 1.6). Briefly, ACh agonists such as carbachol (Zatz and Herkenham, 1981; Wee *et al,* 1992) and nicotine (Trachsel *et al,* 1995) have been reported to cause phase shifts in rhythms as divergent as locomotor activity and neuronal firing rate. Although ACh is not a neurotransmitter of any of the three main SCN afferents, levels were elevated in the SCN after a light pulse at night suggesting a role in light-mediated effects on the circadian timing system (Murakami *et al,* 1984).

With the aim of further investigating the role of ACh in SCN function a unique line of rat was developed based on the breeding program of Overstreet *et al* (1979), who developed the Flinders Sensitive Line (FSL). Animals in the Flinders program were selected on the basis of changes in three physiological variables - drinking behaviour, body weight and core body temperature after challenge with the anticholinesterase di-isopropylflurophosphate (DFP). With respect to the temperature measurement, animals deemed most sensitive exhibited the largest drop in core body temperature after DFP treatment. The Flinders Resistant Line (FRL) was co-selected as a control line for the FSL and was made up of those animals showing the least sensitivity. The FSL animals were reported to have higher densities of muscarinic receptors (while nicotinic receptor numbers were not reported) in some brain regions as well as increased ACh synthesis, accounting partly for their increased sensitivity (Overstreet *et al,* 1984). Most pertinent to this project were the circadian analyses carried out on these animals.

The circadian rhythms of core body temperature, gross motor activity and drinking behaviour were examined in two separate studies under entrained and constant conditions (Shiromani *et al,* 1991; Shiromani and Overstreet, 1994). In the first study transmitters implanted in the abdomen monitored temperature and animals were maintained under normal light/dark conditions for 30 days. Results from this work showed that the peak temperature occurred 2.5h earlier in FSL animals than FRL animals, the authors suggesting this represented a decreased phase angle in the sensitive line (Shiromani *et al,* 1991). The second study monitored drinking behaviour, temperature and gross motor activity under constant dark conditions. The circadian periods of all three rhythms were shorter in FSL than FRL rats by approximately 20 minutes each (Shiromani and Overstreet, 1994). These results implied that

there were inherent differences in the rhythmicity of the circadian timing system between the lines and suggested that these animals might be useful in the study of the role of acetylcholine in SCN function.

The cholinergic upregulation of the FSL animals was inheritable as each new round of offspring displayed increasing sensitivity to acetylcholine (Overstreet, 1991). In addition, FSL and FRL were crossbred to obtain F1 and F2 progeny as well as F1 backcrosses to each parental line. The effects of the muscarinic agonists arecoline and oxotremorine were then recorded in each of the six genetic lines using core body temperature, locomotor activity and responding for water as indices of cholinergic sensitivity. The data indicated that the responses of the genetic crosses were intermediate between the parent lines but resembled more closely the FRL line. This suggested the muscarinic responses were influenced by recessive genes and that the response in the FSL rats had a genetic contribution which was additive (Overstreet, 1991). An advantage exists in using a line of rat with an genetically different brain acetylcholine system in the study of this transmitter in circadian rhythm control. Presently in circadian studies a normal outbred animal (of various species) is subjected to light or drug challenges and any one of a number of circadian rhythms is monitored for phase changes, ranging from locomotor activity to receptor binding levels. An animal such as the FSL rat has an inherently different brain cholinergic system and therefore if ACh is important in SCN function this may be evident in altered timing under LD and/or DD and in altered responses to light and other neurotransmitter agonists involved in the control of circadian rhythms. The possibilities for research into the interaction between acetylcholine and SCN function are extensive.

The FSL and FRL animals have not been maintained in Australia denying further circadian analysis on the lines. Thus, the aim of this section of the project was to establish an independent line based on the approach used to generate the Flinders Sensitive line. The development of a new line of cholinergically sensitive animals will supply the field with a unique opportunity to study acetylcholine and SCN function.

4.2 METHODS

Animals: The breeding program was established with the offspring of 10 pregnant Albino Wistar rats obtained from the Central Animal House, University of Adelaide. As mentioned, the Flinders group substituted the muscarinic agonist oxotremorine as the cholinergic agent and used change in core body temperature as the sole measure of sensitivity. Thus, the present project used only oxotremorine for phenotyping of offspring.

4.2.1 Oxotremorine and temperature

A preliminary study was conducted using 10 normal RB animals implanted with temperature transmitters (section 2.3), to determine the time course of body temperature change after oxotremorine treatment. Animals were housed in individual home cages under a 12L:12D photoperiod for five days to recover from the surgery. At ZT4 (1100h) 5 animals were injected with oxotremorine sesquifumarate (0.25μmol/kg s.c.) and 5 animals with saline. Animals were then left undisturbed for the remainder of the light period. The drop in core body temperature was maximal at 40 minutes post-treatment (Figure 4.1).

4.2.2 Breeding program

The phenotyping procedure was identical to that used by the Overstreet group (Overstreet *et al,* 1979) in that it was carried out over two consecutive days. At 28 days of age each animal was weighed and a baseline core body temperature was recorded. A rectal thermistor inserted 5-6cm was used and temperature was recorded to an accuracy of 1 decimal point. The recording was stable within 30 seconds of insertion avoiding interference of the hyperthermia induced by handling. On the following day animals received a 0.25μmol/kg subcutaneous injection of oxotremorine sesquifumarate (RBI, Columbous, Ohio) and 40 minutes later core body temperature was recorded at the same time as the baseline recording. All phenotyping was carried out between ZT3-5. All offspring from each generation up to G11 were phenotyped in this way. From the founder population the 6 males showing the greatest degree of hypothermia were mated to the 6 females showing the greatest degree of hypothermia to form the Sensitive to OXotremorine (SOX) line. In the same way the 6 males exhibiting the least hypothermia or greatest hyperthermia were mated to the corresponding 6 females to form the Resistant to OXotremorine (ROX) line. When each generation was phenotyped selection for SOX breeders was made only from SOX offspring and ROX breeders only from ROX offspring. No brother-sister pairings were made. After generation 2 seven breeding pairs

were selected for each line to maintain enough animals for experimental work and to decrease the possibility of inbreeding.

Breeders were put together for a period of approximately two weeks at a time. The male was removed before parturition. Offspring were weaned at 21 days of age. When more than one litter was required from any breeding pair the male was returned to the female after offspring were weaned. During the phenotyping, selection and breeding program various physiological parameters were recorded for comparison between the two developing lines.

4.2.2.1 Breeding parameters

Fecundity, fertility and male:female ratio were recorded.

4.2.2.2 Physiological parameters

Body weight at 28 days of age and baseline body temperature were recorded at the time of phenotyping.

4.2.2.3 Selection parameters

Change in body temperature after cholinergic challenge and the difference in this parameter between the sexes of each line was examined.

4.3 RESULTS

4.3.1 Oxotremorine and temperature

Figure 4.1 shows the change in body temperature after treatment with oxotremorine or saline at ZT4. Handling of the animals caused a transient hyperthermia in both the control and treatment groups. A significant drop in temperature was recorded in the oxotremorine group compared to the saline group, which returned to near the pre-treatment value. The maximum decrease in core body temperature occurred 40 minutes post-injection.

4.3.2 Breeding program

4.3.2.1 Breeding parameters

The fertility of SOX and ROX animals was not different, either from each other or RB rats. Of a possible 75 litters born to virgin females, SOX produced 65 and ROX produced 60 litters. The number of possible litters across the entire breeding program including second and third rounds of a single generation was 123. From this number SOX animals produced 108 and ROX 96 litters. The difference was not significant as determined by t-test. The fecundity of the SOX and ROX lines was significantly lower than that of RB rats, which produced an average of 12.3 pups/litter. Virgin SOX and ROX females gave birth to 11.0 pups/litter and 9.9 pups/litter respectively. Across the entire breeding program, SOX parents produced 11.1 pups/litter, a significantly higher number of offspring compared to the fecundity of ROX which was 10.1 pups/litter. The ratio of males to females was identical in each line to that of RB rats (ie 1:1).

4.3.2.2 Physiological parameters

Baseline core body temperature was recorded at 28 days of age at the time of phenotyping. This measure was recorded between ZT3-5 in all generations. There was no significant difference between the lines in baseline temperatures at any generation and the average across all generations was 37.63°C for SOX animals and 37.62°C for ROX animals. The baseline temperature of animals selected as breeders was examined independently. There was no difference between the sexes within either line and thus they were analysed together. Across the entire breeding program the SOX breeders were found to have a higher baseline temperature than the ROX animals (37.68°C and 37.28°C respectively). Planned comparison t-tests on each individual generation indicated differences between the lines in G1, G5, G6, G9 and G11.

Body weight at 28 days of age was also recorded at the time of phenotyping. There was no difference between the lines up to and including generation 5, however from G6, the SOX animals weighed significantly less (13% less in G6) at 28 days than ROX. Figure 4.2 shows the average body weight from generation 5, with males compared to males and females to females. Note that this data is only for the first round of each generation. When litter size was included as a covariate in the statistical analysis it was not found to be a significant factor.

4.3.2.3 Selection parameters

The thermic response of the founder population to the cholinergic challenge could be fitted to a normal distribution (Figure 4.3). The range was from a hypothermic response of 2.6°C to a hyperthermic response of 1.1°C with an average decrease of 1.0°C.

The response of the animals selected as breeders was significantly different between the lines in the first generation (Figure 4.4). Subsequent generations have seen an increase in the hypothermic response of SOX animals, but the response of the ROX animals has remained relatively stable. This is reflected in both the divergence of the breeders response and that of the entire population of each generation (Figure 4.4). The average temperature change between the lines within a generation was significantly different from generation 4 and continued to diverge. There was no difference between the responses of males and females in either line in any generation, nor was there any significant trend between generations.

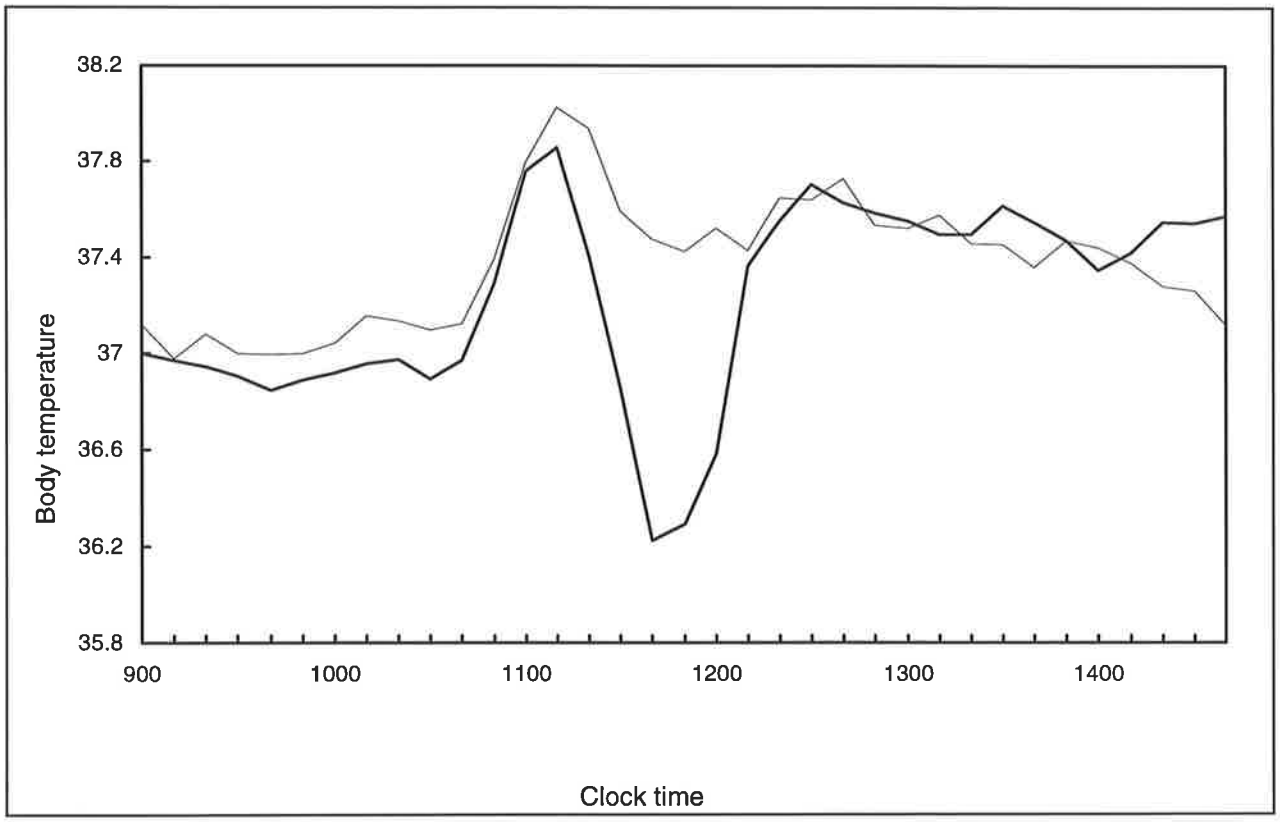

Figure 4.1
The effect of oxotremorine (0.25µmol/kg s.c.) on the core body temperature of randomly bred animals. Five animals were treated with oxotremorine (dark line) at ZT4 (1100h) and five animals received saline treatment (light line). Each data set represents the three point moving average for 5 animals.

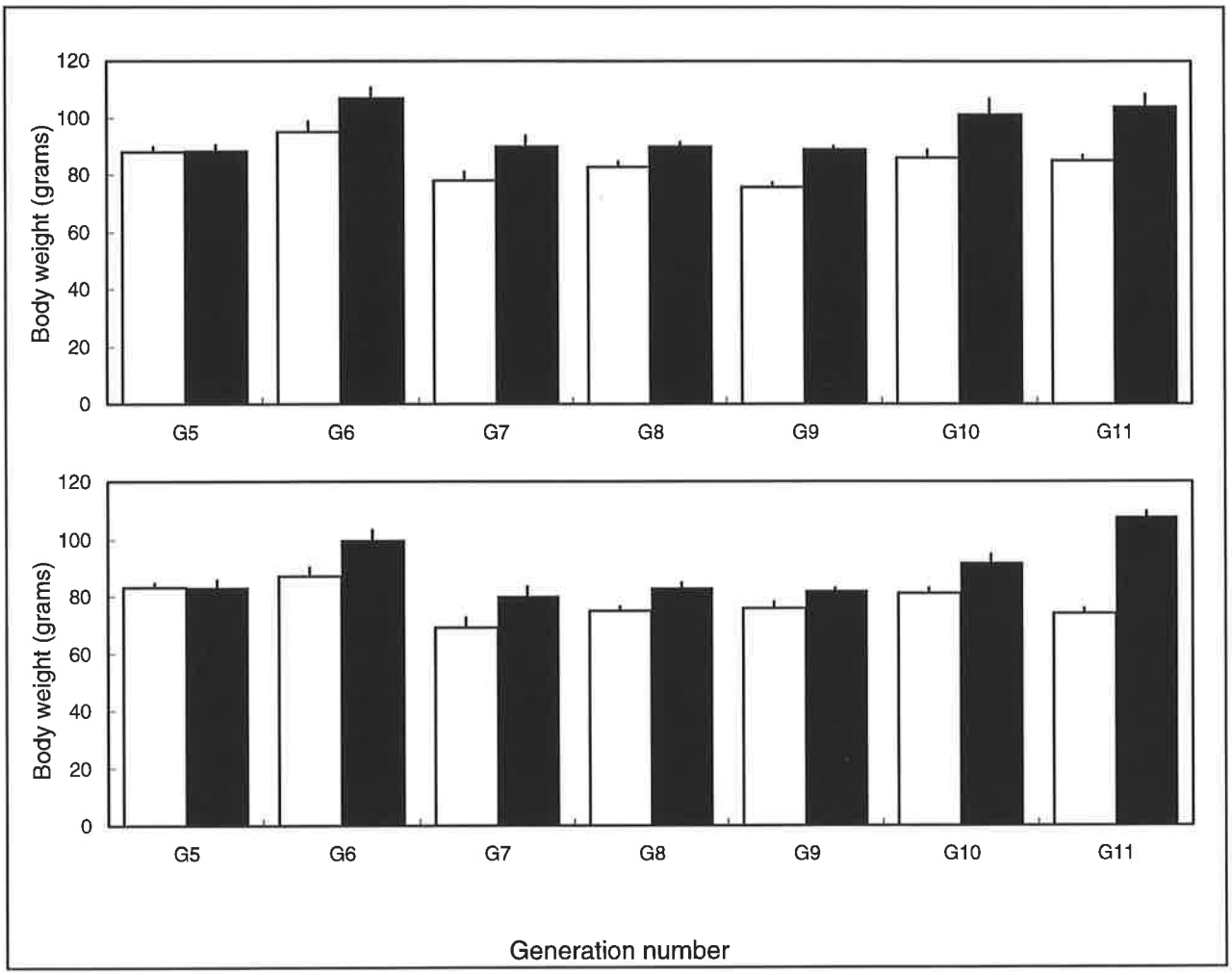

Figure 4.2.
Body weight in grams for all SOX and ROX animals aged 28 days from generation 5 to generation 11. The upper panel contains data from males and the lower panel contains data from females. In each case open bars represent SOX and closed bars represent ROX. Each data set represents the mean ± SEM of 20-50 animals.

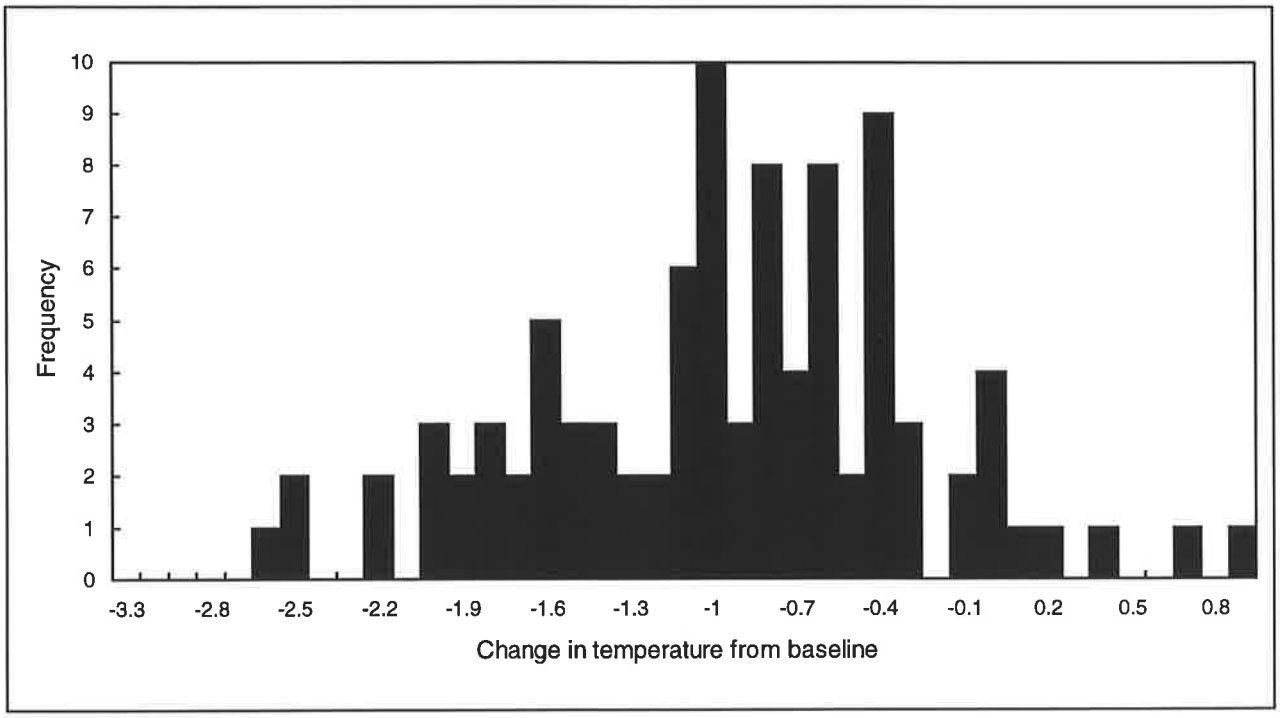

Figure 4.3
The distribution of the temperature response of the founder population to the cholinergic agonist, oxotremorine (0.25μmol/kg). Core body temperature was recorded 40 minutes after oxotremorine treatment and change from individual baseline was calculated. Ninety-four animals were tested and male and female data are combined.

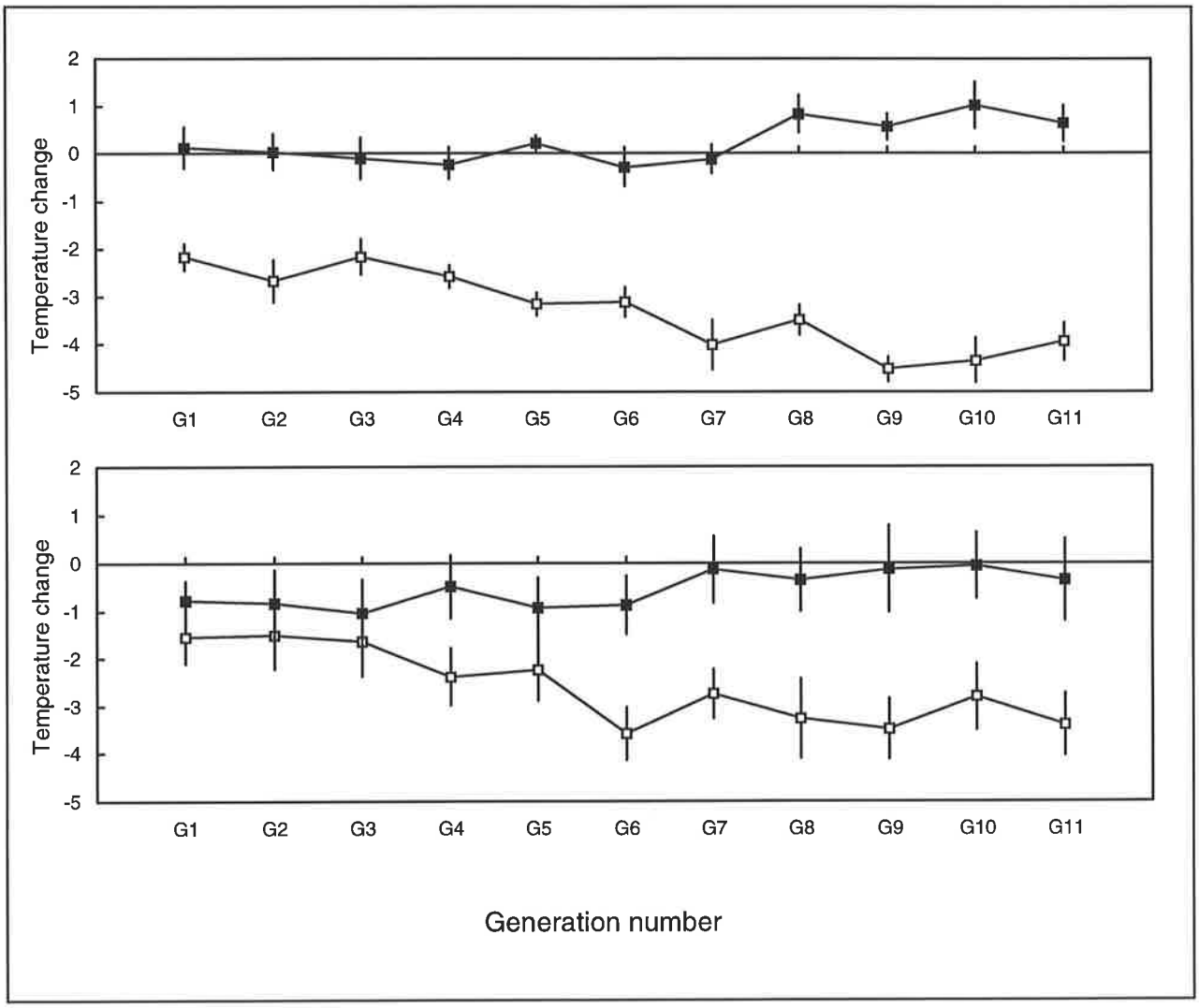

Figure 4.4
Temperature change from baseline in 28 day old animals subjected to cholinergic challenge. The upper panel represents data from animals selected as breeders only (n = 6-7), the lower panel represents data from all offspring for that generation (n = 45-85). Generation number is on the x-axis and change in core body temperature from a baseline recording is on the y-axis. Open symbols represent SOX animals and closed symbols represent ROX animals. Each data point represents the mean ± SD for each group. NB. Male and female data are pooled.

4.4 DISCUSSION

The current breeding program has produced a line of rat with a heightened sensitivity to cholinergic challenge as measured by the thermic response to the acetylcholine agonist, oxotremorine. The Sensitive to OXotremorine (SOX) rats exhibit a large acute drop in core body temperature after cholinergic challenge which increased with each generation to a maximum response of 5.3°C in the eleventh generation. The co-developed Resistant to OXotremorine (ROX) line show a lack of response of the temperature control centres which has changed minimally with each generation. The SOX animals also have a lower body weight at 28 days of age than the ROX animals.

The development of the SOX line of rat was loosely based on previous studies using the Flinders Sensitive Line (FSL) (Overstreet *et al,* 1979). The specific aim of that program was to examine the hypothesis that genetic factors are involved in the sensitivity to the anticholinesterase di-isopropyl fluorophosphate (DFP), as measured by physiological and behavioural variables (Overstreet *et al,* 1979). Subsequently, it was suggested that the FSL were an animal model for the study of human depression (Overstreet and Janowsky, 1991), as the cholinergic system is reported to play an important role in human depressive disorder (Janowsky *et al,* 1972; Janowsky *et al,* 1974). The breeding program employed by the Flinders University group involved the measurement of three independent variables to determine cholinergic sensitivity (Overstreet *et al,* 1979). Core body temperature was used as a measure of the central actions of DFP, drinking behaviour as a measure of peripheral and central regulation by the cholinergic system, and body weight, which was reported to be affected by DFP treatment, would reflect any non-specific effects. Using these parameters two lines exhibiting differing cholinergic sensitivity were developed, and the divergence of the three parameters was significant by F8 (Overstreet *et al,* 1979).

In a later study it was reported that the FSL animals were also more sensitive to the muscarinic cholinergic agonist oxotremorine (Daws *et al,* 1991b) and the phenotyping of further generations was done using this drug as a substitute for DFP and employing core body temperature as the sole measure of cholinergic sensitivity. The development of the SOX line in the present project was done using the hypothermic response to oxotremorine as the only selection parameter as the 1mg/kg dose of DFP reputedly used by the Flinders group was expected to be lethal to approximately 40% of the animals. As oxotremorine was

demonstrated to be as safe and as effective at differentiating between the Flinders lines this program used the cholinergic agonist from the outset.

Throughout the development program of the Flinders lines the animals were maintained under constant light conditions (Overstreet *et al,* 1979). Under such conditions the circadian rhythmicity of melatonin production in the rat is abolished (Honma and Hiroshige, 1978) and other SCN generated cycles can also become arrhythmic (Binkley, 1977; Pangerl *et al,* 1990). It is possible therefore that because of the conditions underwhich the FSL animals were developed there would be some differences between the 2 lines not solely or directly related to the altered cholinergic sensitivity. Animals in the current project were held in a 12L:12D photoperiod to ensure environmental lighting conditions were conducive to proper SCN functioning and the same as those of the randomly bred colony. As a consequence any rhythmicity differences between the lines would not be a result of the lighting regime.

While there was no difference in the baseline temperatures between the lines when all offspring were analysed there was a difference between SOX and ROX animals selected as breeders. Analysis of all breeding animals from eleven generations (male and female data combined) indicated that SOX animals had a higher baseline temperature than ROX animals. This difference was only evident in 5 of the eleven generations and in only 4 of the eight after generation 3, at which the response to the cholinergic challenge had significantly diverged in all offspring. This suggests that the higher baseline was not a factor in the larger temperature change seen in the SOX animals after oxotremorine treatment. If the SOX animals have increased synthesis of acetylcholine (as did the FSL) then it would be expected that their baseline temperatures would be lower as agonists of this neurotransmitter cause hypothermia, however the opposite is evident in the most sensitive SOX animals. Possible mechanisms include an alteration in other neurotransmitter systems impinging on baseline temperature. One mediator of such effects may be the serotonergic neurotransmitter system in the rat. Agonists of the 5-HT_{1a}-receptor such as 8-OH-DPAT cause hypothermia while activation of the 5-HT_{2c} receptors with DOI cause hyperthermia in the rat (Gordon, 1990; Myers *et al,* 1996). If the cholinergic sensitivity was co-selected with altered serotonergic sensitivity or the serotonergic system has been altered as an indirect action then this may be the source of the higher baseline temperature in the SOX animals. This will be discussed further in chapter 9.

The response of the SOX animals to the cholinergic challenge was significantly different from the ROX in generation 4, whereas the divergence between the FSL and FRL animals was only reported to be significant from F8. This discrepancy is likely due to the different selection criteria as the ROX line was not developed as a control line as were the FRL. FRL breeders were selected only from animals showing the least hypothermia in the temperature response parameter, those showing any hyperthermia were not chosen. In contrast, ROX animals showing hyperthermia after oxotremorine treatment were preferentially selected as breeders. As a result, the difference in thermic response between the SOX and ROX lines was more likely to be evident in earlier generations. The ROX animals cannot therefore, be used as control animals in the same way as the FRL animals and it may be that both lines will be equally useful in future studies of the interaction between ACh and SCN function (ie having upregulated and downregulated cholinergic systems). In addition, each set of studies using these lines will need to be done in conjunction with, or compared directly to, studies in normal randomly bred (RB) rats to ensure meaningful interpretation of results.

Early reports on the FSL animals indicated that the S-line males were more sensitive to the DFP treatment than S-line females (Overstreet *et al,* 1979), however in later generations this difference was less evident (Russell *et al,* 1982). In response to this the pairings for the SOX and ROX lines were male-dominated, ie. where rankings indicated the pairing of brothers to sisters, the male animal was used preferentially and paired to a lower ranked non-sibling female. In the early generations it was expected that males of both lines would show heightened thermic response as compared to the females, however this was not the case either in the general population or in the breeding selection. Daws *et al* (1991a) reported no difference between the sexes in response to oxotremorine in FSL and FRL animals 32 days of age taken from the 43rd generation. Thus, at this stage of our breeding program no sex differences are evident in the response to the cholinergic challenge, however this parameter will continue to be monitored.

Body weight was used by the Overstreet group as one of the criterion selection variables to record any non-specific effects of DFP treatment (Overstreet *et al,* 1979) and the FSL animals were reported to have a lower baseline body weight than their control line (Russell *et al,* 1982). The authors suggested that this physiological variable correlated with the tendency for human depressives to also have lower body weight. Although the SOX animals have not been examined as an animal model for the study of human depression, they do exhibit similar

characteristics to the FSL animals. From generation 6 the SOX animals weighed significantly less at 28 days of age than the ROX animals. The other physiological parameter measured in the Flinders lines was locomotor activity as recorded by line crossings in an open field chamber. This has not been looked at as such in our animals, however general activity will be discussed in chapter 11.

The FSL animals were reported to be more sensitive to the effects of serotonergic as well as cholinergic agents as measured by larger thermic responses to the serotonergic antagonist cyproheptadine and the agonist mCPP (Wallis *et al,* 1988). Serotonin is reported to play a vital role in the satiety/hunger regulation system (Kitchener and Dourish, 1994; Dryden *et al,* 1996) and effects the amount of food intake possibly via interaction with neuropeptide Y (Myers *et al,* 1996). The lower body weight in the SOX animals may therefore be an indirect result of altered levels of serotonergic sensitivity in these animals. Lower body weight was also reported in the FSL animals however possible mechanisms were not discussed (Russell *et al,* 1982). As acetylcholine is not reported to have any specific role in satiety mechanisms this may provide evidence for altered serotonergic sensitivity in the SOX animals.

The similarities between the SOX and FSL animals with respect to the continued divergence of the temperature response and difference in body weight suggest that the changes made to the Overstreet protocol have resulted in the development of an independent model of genetic upregulation (and possibly downregulation) of the cholinergic system.

Chapter 5

Circadian rhythms in SOX and ROX in normal lighting

5.1 INTRODUCTION

The Flinders Sensitive Line (FSL) of rat was reported to have an upregulated brain cholinergic system manifested in a large drop in core body temperature as a response to cholinergic challenge (Overstreet, 1991). An increased number of brain muscarinic receptors in addition to increased ACh synthesis were thought to be factors partially responsible for this increased sensitivity (Overstreet *et al,* 1984). It was also reported that the timing of the circadian rhythms of temperature, locomotor activity and drinking behaviour was altered in the FSL rat (Shiromani *et al,* 1991; Shiromani and Overstreet, 1994). These studies provided the first indication that genetically altered sensitivity to ACh agents could be correlated with changes in the functioning of the circadian timing system.

There is an ever expanding literature base intimating a role for acetylcholine in SCN function (for detailed review see section 1.6). Carbachol, a non-specific ACh agonist caused phase shifts in the circadian rhythm of locomotor activity in hamsters (Wee *et al,* 1992), rats (Furukawa *et al,* 1987) and mice (Zatz and Herkenham, 1981). Nicotine, a nicotinic cholinergic agonist, changed the phase of the melatonin rhythm in rats (see chapter 3) and neuronal firing rate rhythm in rats (Trachsel *et al,* 1995). Taken together these studies suggest an important role for ACh in SCN function and suggest that the FSL rat was a unique model for the study of that interaction. The development of the independent SOX line has produced a similar line of rat with inheritable cholinergic sensitivity. Importantly, this has enabled us to monitor differences in rhythmicity of the two lines from the outset of the breeding program. The Flinders animals were bred under conditions of constant light, and it is not possible to predict what effects such a lighting schedule had on the development of the circadian timing system. It was the aim of this study to examine aspects of circadian rhythmicity of SOX animals in each generation under a 12L:12D photoperiod compared to both ROX and randomly bred rats.

Most widely used to monitor the functioning of the circadian timing system are the rhythms of body temperature and locomotor activity as in most species these are very robust rhythms and phase shifts are easily assessed. Another rhythm commonly used is the production of the hormone melatonin. Melatonin is produced by the pineal gland in the brain and can be measured in plasma, saliva, directly from the pineal or via it's metabolite, 6-sulphatoxymelatonin (aMT.6S) in the urine. In animals, melatonin rhythmicity is solely

controlled by output from the SCN and as such no other factors influence the timing of production. In rats, aMT.6S in urine has been used very successfully as an accurate marker of the response of the SCN to light and a variety of neurotransmitter agonist/antagonists (Kennaway, 1993; Kennaway *et al,* 1996; Rowe and Kennaway, 1996). In addition, the aMT.6S excretion rhythm has been mapped in various light/dark cycles in normal rats (Kennaway and Rowe, 1994). For these reasons, aMT.6S was used as the major marker for SCN timing in the SOX and ROX rats. Melatonin rhythmicity was mapped in a light/dark cycle in animals of each generation to determine the stage in the breeding program at which differences in timing emerged (if at all) between the lines. To reinforce that differences seen between the lines were SCN-driven, the temperature and general activity rhythms (clock-controlled rhythms) were also monitored for timing changes in a later generation.

5.2 METHODS

5.2.1 Melatonin 12L:12D

The melatonin rhythms in breeding males from each generation were recorded at 35 days of age (ie one week after phenotyping by cholinergic challenge). Animals were placed in individual metabolism cages in light-controlled environment chambers, fed a liquid diet of Osmolite HN (Ross Laboratories, Columbus, Ohio) *ad lib* to promote high urine flow, and allowed to acclimatise for 4 days. The lighting schedule was 12L:12D with lights off at 1900h.

Urine for aMT.6S analysis was collected over 2 consecutive nights into hourly samples from 1800-0900h (section 2.1). Urine vials were weighed and stored frozen for RIA (section 2.2). For each animal, two overnight profiles were produced and the timing of melatonin onset was determined as the time at which the aMT.6S excretion rate rose above 20pmol/h. The average was taken for each animal and the difference in time of onset between the lines in each generation was analysed by t-test.

5.2.2 Temperature and general activity 12L:12D

An assessment of the rhythms of body temperature and general activity of SOX and ROX rats was conducted to determine whether other SCN-controlled rhythms exhibited similar timing. This was only done in animals of the eleventh generation.

Five SOX and five ROX males from G11, aged 35 days were implanted with temperature transmitters which recorded both core body temperature and body movement (section 2.3) and placed in individual home cages in a light-controlled environment chamber in a 12L:12D photoperiod (lights off 1900h). Temperature and activity were recorded automatically under a light/dark cycle for 5 days. The marker used to determine the timing of the temperature rhythm was the morning decline, taken as the time the temperature dropped below the daily average for that animal. The phase marker used to assess the timing of general activity was the activity offset as the transition from light to darkness generally initiates a burst of activity from all animals which masks the onset of both activity and temperature.

5.2.3 Melatonin 8L:16D

To ensure that the differences seen in onset time of aMT.6S excretion between the lines were clock-derived and not a result of altered pineal sensitivity, SOX and RB rats were exposed to an advance of the time of lights off of 4 hours to remove any masking of the melatonin excretion rate by light. Using the automatic collection of urine and RIA for aMT.6S, advances of the aMT.6S excretion rhythm have not been achieved in our laboratory. An advance of the melatonin onset time with the advanced dark onset would therefore imply a pineal mechanism while no change would indicate the early onset in SOX animals was clock-driven.

Fifteen SOX males from G10 and in the top 30% of their respective screens and 5 RB males were housed in individual metabolism cages in light-controlled environment chambers in a 12L:12D photoperiod, with lights off at 1900h. Animals were fed a liquid diet and allowed to acclimatise for 4 days. The experiment was carried out over 4 consecutive nights. The first two nights were used as controls, with lights off at 1900h and on at 0700h and urine collected hourly from 1800 to 0900h. On the following two nights, 10 SOX and 5 RB rats underwent a photoperiod change with lights off at 1500h and on at 0700h. The remaining 5 SOX rats were maintained in 12L:12D. Collections for all animals during the second phase were hourly from 1300-0800h. Urine vials were weighed and stored frozen for RIA (section 2.2).

5.3 RESULTS

5.3.1 Melatonin 12L:12D

The time of onset of the 6-sulphatoxymelatonin excretion rate in SOX animals under a normal 12L:12D photoperiod was significantly earlier in the third generation than both ROX and RB rats (Figure 5.1). The timing of the onset of aMT.6S excretion continued to diverge between the SOX and ROX rats in both directions. Interestingly, ROX animals from G8 and G9 had a later onset than both the SOX and RB rats (Figure 5.2).

The average overnight profile for SOX and ROX animals of the 8th and 9th generations and RB rats shows the earlier onset in the SOX animals and later onset in the ROX than RB animals (Figure 5.2). The peak production is not significantly different ($p < 0.05$) between the groups, however, the total overnight levels differ significantly. SOX animals excreted 520 ± 31 pmol, ROX animals 429 ± 28 pmol and RB rats 544 ± 32 pmol. ANOVA indicated that the ROX animals produced significantly less aMT.6S than the other two groups ($p < 0.05$). While the offset as depicted in Figure 5.2 appears different in the SOX animals this may be an artefact of the collection system. Animals tend to urinate in smaller volumes and less regularly in the latter stages of the collection period making the use of the offset as a phase marker inappropriate.

5.3.2 Temperature and general activity 12L:12D

Under entrained conditions the timing of the morning decline of core body temperature was significantly earlier in SOX animals than ROX animals (Figure 5.3). The temperature offset in SOX animals occurred at zeitgeber time 23.1 ± 0.3h, and in ROX animals at 23.9 ± 0.2h (where zeitgeber time 24 = lights on).

There was no difference in the timing of the offset of the general activity patterns between the groups (Figure 5.4). Both SOX and ROX animals exhibited activity offset in the hour after lights on in the morning (24.7 ± 0.1 and 24.5 ± 0.0 respectively).

5.3.3 Melatonin 8L:16D

The average onset time of aMT.6S excretion rate on nights 1 and 2 was significantly earlier in SOX animals than the RB animals in the 12L:12D photoperiod (15.9 ± 0.1h and 16.5 ± 0.3h respectively). After the lights were turned off 4 hours earlier than normal there was no significant advance seen in the timing of the aMT.6S excretion rhythm in either of the treatment groups, SOX or RB rats (Figure 5.5). The onset times of aMT.6S excretion were the same between the groups after two treatment nights.

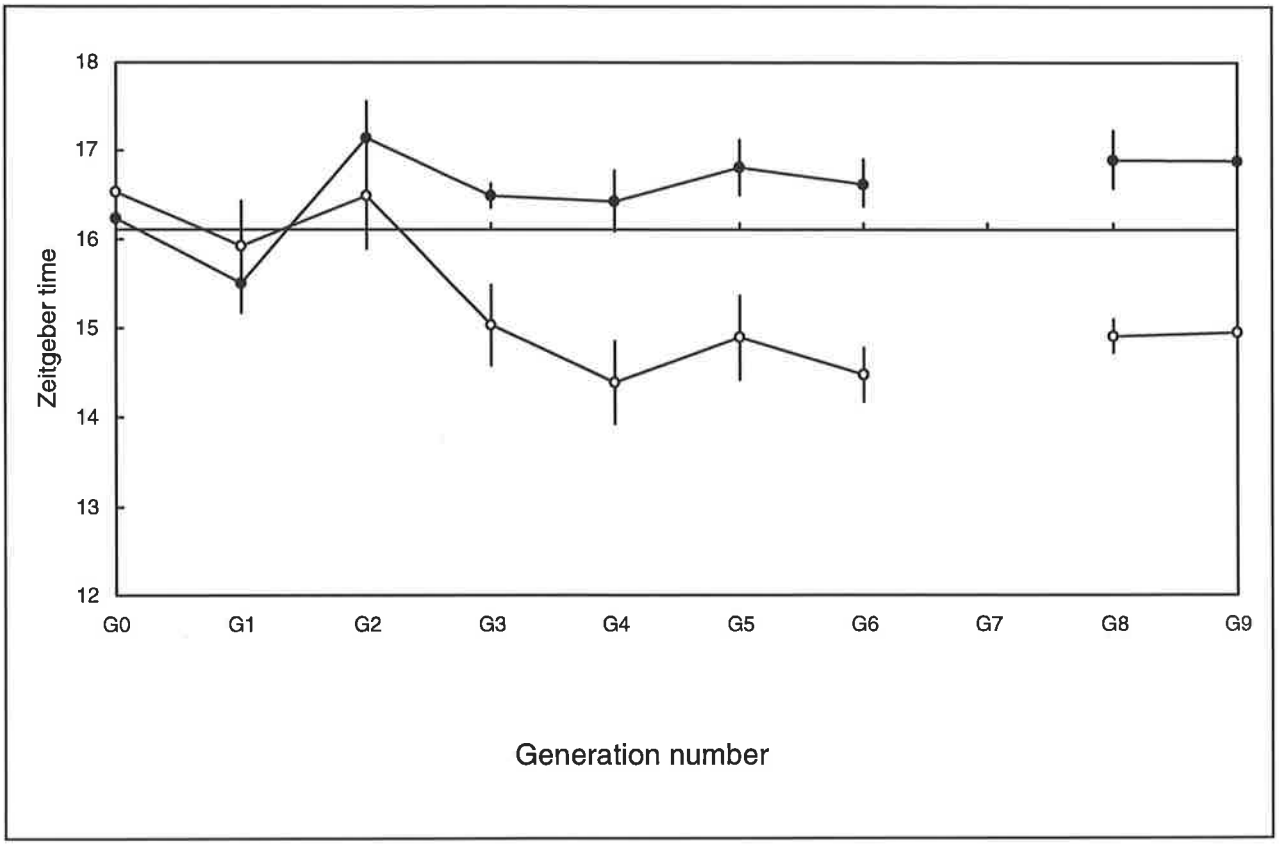

Figure 5.1.
Onset time for SOX (open symbols) and ROX (closed symbols) males as recorded by 6-sulphatoxymelatonin excretion rate onset. Generation number is on the x-axis and refers to the generation the animals were selected from. The x-axis crosses at the onset time for RB rats (ZT16.1). Onset time was defined as the time at which the aMT.6S excretion rate rose above 20pmol/h. Light cycle was 12L:12D with lights off at 1900h (ZT12). All points are mean ± SEM for 5-10 animals.

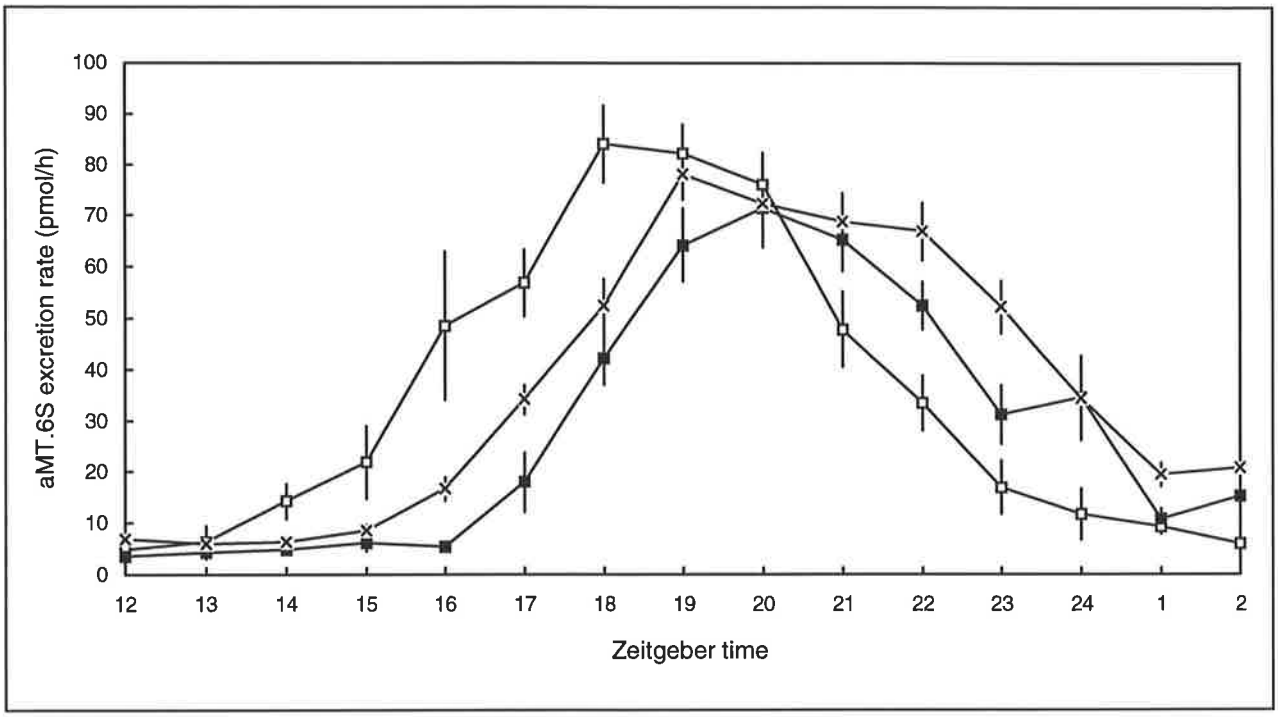

Figure 5.2
6-sulphatoxymelatonin excretion profiles in 12L:12D for SOX (n=20) and ROX (n=20) males selected from G8 & G9 and RB males (n=20). Open symbols represent SOX animals, closed symbols represent ROX animals and crosses represent RB animals. Collections ran from 1800h to 0900h with lights off at 1900h (ZT12). Each data point represents the mean ± SEM. Where SEM bars are not visible they are obscured by the symbol.

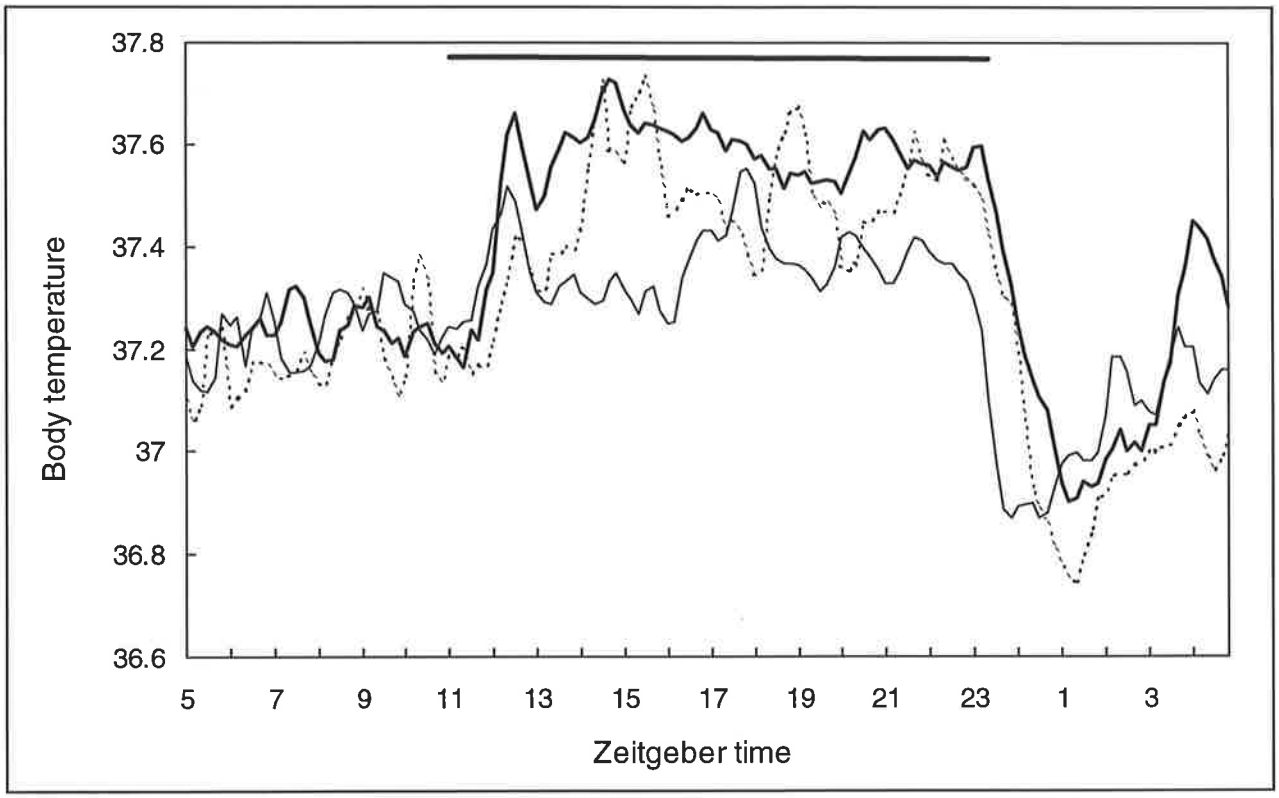

Figure 5.3
Average 24 hour profiles for SOX (n=5), ROX (n=5) and randomly bred (n=10) male rats. Recordings were taken over 5 consecutive days and an average profile was produced for each animal. The group average was then calculated for each time point. The profiles represented are three point moving average 24 hour profiles for each group. The heavy line represents ROX animals, the light line represents the SOX animals and the dashed line represents the normal animals. Light cycle was 12L:12D with lights off at 1900h (ZT12).

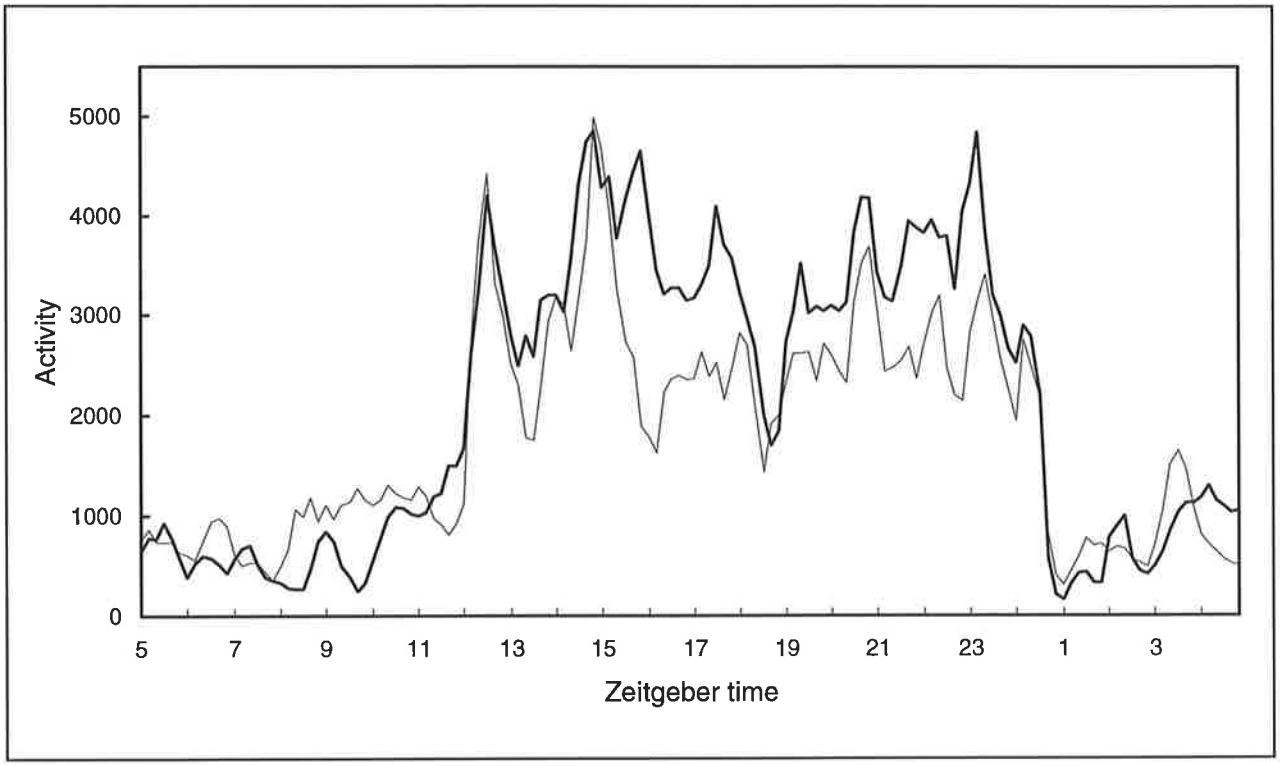

Figure 5.4
Average 24 hour general activity profiles for SOX (n=5) and ROX (n=5) male rats. Recordings were taken over 5 consecutive days and an average profile was produced for each animal. The group average was then calculated for each time point. The profiles represented are three point moving averages of each group. The heavy line represents ROX and the light line represents the SOX animals. Light cycle was 12L:12D with lights off at 1900h (ZT12).

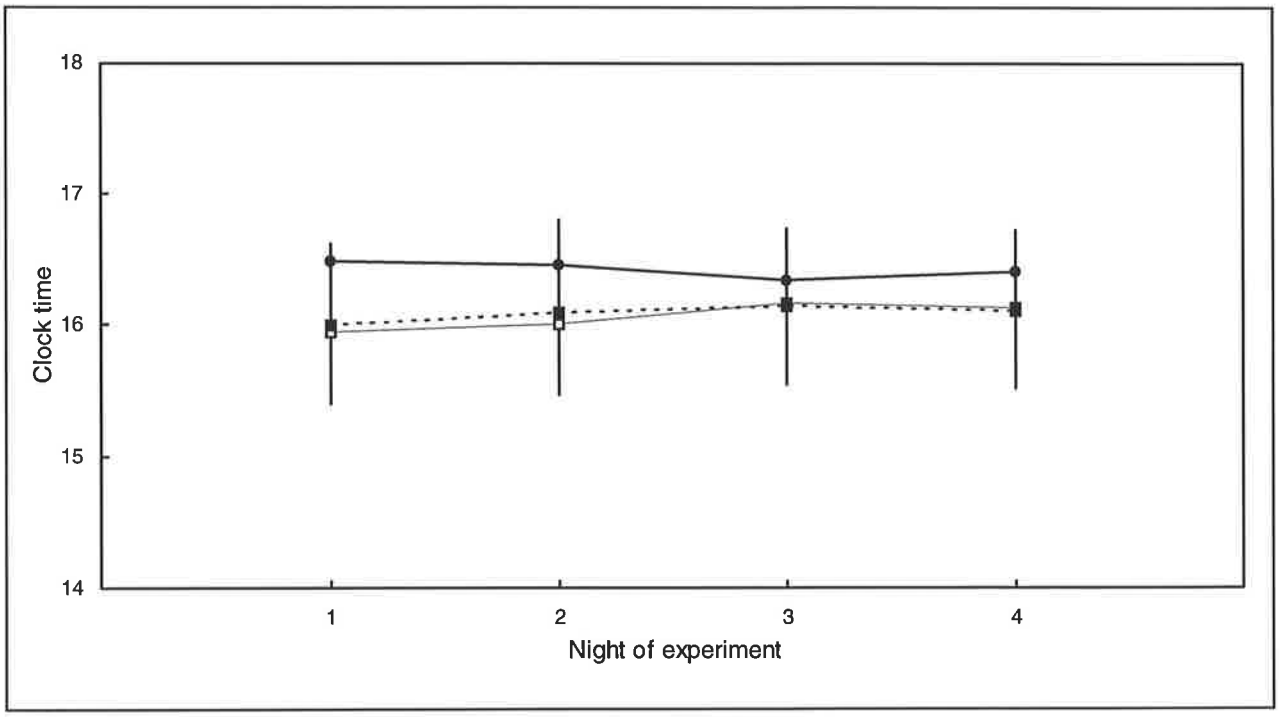

Figure 5.5
6-sulphatoxymelatonin excretion rate onset for SOX and RB rats. Open squares represent SOX animals maintained in a 12L:12D photoperiod with lights off at 1900h (ZT12) for 4 nights. Closed circles represent RB animals exposed to a normal 12L:12D photoperiod for the first two nights of the experiment followed by a 4 hour advance of the time of lights off (ZT8) on the last two nights. Closed squares represent SOX animals exposed to the same conditions. Each data point represents the mean onset time ± SEM for 5-10 animals.

5.4 DISCUSSION

The timing of melatonin rhythmicity in the SOX animals was different from ROX animals from generation 3 and remained so to generation 9. The onset time of excretion of the melatonin metabolite 6-sulphatoxymelatonin was earlier in SOX males than both ROX and RB rats and this altered timing is postulated to be associated with the heightened sensitivity of SOX animals to cholinergic stimuli. In addition, this study showed that the altered timing of aMT.6S excretion was mirrored in the timing of the rhythm of core body temperature, with the SOX animals having an earlier morning decline in temperature than both ROX and RB. Together with the data showing no change in aMT.6S production after early dark onset we conclude that the decreased phase angle of the aMT.6S rhythm and core body temperature rhythm in SOX rats are related to altered cholinergic sensitivity affecting the SCN rather than changes in pineal gland sensitivity.

The Flinders Sensitive Line (FSL) of rat provided the first evidence that cholinergic supersensitivity could be correlated with a shorter free-running period of core body temperature (Shiromani *et al,* 1991). Subsequently, locomotor activity and drinking behaviour rhythms were also demonstrated to cycle with a shorter free-running period in the FSL animals (Shiromani and Overstreet, 1994). The SOX animals were developed specifically to examine the interaction of the cholinergic neurotransmitter system with the SCN, based on the circadian studies in the FSL rat. As discussed in chapter 4, the development of the SOX animals has closely followed that of the FSL with the SOX rats demonstrating similar physiological characteristics of temperature responses and body weight. It was expected that the circadian timing systems of the SOX animals would also function differently and interestingly, the divergence in timing of aMT.6S excretion between the SOX and ROX lines and randomly bred animals has occurred in both directions.

Throughout the Flinders breeding program the FRL were reported to be identical in every parameter to normal outbred animals (up to ~ F60), and were predominantly used as a control line for the FSL animals (Overstreet, 1991). In contrast, the ROX animals have shown an increasing resistance to the cholinergic agonist oxotremorine, as measured with core body temperature response. Further, in this study they have also exhibited a later onset of aMT.6S excretion than both SOX and RB. Animals from generations 8 and 9 show an increased phase angle difference in the rhythms of aMT.6S and temperature than both SOX and RB rats. The

ROX animals were not selected in the same manner as the FRL animals, and therefore have not been used as a control line as such. As mentioned, the FRL animals were bred only from animals showing the least hypothermia, whereas ROX line breeders were selected from those showing the most hyperthermia or least hypothermia. The more extreme temperature responses in the ROX animals may represent a true resistance, unlike the FRL, which Overstreet suggested were not literally a resistant line (Overstreet, 1991). It appears that while the increased sensitivity of the SOX animals is correlated with a decreased phase angle, the decreased sensitivity (or increased resistance) of the ROX animals may be correlated with an increased phase angle.

The FSL animals were reported to have a decreased phase angle difference in temperature under LD conditions (Shiromani *et al,* 1991) and a shorter free-running period of the temperature and locomotor activity rhythms than FRL (reported to have identical period to RB animals) (Shiromani and Overstreet, 1994). Aschoff was the first to associate the phenomenon of a short (or negative) phase angle difference (as measured by locomotor activity in LD conditions) with a shorter free-running period of activity in finches (Aschoff, 1965b). The smaller phase angle difference seen in both aMT.6S excretion and core body temperature rhythms in the SOX animals will be examined further with respect to concurrent changes in free-running period in the next chapter. At this point however, the changes seen in the circadian timing system of SOX animals mirror those reported in the FSL model.

The early study of diurnal rhythmicity of body temperature of FSL and FRL rats by Shiromani *et al* (1991) indicated a 2.5 hour difference in the timing of peak temperature. This suggested that the FSL animals had a phase advance of the temperature rhythm compared to the FRL animals. However, the data in that paper suggest that the peak temperature determined by COSINOR analysis was not an appropriate phase marker in that model. The authors clearly state that the temperature of the FRL 'peaks towards the end of the animals active period' while the FSL temperature 'peaked early'. It would appear from the average temperature profiles that either onset or offset would be a more appropriate marker and would suggest an advance of 1-1½ hours depending on criteria selected. While there is still an obvious phase advance of the temperature rhythm in FSL is may not be truly represented by the marker chosen in this model and was thus exaggerated.

The increased sensitivity to cholinergic stimulation in the SOX animals is as yet unexplained at the molecular level. In the FSL animals, levels of muscarinic receptors were different in the hippocampus and striatum but not the hypothalamus. Such changes in receptor number may also have occurred in brain regions that did not undergo specific study, such as the SCN or pineal gland. The enzymes responsible for synthesis and degradation of acetylcholine (choline acetyltransferase and acetylcholinesterase respectively), are found in the pineal gland of rats (Rodriguez de Lorez Arnaiz and Pellegrino de Iraldi, 1972; Finocchiaro *et al,* 1990) together with both classes of cholinergic receptor, (muscarinic and nicotinic) (Taylor *et al,* 1980; Finocchiaro *et al,* 1989; Finocchiaro *et al,* 1990; Reuss *et al,* 1992; Stankov *et al,* 1993). Cholinergic stimulation of rat pineal glands with the cholinergic agonist carbachol caused increased secretion of melatonin (Finocchiaro *et al,* 1989; Finocchiaro *et al,* 1990; Laitinen *et al,* 1992). It may be argued therefore that the early onset of melatonin secretion in SOX animals is merely a reflection of the increased sensitivity of the pineal to existing cholinergic stimulation. We believe this is not the case for two reasons.

The first is the similar phase angle of the core body temperature and melatonin excretion rhythms of these animals. Temperature, a second clock-controlled rhythm cycles with a similar phase angle as the melatonin secretion rhythm. This suggests that both rhythms are a direct reflection of the timing of the SCN under a light/dark cycle. The second piece of evidence comes from the early dark onset experiment. If the pineal gland was secreting melatonin earlier in the dark phase as a response to increased intrinsic cholinergic stimulation, the inhibition of which is only removed after the lights go out, then we would expect to see a concurrent increase in melatonin excretion rate with an earlier lights off compared to control animals under an identical photoperiod. The SCN would not respond to a phase advance of the light/dark cycle immediately, and in fact using this system we have as yet been unable to obtain a phase advance of the aMT.6S rhythm within our normal period of study (ie – 2 days). Therefore, an earlier onset of melatonin secretion would imply a pineal-driven mechanism, whereas no change would imply a clock-driven mechanism. As reported, the onset of melatonin secretion did not change with early dark onset, implying the decreased phase angle of aMT.6S excretion, together with the altered phase of the temperature rhythm, is indeed a marker of altered SCN timing in these animals.

This is further validated with analysis of the total overnight production of melatonin in each group. Although the SOX animals produce more overnight than the ROX animals, this can be

directly attributed to the earlier onset in these animals. As can be seen in the overnight profiles there is no difference in the peak production, suggesting that the pineal gland is not secreting more melatonin in the cholinergically sensitive SOX animals. While it also appears that the SOX animals have an earlier offset of the rhythm as well, this may be an artefact of the collection system. The animals tend to eat the majority of their food in the first half of the night and as urine retention is minimal, flow is reduced and less regular in the second half of the night. It is for this reason that onset of aMT.6S excretion rate is used preferentially as a phase marker. However, in this experiment the timing of urine flow was not different between the lines (data not reported) and thus the early decline in aMT.6S excretion rate may not be masked by urine excretion rate.

Alternatively, the earlier offset of melatonin excretion levels may be mediated by general activity. This also appears not to be the case as there was no significant difference between the lines with respect to the timing of onset or offset of general activity. The onset coincides with the time of lights off in the evening and in both lines the activity offset is approximately an hour after lights on in the morning. While activity was apparently masked by the LD cycle, both melatonin and temperature continued to exhibit altered timing under cycling light/dark conditions, suggesting that these rhythms were not affected by activity or the light/dark transitions but rather by the biological clock. Thus, the earlier offset in melatonin excretion rate in the SOX animals appears to be real and therefore also clock-driven.

In humans the temperature and melatonin rhythms are inversely correlated and have a very strong yet still not completely defined interaction. In rodents the relationship is directly correlated, such that both melatonin and temperature are elevated during the dark hours and low in the light phase. Human studies have shown that melatonin administration can have direct effects on core body temperature at certain times of the day. Administration of melatonin to rats at ZT3 (3 hours after lights on) caused a significant and sustained drop in core body temperature in a small cohort (n=4) (Morton, 1987). Subsequent to this study very little work has been published on the melatonin/temperature interaction in rodents. The possibility exists that the early offset in the core body temperature rhythm of SOX animals is a result of a pineal/thermoregulation interaction such that the temperature rhythm is masked by the early onset of aMT.6S excretion. This is very unlikely however, as the early onset of aMT.6S excretion was not associated with a concurrent increase in core body temperature in these animals early in the dark period. Indeed, the onsets, although not easily defined are not

different in the profiles of the 3 groups. This is most likely due to increased temperature as a result of the short burst of activity that follows lights off in all animals, resulting in the temperature increase in the early evening being masked by general activity and thus the light-dark transition. Therefore the earlier decline in SOX animals temperature is a reflection of an accelerated rhythm of SCN output.

The endogenous self-sustained rhythm generated by the SCN in mammals is entrained to the external environment via light input. Studies in *Drosophila* gave the earliest indication of the precise mechanisms by which circadian rhythmicity is maintained (outlined in detail in section 1.4.1). It is now well established in *Drosophila* that the circadian period of the animal is governed by the oscillation of mRNA and protein products of the *period* gene and the *timeless* gene. In the morning the *per* and *tim* genes are switched on and the translation of mRNA begins. The proteins are transcribed during the day and around dusk they combine and begin nuclear entry. The peak levels of *per* mRNA occur around ZT16 in normal flies maintained in 12L:12D conditions. The proteins feed back onto the DNA to switch off the genes until the levels become low enough for the genes to be switched on again around lights on. The length of this translation/transcription cycle determines the period length of the organism (Hardin *et al,* 1990). Research has further characterised the molecular mechanisms underlying light-induced phase changes. It was found that light destroys the TIM protein and this effects *per* cycling as the PER protein cannot enter the nucleus without TIM. A light pulse in the evening destroys TIM at a time when the protein products are accumulating and preparing to move into the nucleus. Removal of TIM at this time sets the process back, causing phase delays. Similarly, a pulse in the morning removes TIM at a time when the protein levels are low but still inhibiting translation of the mRNA, so the genes are turned on earlier, causing phase advances (Hunter-Ensor *et al,* 1996). This is suggested as the mechanism for the phase response curves described in mammals.

Under entrained conditions the SOX animals have an earlier onset of aMT.6S excretion and an earlier offset of the temperature rhythm than ROX animals. If the transition from dark to light in the morning is interpreted differently in the SOX animals causing TIM to be depleted faster then this may explain the decreased phase angle in these animals at the molecular level. In light-dark conditions the SCN is under the entraining effects of light and as such the neural pathways and associated neurotransmitters play a role in the timing of the body's rhythms. An upregulated cholinergic system may be directly causing changes in the perception of light by

the SCN, or indirectly through the interactions with other neurotransmitter systems such as serotonin. The serotonergic system has recently been hypothesised as an important mediator of SCN timing in rats (Kennaway *et al,* 1996; Moyer *et al,* 1997) and the projection from the raphe nucleus is suggested as the likely route of this action in the system. The FSL animals were reported to have increased sensitivity to both cholinergic and serotonergic agents (general antagonist cyproheptadine and 5-HT_{2c} agonist mCPP) (Wallis *et al,* 1988) and the altered reception of light information hypothesised in the SOX rats may therefore also be occurring via altered serotonin stimulation. Endogenous changes in the cholinergic (and/or serotonergic) neurotransmitter systems in the SOX animals may cause the perception of the signal of lights on (or off) to be altered as well, resulting in changes in SCN rhythmicity.

This section of the project has demonstrated that the inherited cholinergic sensitivity of the SOX animals is related to a decreased phase angle difference in the onset of the aMT.6S excretion rate and the offset of the core body temperature rhythms. In the most recent generations studied it also appears that the ROX animals, bred for a reduced sensitivity to the cholinergic agonist oxotremorine, exhibit an increased phase angle difference in the aMT.6S excretion rate rhythm onset, as compared to RB animals. This suggests that both lines will supply unique opportunities for the study of the interaction between the acetylcholine neurotransmitter system (and other key transmitters) and the circadian timing system of rats.

Chapter 6

Circadian rhythms in SOX and ROX rats in constant conditions

6.1 INTRODUCTION

The timing of mammalian circadian rhythms is dependent on the interaction between the environmental photoperiod and the endogenous rhythm of neural output from the suprachiasmatic nucleus (SCN). When light cues are removed the circadian rhythms of animals cycle with the phase and period of the self-generated rhythm of the SCN. Human subjects deprived of sunlight for 126 days showed a lengthened free-running period of cortisol excretion (24.8h) (Kennaway and van Dorp, 1991). Hamsters having a lesioned retino-hypothalamic tract, preventing the transfer of light information to the SCN, exhibit a free-running period of locomotor activity, and under constant conditions show varying periods (from $<$24h to $>$24h) of wheel running activity (Johnson *et al,* 1988). Rats in constant dark conditions exhibit periods of temperature and drinking behaviour rhythms of about 24.2h (Stephan, 1983; Kennaway *et al,* 1996; Rowe and Kennaway, 1996). Thus, isolation of the SCN from environmental zeitgebers supplies an indication of the intrinsic, genetically derived rhythm of the pacemaker.

The Tau mutant hamster exhibits a free-running period of locomotor activity of approximately 20h and this is mirrored in the period of the neuronal firing rate rhythm from an SCN isolated from these animals (Davies and Mason, 1994). Thus, the cycle of genetic material responsible for the generation of circadian rhythms in these animals is manifested in the periods of the rhythms of neuronal firing rate and locomotor activity in an identical manner. As outlined in chapter 5, the SOX animals appear to have an SCN which functions differently than ROX and RB rats under cycling light/dark conditions, the SOX rats having a smaller phase angle of the melatonin and temperature rhythms compared to the other groups. The FSL animals, on which the development of the SOX line was based, similarly showed both a decreased phase angle of the temperature rhythm as well as a shorter free-running period of temperature, drinking behaviour and locomotor activity (Shiromani and Overstreet, 1994). It is necessary therefore, to 'isolate' the SCN of the SOX and ROX rats from environmental light cues in an *in vivo* situation to determine the free-running periods of their clocks.

While the early phasetyping of these animals was done using 6-sulphatoxymelatonin as a marker of SCN timing under entrained conditions, in constant darkness the system has some limitations. The liquid diet fed to the animals needs daily replenishment and while every effort is made to prevent this action from providing a possible entraining signal, together with daily

cage maintenance it involves disturbance of the animals. Other systems that escape such limitations include locomotor activity monitoring and core body temperature rhythm monitoring. These rhythms were monitored in SOX and ROX rats at various stages of the breeding program to assess the free-running periods of their circadian rhythms.

6.2 METHODS

Part 1.

Five SOX males and 5 ROX males from generation 2a in the top 5% of their respective screens were selected for this experiment. Animals were placed in individual cages inside a light-controlled environment chamber. Each cage was connected by a tunnel to a running wheel to which the animals had free access (section 2.4). Animals were provided with rat chow and water *ad lib* which required replacing once a week and cages were also cleaned once a week. All cage maintenance was carried out in complete darkness using infrared viewers and an infrared light source.

The experiment ran for four weeks; the first week animals were in a 12L:12D photoperiod with lights off at 1900h after which they were put into constant darkness for a further three weeks.

Part 2.

Five SOX and 5 ROX males from generation 8a in the top 5% of their respective screens were selected for this experiment. Under a 3% halothane/oxygen anaesthesia each animal was implanted with a temperature transmitter in the peritoneum (section 2.3). They were then placed in individual home cages in a light-controlled environment chamber and supplied with rat chow and water *ad lib*. After three days in a 12L:12D photoperiod the lights were switched off and animals remained in darkness for 6 days.

Part 3.

Five SOX and 5 ROX males from generation 11 in the top 10% of their respective screens were selected for this experiment. Temperature transmitters were implanted into the peritoneum of each animal under 3% halothane/oxygen anaesthesia (section 2.3). Animals

were housed in individual cages connected by a tunnel to a running wheel in a light-controlled environment chamber (section 2.4). They were supplied with rat chow and water *ad lib*. After a three day acclimation/recovery period the lights were switched off in the chamber and animals were left in darkness for 5 days during which both temperature and running activity were measured continuously (sections 2.3 and 2.4).

Part 4.

Five SOX and 5 ROX males from generation 12 and in the top 20% of their respective screens were selected for this experiment. Animals were implanted with temperature transmitters under a 3% halothane/oxygen anaesthesia and placed in individual home cages in a light controlled environment chamber (section 2.3). The initial photoperiod was 12L:12D (lights off 1900h) followed by 14 days of constant darkness to assess free-running period. Feeding, watering and cage maintenance were carried out in complete darkness every 5 days using infrared viewers and an infrared light source.

6.3 RESULTS

Part 1.

The average free-running periods for wheel running activity in the two groups of animals from generation 2a were not different from each other. SOX animals free ran with a period of 24.2 ± 0.02h and ROX animals free ran with a period of 24.2 ± 0.03h (Table 6.1). Representative actograms of SOX and ROX animals are shown in Figure 6.1.

Part 2.

The period of the temperature rhythm under free-running conditions was significantly shorter in G8 SOX animals (23.9 ± 0.1h) than G8 ROX animals (24.3 ± 0.1h)(Table 6.1). Figure 6.2 shows the average temperature profiles for both groups from generation eight after 6 days of constant darkness.

Part 3.

SOX and ROX animals from generation 11 also showed different free-running periods of their core body temperature rhythms. SOX animals had a free-running period of 23.9 ± 0.1h and

ROX animals had a period of 24.4 ± 0.1h.(Figure 6.3). The free-running period of wheel running activity was also found to be significantly shorter in SOX animals than ROX animals (Table 6.1) (Figure 6.4).

Part 4.

The average free-running periods of both temperature and general activity were significantly shorter in the SOX animals than ROX animals of generation 12 (Table 6.1). Figure 6.5 shows representative actograms of the temperature rhythms of SOX and ROX animals.

Table 6.1
The free-running period of wheel running, temperature and general activity rhythms of SOX and ROX animals maintained in constant darkness.

Generation	Line	Rhythm	Period
2a	SOX	Wheel running	24.2 ± 0.02
2a	ROX	Wheel running	24.2 ± 0.03
8	SOX	Temperature	23.9 ± 0.1*
8	ROX	Temperature	24.3 ± 0.1
11	SOX	Temperature	23.9 ± 0.1*
11	ROX	Temperature	24.2 ± 0.1
11	SOX	Wheel running	24.0 ± 0.1*
11	ROX	Wheel running	24.4 ± 0.1
12	SOX	Temperature	23.9 ± 0.04*
12	ROX	Temperature	24.3 ± 0.1
12	SOX	General activity	24.0 ± 0.03*
12	ROX	General activity	24.3 ± 0.05

The data show the mean ± SEM (hours, n=5) period of the 3 circadian rhythms studied in
* SOX and ROX animals from various generations. Stars indicate significant difference from RB free-running period.

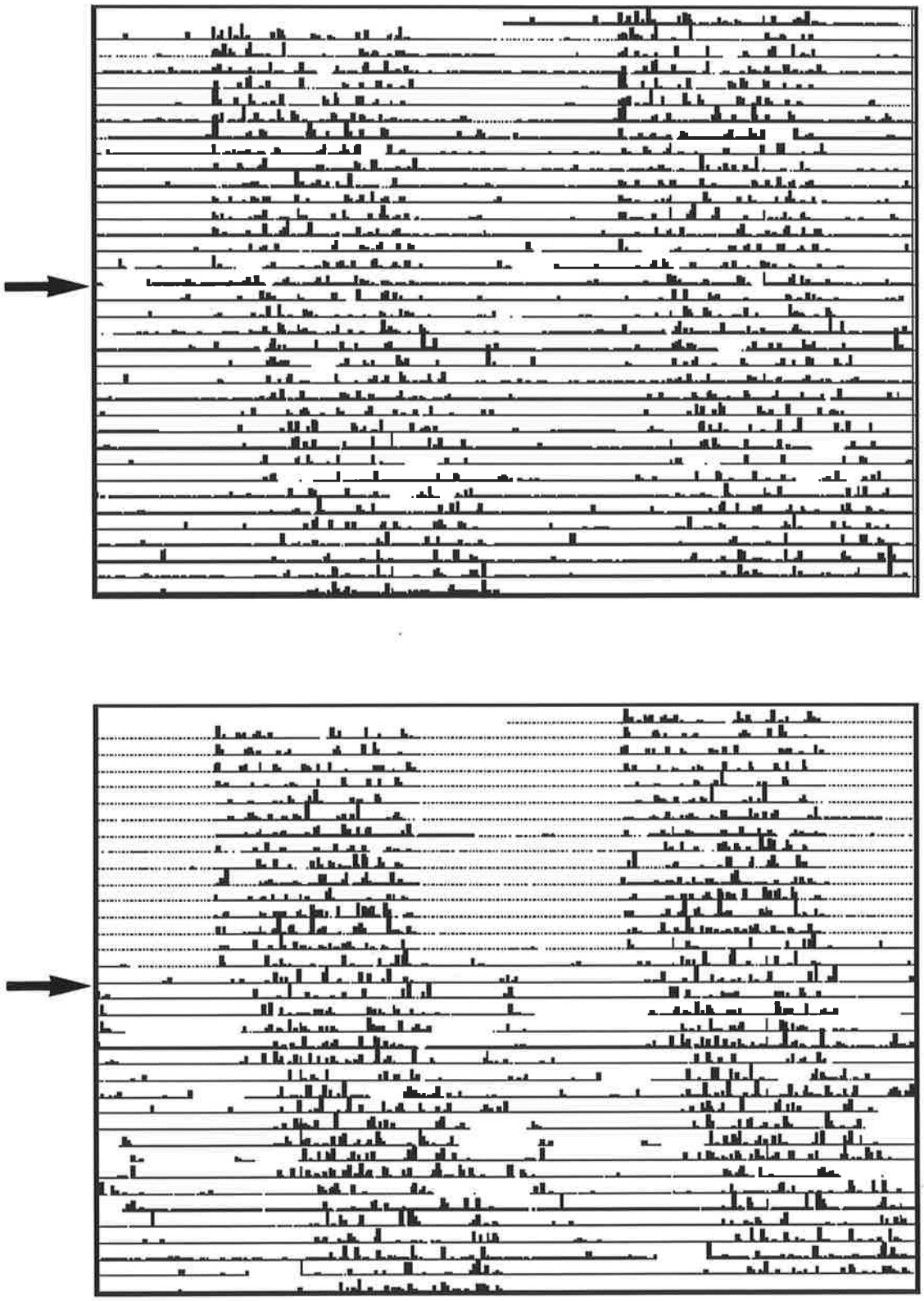

Figure 6.1
Representative wheel running activity actograms of SOX (upper panel) and ROX (lower panel) animals from G2a. Arrows indicate change in photoperiod from 12L:12D (lights off 1900h) to DD.

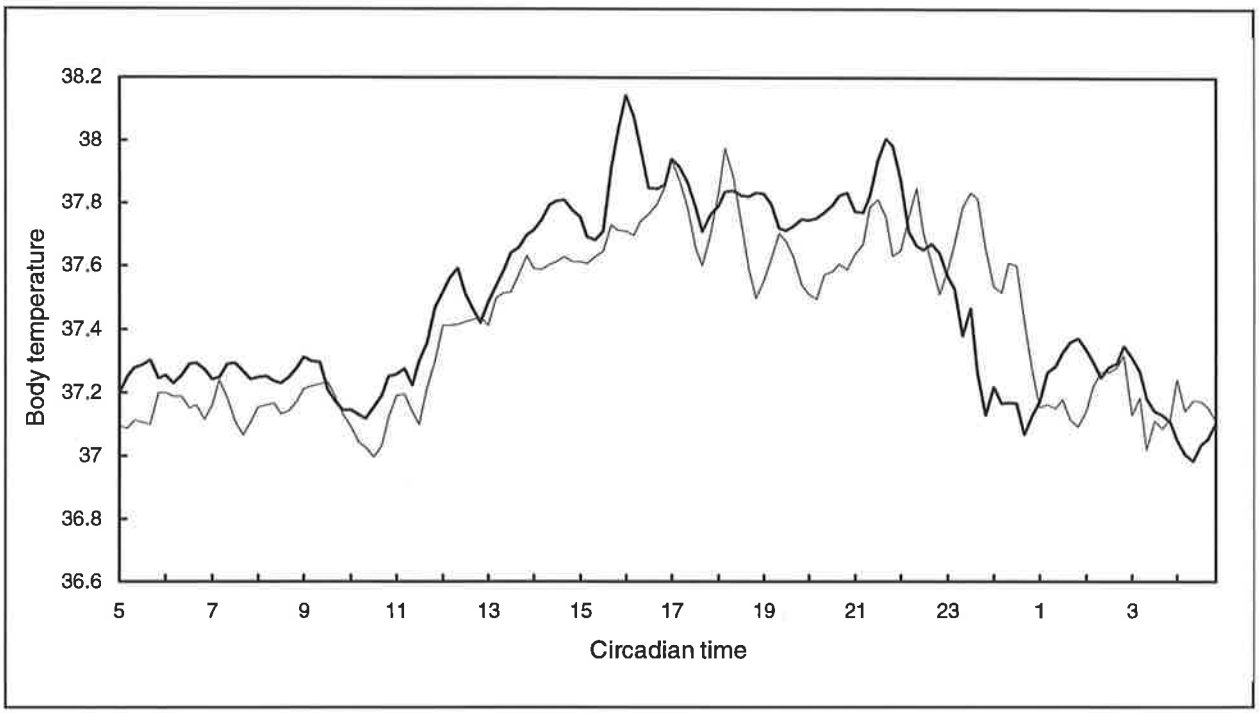

Figure 6.2
Average core body temperature profiles for 5 SOX (heavy line) and 5 ROX (light line) males after six days of constant darkness. Each data set represents a 3 point moving average of the temperature profiles of five animals.

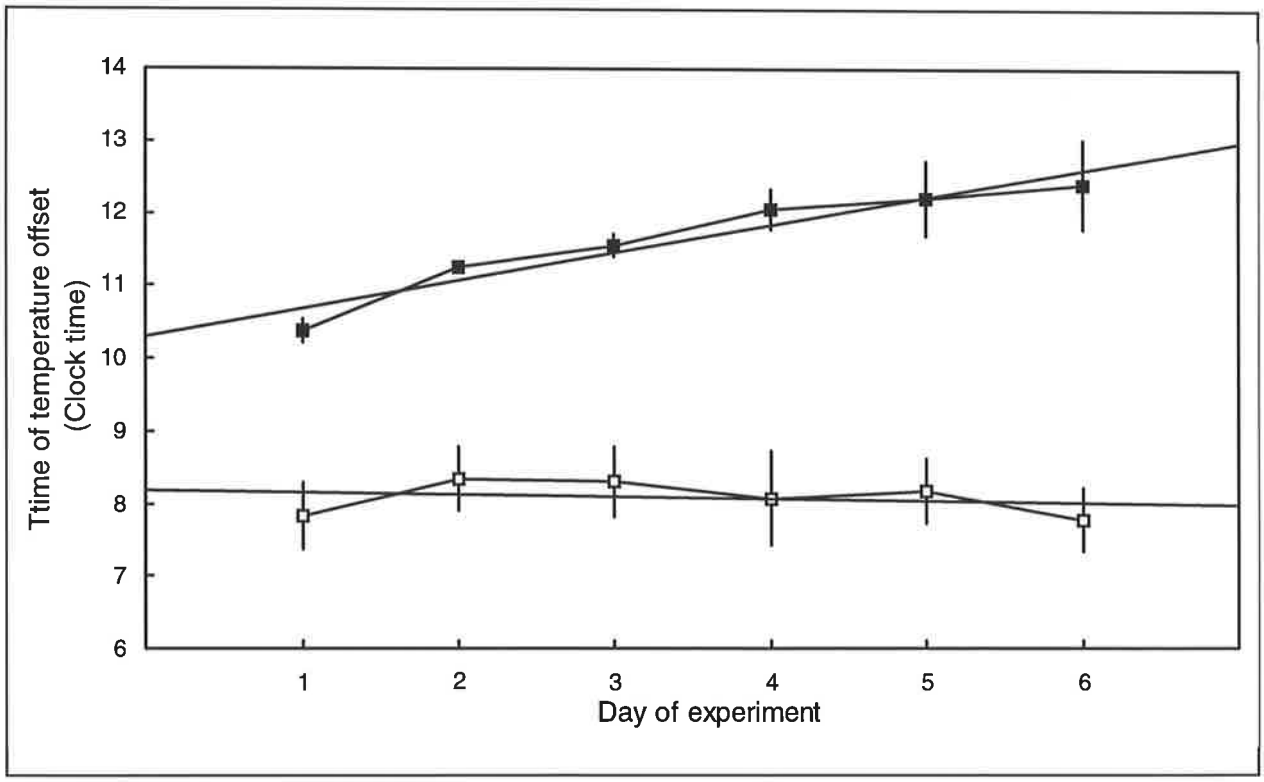

Figure 6.3
Time of the morning decline of core body temperature in 5 SOX (open squares) and 5 ROX (closed squares) under constant dark conditions. Temperature offset was estimated for each animal for each 24 hour period, as the time at which the body temperature dropped below the average for that animal. Each data point represents the mean ± SEM for 5 animals. The line through each data set represents the trendline for the data.

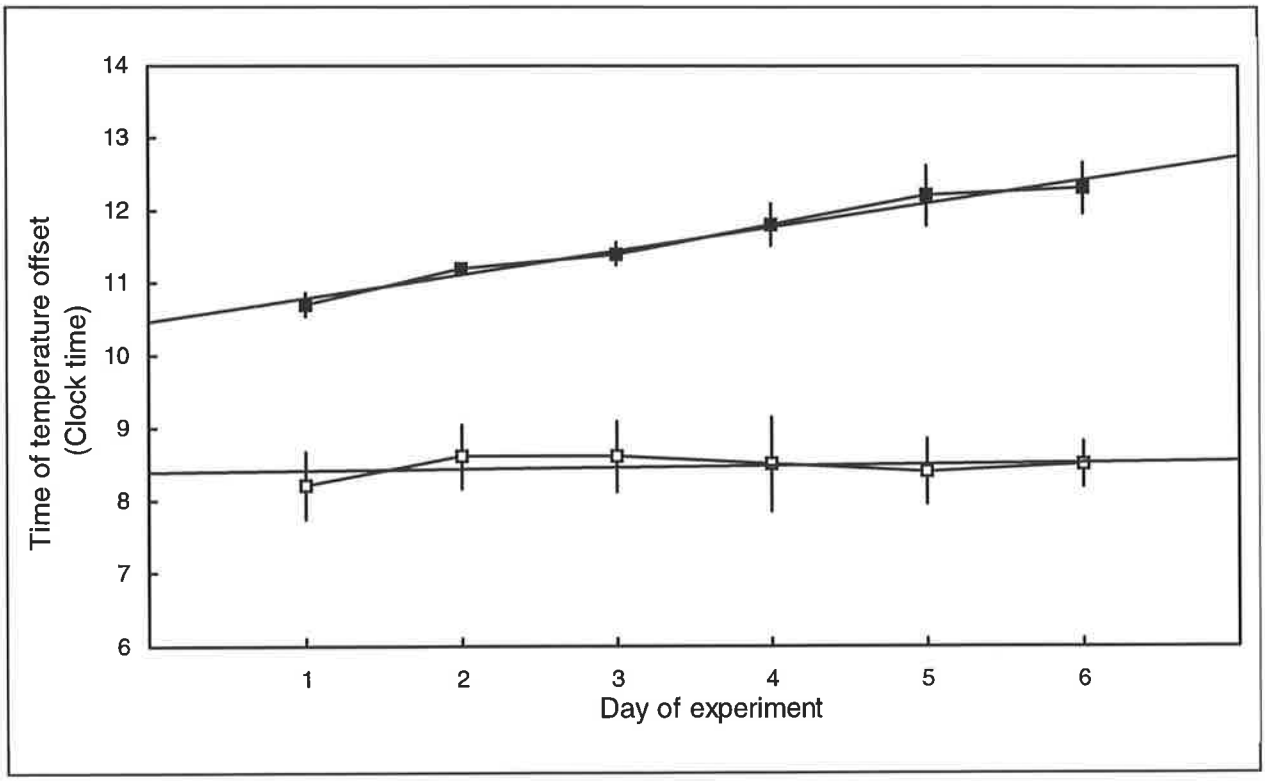

Figure 6.4
Time of the morning decline of wheel running activity in 5 SOX (open squares) and 5 ROX (closed squares) under constant dark conditions. Activity offset was estimated for each animal for each 24 hour period, as the time at which the wheel running activity level dropped below the average for that animal. Each data point represents the mean ± SEM for 5 animals. The line through each data set represents the trendline for that data.

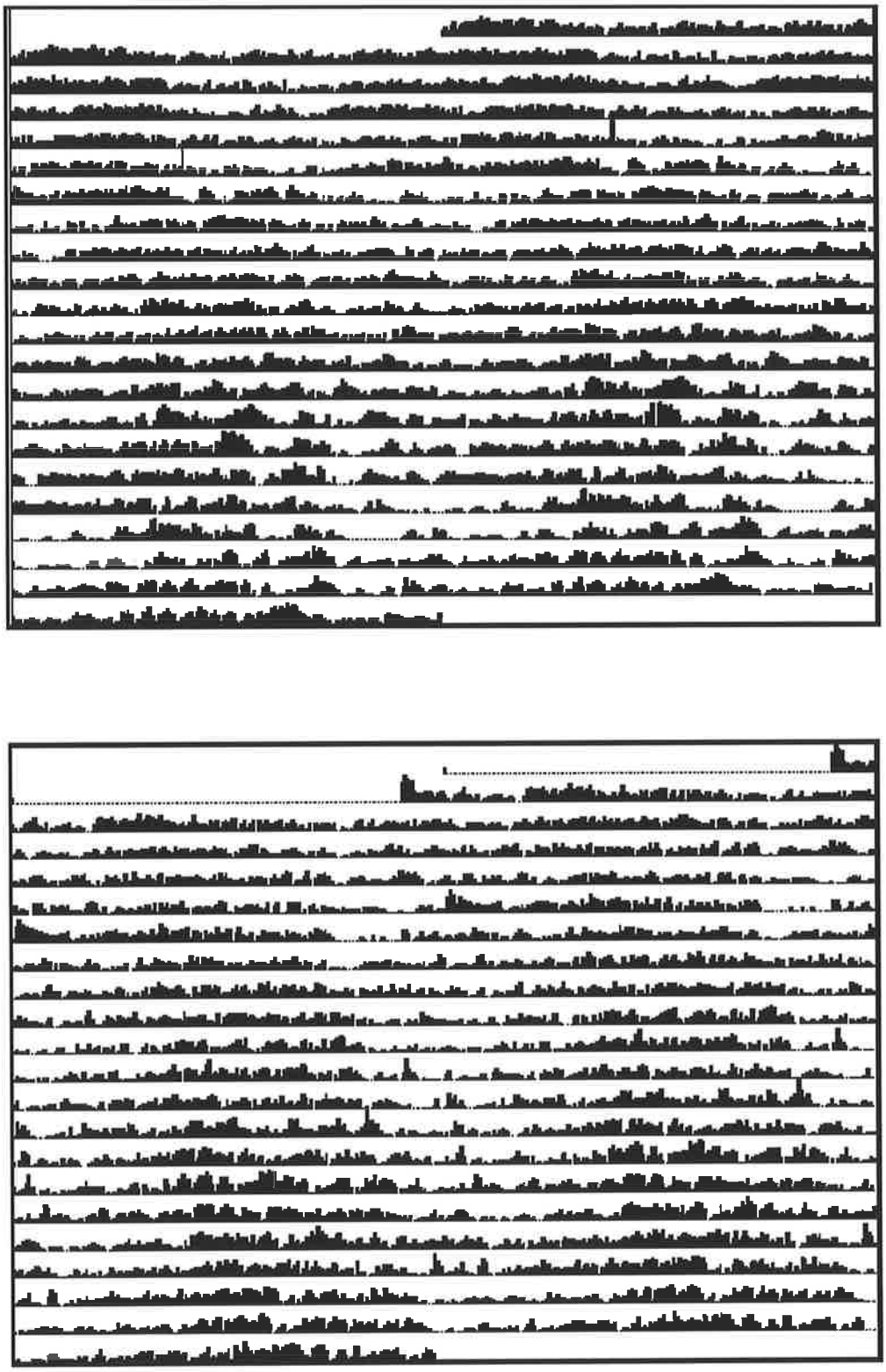

Figure 6.5
Representative actograms of temperature rhythms of SOX (upper panel) and ROX (lower panel) animals selected from generation 12. Animals were maintained in constant darkness.

6.4 DISCUSSION

The free-running period of the temperature and locomotor activity rhythm in SOX and ROX animals was assessed at different stages of the breeding program. The SOX animals from generations 8 and 12 showed significantly shorter free-running periods of temperature and G11 SOX a shorter period of wheel running activity than ROX animals, a difference not evident in animals selected from generation two. Thus, there appears to be an inherent difference in the functioning of the circadian timing system between the lines under constant conditions.

Normal rats maintained in constant conditions (DD) generally display circadian periods of about 24.2h. Our laboratory has previously reported free-running locomotor activity rhythms in Wistar rats with a period of 24.24h (Rowe and Kennaway, 1996) and temperature rhythms of 24.2-24.3h (Kennaway *et al,* 1996) in DD. Thus, in the early generations of the breeding program the SOX and ROX animals had a free-running period of the circadian rhythm of locomotor activity identical to normal RB Wistar rats. After several rounds of selective breeding the SOX animals have shown a significant shortening of the free-running period of both temperature and general activity while the ROX animals have tended to display a slight lengthening of the free-running period evident only in the animals selected from generation 12. The increased cholinergic sensitivity therefore appears to be correlated with a shortening of the free-running period in the SOX animals in addition to the previously discussed smaller phase angle of the melatonin and temperature rhythms in a cycling photoperiod.

The cloning of the mammalian period gene has been one of the most significant developments in the circadian rhythm field in recent times (Tei *et al,* 1997). It brings us a step closer to precisely defining the way that circadian rhythms are generated in the SCN and the specific genes involved in the process. Further, it supplies conclusive evidence that in mammals, the period of SCN rhythmicity is largely genetically controlled. While this fact is widely recognised through genetic work with the *tau* hamster and the *clk* mouse, this latest development is a defining moment in the area.

The intrinsic period of Drosophila locomotor activity and eclosion cycles is a reflection of the cycles of DNA→mRNA→protein products of the genes *period* and *timeless*. If both of these genes are intact in the fly then the periods of eclosion and locomotor activity are approximately 24 hours. When the genes are altered due to mutation or deletion the length of the cycle of

mRNA to protein product is also altered (see section 1.4). The shorter free-running period in SOX animals however, is not likely a result of a single deletion or mutation as circadian parameters were not taken into account in the selection protocol. The question remains then what factors are influencing the intrinsic rhythm of the SCN under free-running conditions. Possible explanations for the altered SCN rhythmicity rely on the idea of neurotransmitters exerting some 'tonic' control on SCN period even under constant conditions.

The change in the free-running period of SOX animals is in accordance with reports that continuous carbachol produces a shortening of circadian free-running periods. Murakami *et al* (1986) reported that implantation of a pellet containing carbachol near the SCN of rats caused a shortening of the free-running period of drinking behaviour under DD. This was opposite to the results reported previously for intact animals under constant light conditions in which the free-running period is reported to lengthen (Honma et al, 1978; Summer et al, 1984). There was some doubt however, whether the SCN was receiving a constant input of light as the closing of the eyelids would have caused fluctuations in intensity, changes in which are known to alter free-running periods (Aschoff, 1960). Thus, the same group studied animals that had undergone removal of eyelids under constant light using both drinking behaviour (Murakami et al, 1986) and locomotor activity (Furukawa et al, 1987). The free-running periods of the rhythm were significantly shorter than animals in DD and correlated with the periods of animals kept in DD and implanted with carbachol pellets. These reports suggest that constant light and constant carbachol provide similar signals to the pacemaker of rats and indicate a possible mechanism for the shorter free-running period in SOX animals. The upregulated brain cholinergic system may be causing an increased level of cholinergic stimulus at the level of the SCN and having effects directly on the timing of output from the pacemaker.

The increased cholinergic sensitivity of SOX animals is most likely a result of global changes in cholinergic mechanisms throughout the brain, as the FSL rats were reported to have increased muscarinic receptor numbers in several brain regions. It is accepted that select brain regions outside the SCN have a role in the mediation of light effects on SCN timing (IGL, raphe nucleus) but that the intrinsic period of the clock is entirely governed by the transcription/translation of genetic material within the SCN itself. This view may be somewhat limiting. It seems improbable that the environment surrounding the SCN, which includes neural connections (major and minor) to other brain regions, has no effect on the intrinsic rhythm of gene turnover. A more likely scenario is that cycling hormone and

neurotransmitter levels in the surrounding milieu moderate the genetically generated rhythm of SCN output. We hypothesise that the normal level of cholinergic stimulus impinging upon the intrinsic cycle of genes in the SCN is altered in the SOX animals resulting in altered period length. Regions such as the brainstem and basal forebrain extend cholinergic projections to the SCN which could account for the altered level of stimuli. In turn, this altered cholinergic tone may act on the SCN via alterations in the level of serotonergic stimulus, a neurotransmitter reported to affect SCN function in rats (Kennaway *et al,* 1996; Moyer *et al,* 1997).

The rhythmicity of core body temperature was not monitored in each generation as melatonin production was, however the divergence of SCN timing as evident in the two rhythms appears to have followed a similar pattern. SOX animals were found to have an earlier melatonin onset in light/dark from generation 3 indicating no difference in SCN function in the two previous generations. In this part of the study SOX and ROX animals from generation 2 were found to have identical free-running periods, whereas by generation 8 the lines had diverged significantly. In the FSL rat, circadian studies weren't carried out until generation 32 making it impossible to determine when divergence occurred in that breeding program. Further, the SOX and ROX animals have been in a 12L:12D photoperiod since their inception, unlike the Flinders animals which were initially bred under constant light conditions. The present data suggests that the circadian period differences seen between the FSL and FRL animals were likely due to changes in cholinergic stimulation of the SCN rather than changes in the circadian timing system due to the early development in constant light.

In generation 2 animals the free-running periods of wheel running of the SOX and ROX animals were identical to RB animals (24.2h) while SOX animals from G8, 11 and 12 displayed significantly shorter free-running periods. In turn, ROX animals selected from generation 12 tended to display a lengthening of the free-running period as compared to RB and G2 ROX animals. Thus, as with the timing of onset of melatonin production in entrained conditions, the divergence between the lines appears to have occurred in both directions such that both lines have circadian systems which function differently from the norm. The aforementioned hypothesis of increased cholinergic 'tone' having an affect, directly or indirectly, on circadian rhythmicity would also extend to decreased levels of cholinergic stimulus. If increased cholinergic sensitivity is correlated to a shorter free-running period, then decreased sensitivity may be correlated to a longer period under constant conditions. Regardless, these results indicate altered circadian timing in SOX animals, similar to that

reported in the Flinders line, and make the SOX line potentially invaluable in the study of cholinergic action in SCN function.

Chapter 7

Circadian rhythms and light pulses in SOX and ROX rats

7.1 INTRODUCTION

The most important external factor that affects SCN rhythmicity in mammals is light. Termed a zeitgeber (time-giver), light entrains the endogenous rhythm of the clock to the cycle of night and day. For animals in the wild, the dawn each day resets the body's clock to allow for adaptation to a constantly changing environment. Humans are more likely to ignore or override this daily re-entraining signal due to the constraints of our work and social lives. The rhythms of the body become desynchronised with the external environment as a result of rapid shifting of time zones or pressure to work abnormal hours.

The neural pathways that are primarily responsible for the mediation of light effects on SCN function are, at present thought to number three. A single, direct, mono-synaptic projection from the retina to the suprachiasmatic nucleus, termed the retino-hypothalamic tract (RHT), and two indirect pathways, one via the intergeniculate leaflet and ventral lateral geniculate nucleus, called the retino-geniculo-hypothalamic tract (R-GHT), and one via the raphe nucleus, make up the major SCN afferents (for detailed neurochemistry see section 1.5). The relative importance of each of these tracts is reported to vary between species as recent studies have shown. Rowe and Kennaway (1996) demonstrated the lack of an effect of the EAA antagonist MK-801 on the light-induced phase delay of the aMT.6S excretion rhythm in the rat and the lack of phase shift after treatment with the EAA agonist, NMDA. This data was in contrast to the widely held idea that the EAA of the RHT were the main transmitters mediating light effects in all species (Liou *et al,* 1986; Gauer *et al,* 1994). Further studies showed that serotonin acting through the raphe projection to the SCN may be more important in light-induced phase changes in the rat (Kennaway *et al,* 1996; Moyer *et al,* 1997).

The SCN of animals (Daan and Pittendrigh, 1976) and humans (Minors *et al,* 1991) responds differently to light stimuli according to the time of the cycle the stimulus is applied. Using the melatonin excretion rhythm as a marker of SCN output Kennaway and Rowe (1994) showed that phase delays were achieved with 15 minute light pulses given at 4, 6 or 8 hours after lights off, but not at 2 or 10 hours after lights off. This partial phase response curve for melatonin is in agreement with the full PRC for running activity of rodents (Daan and Pittendrigh, 1976), and for human body temperature (Minors *et al,* 1991). The largest phase delay in aMT.6S excretion occurred after light treatment at 4 or 6 hours after lights off (CT16 - CT18) (Kennaway and Rowe, 1994). The induction of the immediate early gene, *c-fos* in the SCN is

also time-gated. Rea (1989) demonstrated that gene activation could only be detected after light treatment during the subjective night and not during subjective day, an identical pattern to the response of melatonin, activity and temperature rhythms.

The development of the SOX line of rat was pursued with the specific aim of examining the role of ACh in SCN function via a unique endogenously cholinergically upregulated rat. Acetylcholine is not found in any of the main neural pathways to the SCN, although there is compelling evidence for some role for this transmitter in the circadian timing system (see section 1.6). Several minor tracts project to the SCN from other brain regions (Rao *et al,* 1987; Tago *et al,* 1987; Bina *et al,* 1993; Kiss and Halasz, 1996), some of which also receive retinal inputs (Bina *et al,* 1993). It is possible that ACh could play a modulatory role in the mediation of light effects on SCN function via these minor projections. We have already reported that the SCN of the SOX rats functions differently from both ROX and RB rats, with early onset of melatonin excretion and early offset of temperature under entrained conditions, as well as a shorter free-running period of the temperature rhythm in constant darkness (chapters 5 and 6). This section of the study examined the effect of a light pulse at various circadian times on the melatonin rhythm, the temperature rhythm and the induction of *c-fos* in the SCN of SOX, ROX and RB rats.

7.2 METHODS

7.2.1 Melatonin studies

Part 1.

Two groups of animals were used in this part of the study. Ten SOX and 10 ROX males from generations 4 and 5 aged 35 days and in the top 20% of their respective screens, were used in the first part of the study and 5 SOX and 5 ROX males from generation 10 aged 35 days and in the top 10% of their respective screens were selected for the second part. The two parts were separated by approximately 18 months. Animals were placed in metabolism cages, onto liquid diet and allowed to acclimatise for 4 days. Protocol followed that of the 4-day test as outlined in section 2.1. After the control collection on night 1 animals were treated with a 1 minute/2 lux light pulse at CT16 (4 hours after subjective lights off). The animals were then left for a

further two post-treatment nights. Urine was collected continuously each subjective night in hourly samples. Collection vials were weighed and urine stored frozen for RIA (section 2.2).

Part 2.

Fifteen SOX and 15 ROX males from generations 8 and 9 aged 35 days and in the top 20% of their respective screens were selected for this experiment. Protocol followed the 4-day test as outlined in section 2.1. The intervention on night 2 was a 1 minute/2 lux light pulse administered at CT18 (6 hours after subjective lights off). Urine was collected continuously each subjective night into hourly samples. Collection vials were weighed and urine stored frozen for RIA (section 2.2).

Part 3.

Ten SOX and 10 ROX males from generation 10 aged 35 days and in the top 20% of their respective screens, and 4 RB males aged 35 days were selected for this experiment. Protocol followed the 4-day test as outlined in section 2.1. The intervention on night 2 was a 15 minute/2 lux light pulse at CT16 (4 hours after subjective lights off). Urine was collected continuously each subjective night into hourly samples. Collection vials were weighed and urine stored frozen for RIA (section 2.2).

Part 4.

Five SOX and 5 ROX males from generation 10b aged 35 days and in the top 20% of their respective screens were selected for this experiment. Protocol followed the 4-day test as outlined in section 2.1. Night 1 was used as a control night. A 1 minute/2 lux light pulse was administered at CT6 on subjective day 2 (after 18 hours of continuous darkness). Collection periods were identical to those described in Part 1. Urine was collected continuously each subjective night into hourly samples. Collection vials were weighed and urine stored frozen for RIA (section 2.2).

7.2.2 c-FOS studies

Part 1.

Thirty-three SOX males and thirty-three ROX males from generations 7 and 8 in the top 50% of their respective screens and 10 RB males were selected for this experiment which was conducted over three separate sessions. Animals were housed 4 or 5 to a cage in a 12L:12D photoperiod with lights off at 1900h. A total of 23 SOX, 23 ROX and 5 RB animals were

treated with a light pulse (1 minute/2 lux) at ZT16 (4 hours after lights off) and the remaining animals were left untreated as dark controls. Two hours after treatment animals were decapitated and brains removed within 30 seconds. Brains were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For processing protocol see section 2.5.

Part 2.

Seventeen SOX males and thirteen ROX males from generations 10 and 11 in the top 40% of their respective screens and 8 RB males were selected for this experiment which was conducted over two sessions. Animals were housed 4 or 5 to a cage in a 12L:12D photoperiod with lights off at 1900h. A total of 12 SOX, 8 ROX and 4 RB rats were treated with a light pulse (1 minute/2 lux) at ZT18 (6 hours after lights off) and the remaining animals were left untreated as dark controls. Two hours after treatment animals were decapitated and brains removed within 30 seconds. Brains were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For processing protocol see section 2.5.

Part 3.

Ten SOX males and 10 ROX males from G12 were selected for this experiment. Animals were housed 5 to a cage in a 12L:12D photoperiod with lights off at 1900h. On the experimental day 6 SOX and 6 ROX animals were exposed to a 1 minute/2 lux light pulse at CT2 (after 14 hours of darkness) and the remaining animals were left as untreated controls. Two hours after treatment animals were decapitated and brains removed within 30 seconds. Brains were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For processing protocol see section 2.5.

7.2.3 Temperature studies.

Five SOX and five ROX males from generation 8 in the top 20% of their respective screens were selected for this experiment. Animals were anaesthetised under 3% halothane/oxygen and a temperature transmitter was implanted in the peritoneum. Animals were then placed in individual cages inside a light-controlled environment chamber on a 12L:12D photoperiod, lights off at 1900h, for three days to recover. The protocol followed that of the melatonin studies with a control night, a treatment night and two post-treatment nights to assess phase changes. Data was collected for five days before the treatment night in a light/dark cycle to get an assessment of the normal rhythm of the animals. The lights were switched off at 1900h

on the fifth night and remained off for the rest of the experiment. The animals received a 1 minute/2 lux light pulse at CT18 (6 hours after subjective lights off) on experimental night 2. Data was then collected for a further two days.

7.3 RESULTS

7.3.1 Melatonin studies

Part 1.

On the control night the SOX animals from generation 4/5 had a significantly earlier time of onset than the ROX animals (14.8 ± 0.3h and 15.9 ± 0.4h, respectively). The light pulse (1 minute/2 lux) on night 2 caused an acute suppression in the aMT.6S excretion rate in both groups. Melatonin production recovered in the SOX animals after a brief drop (approximately 1.5 hours) however, the ROX animals showed sustained suppression of aMT.6S of more than 3 hours (Figure 7.1). A significant phase delay was seen on nights 3 and 4 as compared to the control night 1 in the ROX animals, however the SOX animals did not show any phase delay from night 1 (Figure 7.1) (Table 7.1).

In the animals selected from generation 10 the timing of aMT.6S excretion onset was again earlier in the SOX group (15.1 ± 0.2h) than the ROX group (17.1 ± 0.1h). The 1 minute/2 lux light pulse caused an acute suppression that lasted about 2 hours in the SOX and 5 hours in the ROX (Figure 7.2). The SOX animals showed a small but significant delay in the timing of onset of aMT.6S excretion rate as compared to night 1 (Figure 7.2) (Table 7.1). In contrast the ROX animals exhibited a large delay of 3.0 ± 0.5h on night 3 compared with night 1 (Figure 7.2) (Table 7.1).

Part 2.

The onset time on the control night was again significantly earlier in the G8/9 SOX group (15.8 ± 0.3h) than the ROX group (17.0 ± 0.3h). The light pulse at CT18 caused an acute suppression of the melatonin excretion rate on the night of treatment in both groups (Figure 7.3). A significant phase delay in the timing of onset of aMT.6S excretion rate on nights 3 and 4 was also seen in both groups as compared to night 1 (Table 7.1). There was no difference in response times between the groups. On night 3 however, melatonin production in the ROX

animals was reduced by 54% from that produced on night 1, compared to a 27% reduction in SOX animals (Figure 7.3).

Part 3.

The onset times of the three groups on the control night were significantly different from each other group. SOX animals had an onset of 15.5 ± 0.2h, RB animals of 16.2 ± 0.1h and ROX animals had an onset of 17.0 ± 0.3h. A 15 minute light pulse at CT16 caused a significant acute suppression of the melatonin excretion rate in all groups (Figure 7.4). On nights 3 and 4 all groups showed a significant phase delay in the timing of onset of aMT.6S excretion as compared to night 1. There was no significant difference in the degree of delay between the groups.

Part 4.

In this experiment the timing of onset of melatonin production was again earlier in SOX animals when compared to ROX (14.9 ± 0.3h and 16.9 ± 0.1h respectively). A light pulse at CT6 on subjective day 2 (18 hours after continuous darkness onset) had no effect on the timing of the aMT.6S excretion rhythm in either SOX or ROX animals (Figure 7.5).

7.3.2 c-FOS studies.

Part 1.

A light pulse at ZT16 caused a significant number of c-FOS-immunopositive cells to be identified in each group compared to the control animals (Figure 7.6). However, SOX animals had significantly less cells stained than both ROX and RB rats (Table 7.2) (Photo 7.1).

Part 2.

Treatment at ZT18 resulted in similar numbers of cells being labelled in each of the three groups (Table 7.2). The number of immunopositive cells in the SCN of the 3 groups were not significantly different (Figure 7.7) (Photo 7.2).

Part 3.

Treatment with a 1 minute/2 lux ;light pulse at CT2 after 14 hours of darkness failed to cause c-FOS positive cells to be identified in the SCN of SOX or ROX animals (Table 7.2).

7.3.3 Temperature studies.

The timing of the morning decline in core body temperature was significantly different between the two lines on the control night. SOX animals exhibited a decline at 23.1 ± 0.1h and ROX at 24.0 ± 0.2h. The light pulse at CT18 on the second experimental night did not cause any acute effect at the time of administration however, the SOX animals did show an acute delay on the morning following the light treatment. Subsequently, on the two post-treatment nights both groups showed significant phase delays in the timing of the morning decline. SOX animals had delays of 2.0 ± 0.1h and 2.4 ± 0.2h on nights 3 and 4 respectively and ROX showed delays of 1.1 ± 0.1h and 1.4 ± 0.1h respectively.

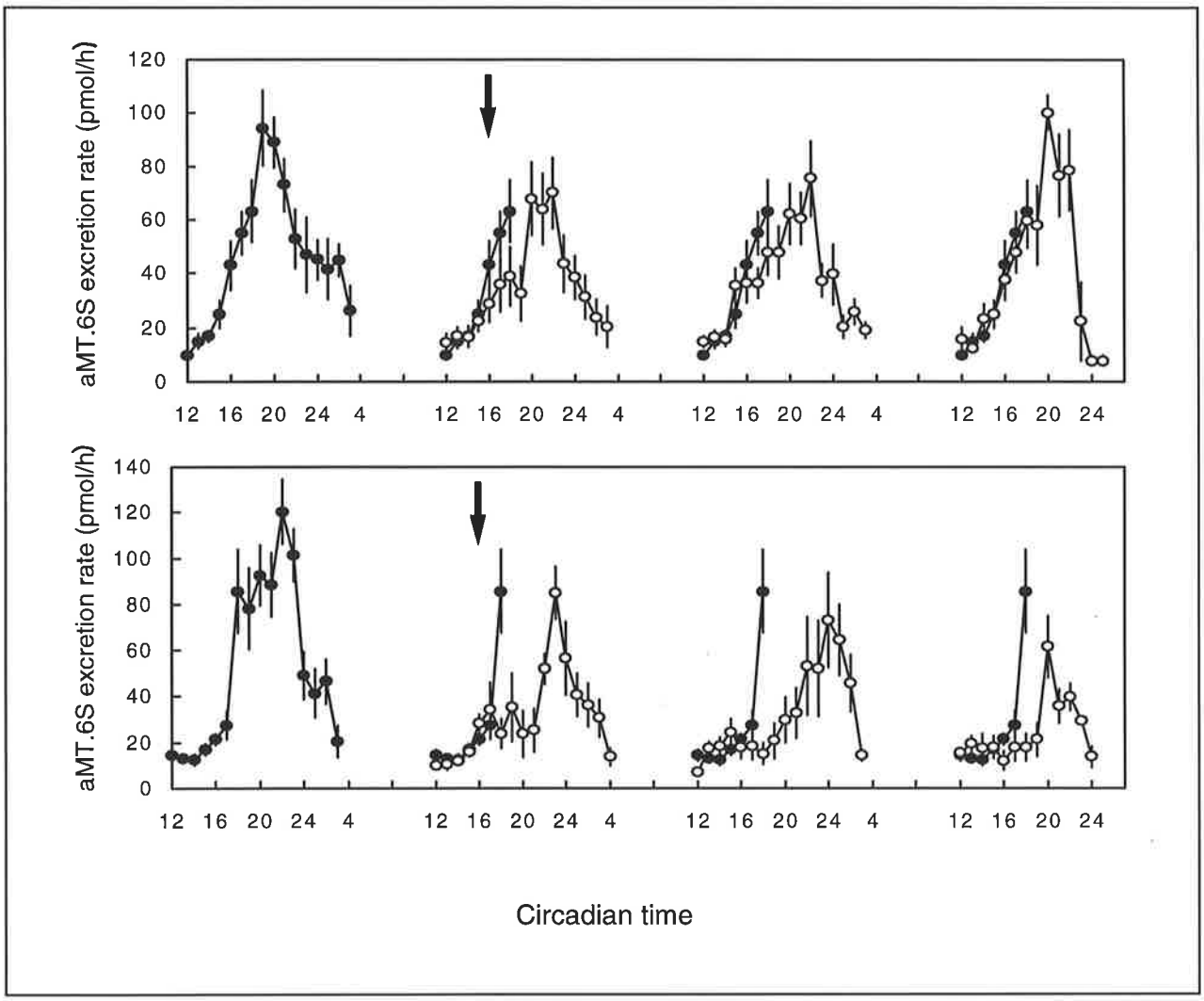

Figure 7.1
6-sulphatoxymelatonin excretion profiles for G4/5 SOX (upper panel) and ROX (lower panel) treated with a 1 minute/2 lux light pulse at CT16. Four consecutive nights are shown. In each panel data from night 1 represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for 10 animals.

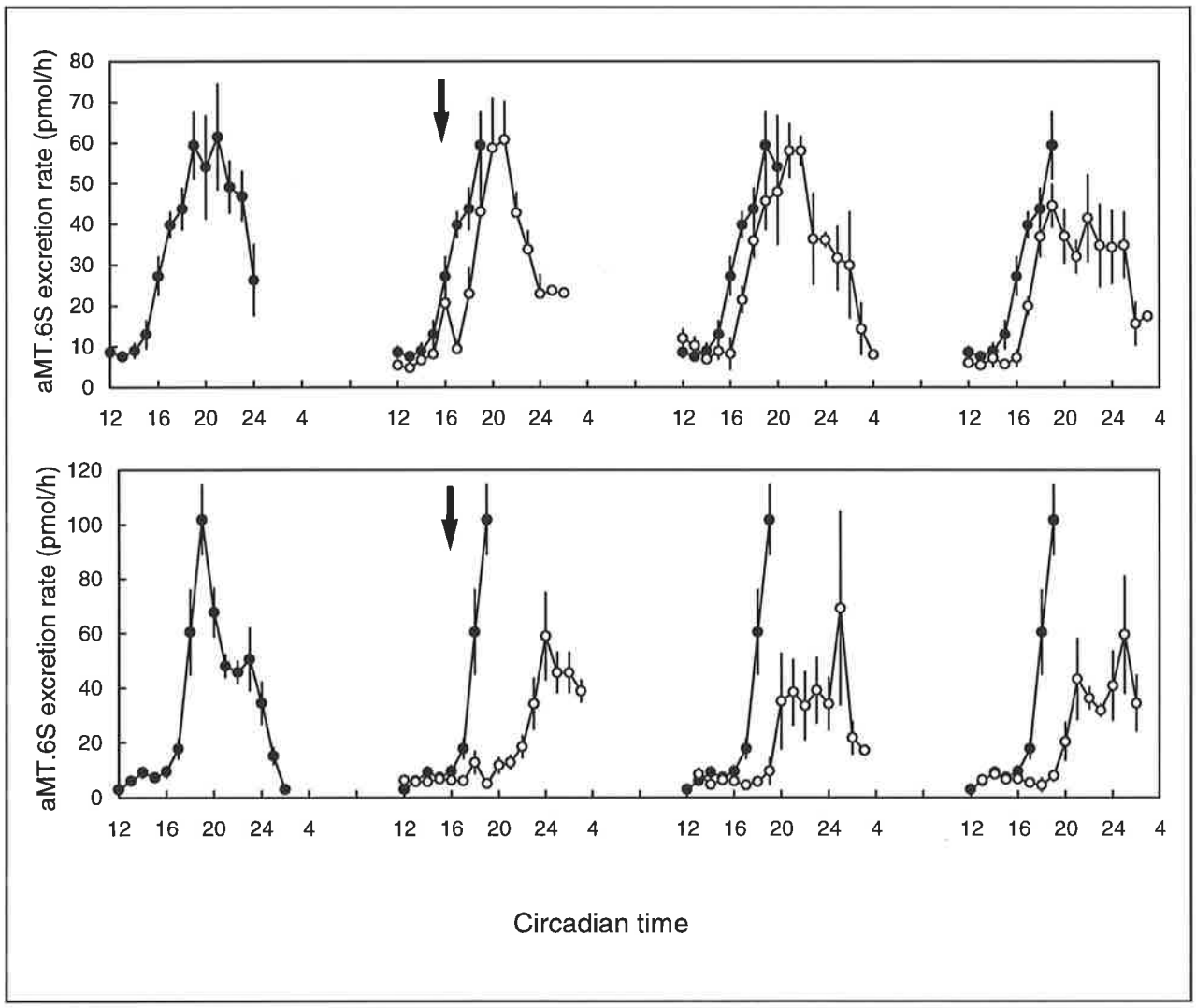

Figure 7.2
6-sulphatoxymelatonin excretion profiles for G10 SOX (upper panel) and ROX (lower panel) treated with a 1 minute/2 lux light pulse at CT16. Four consecutive nights are shown. In each panel data from night 1 represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for 5 animals.

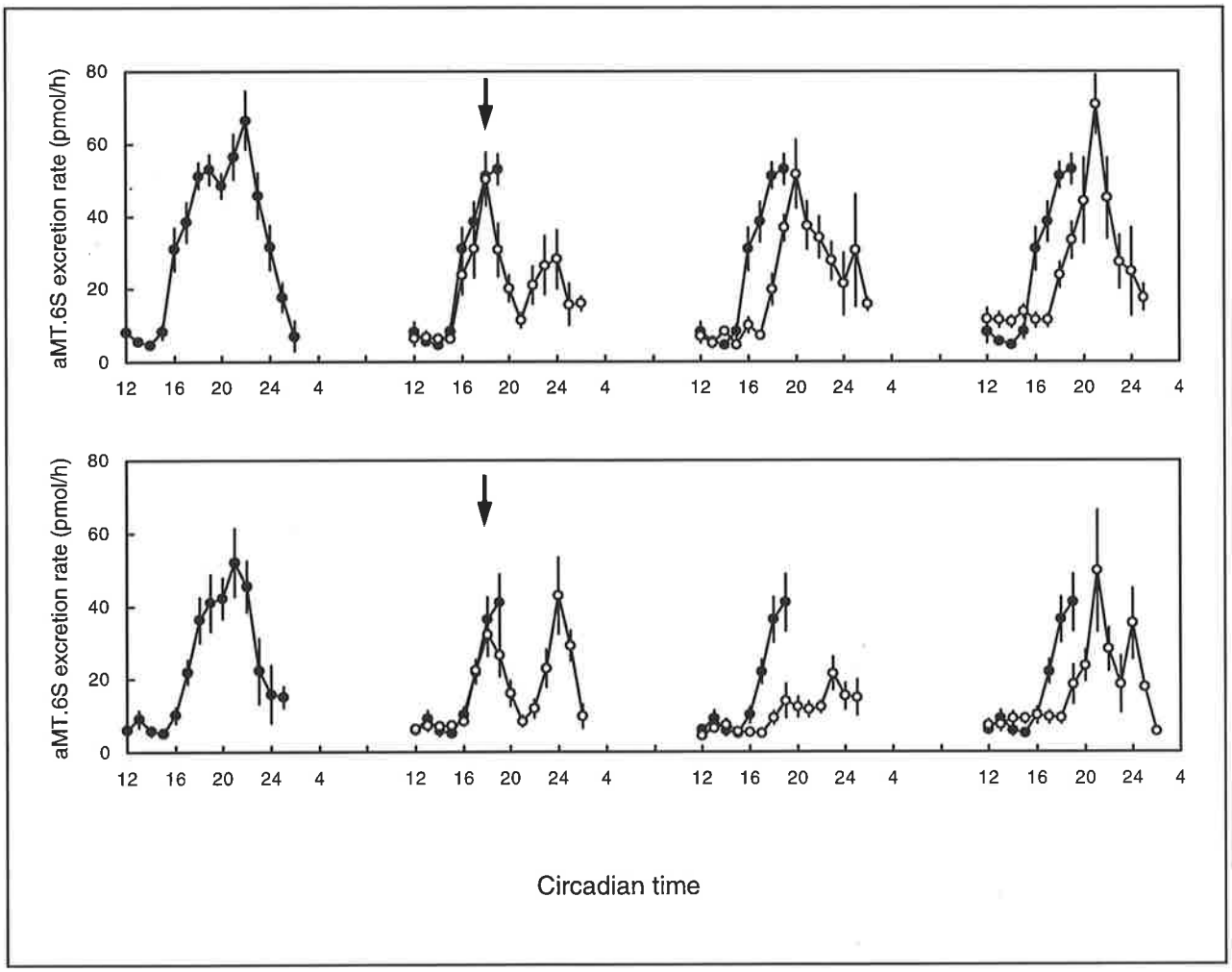

Figure 7.3
6-sulphatoxymelatonin excretion profiles for G10 SOX (upper panel) and ROX (lower panel) animals treated with a 1 minute/2 lux light pulse at CT18. Four consecutive nights are shown. In each panel data from night 1 represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for 15 animals.

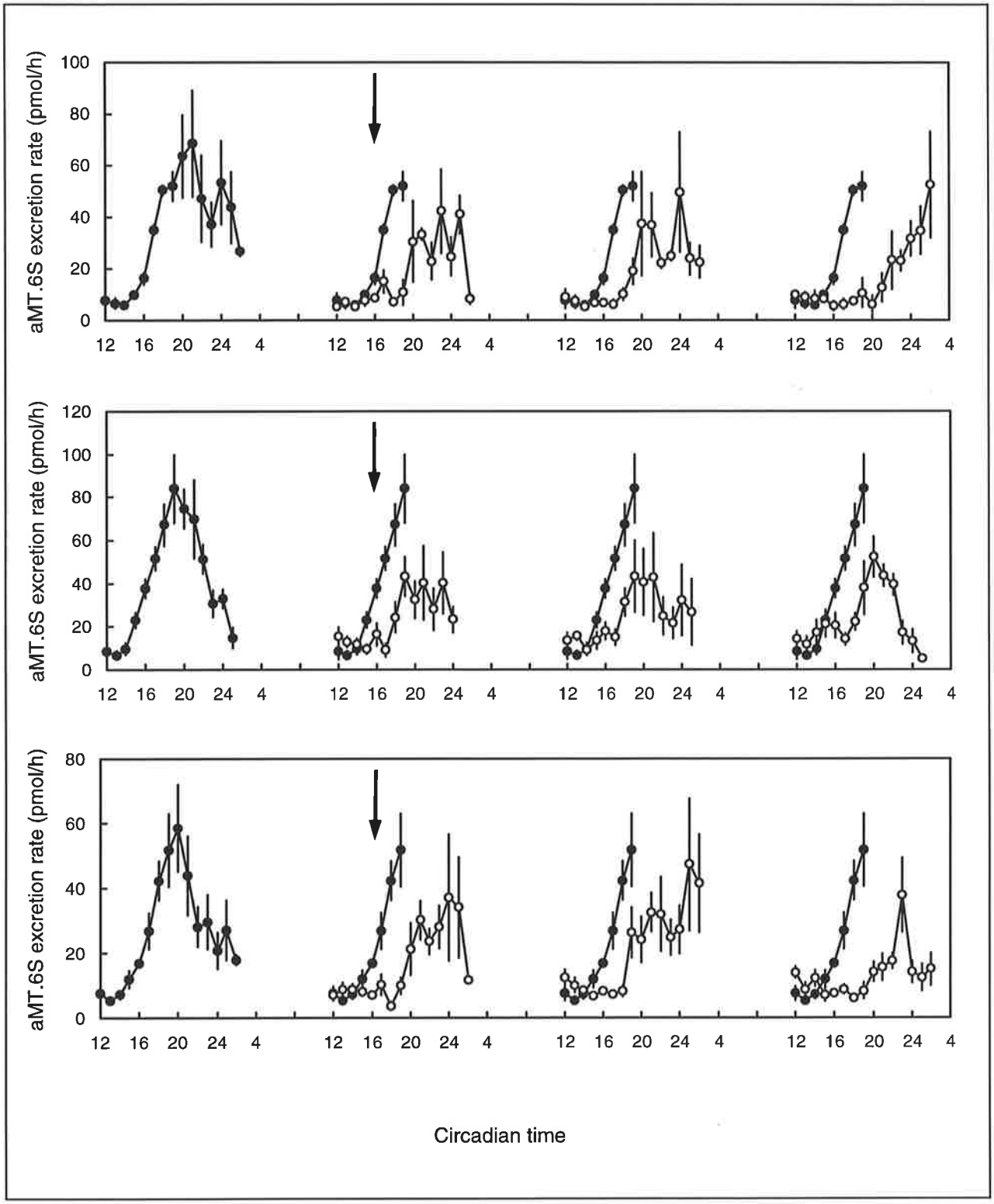

Figure 7.4
6-sulphatoxymelatonin excretion profiles for 4 RB males (upper panel), 10 SOX (centre panel) and 10 ROX (lower panel) treated with a 15 minute/2 lux light pulse at CT16. Four consecutive nights are shown. In each panel data from night 1 represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM.

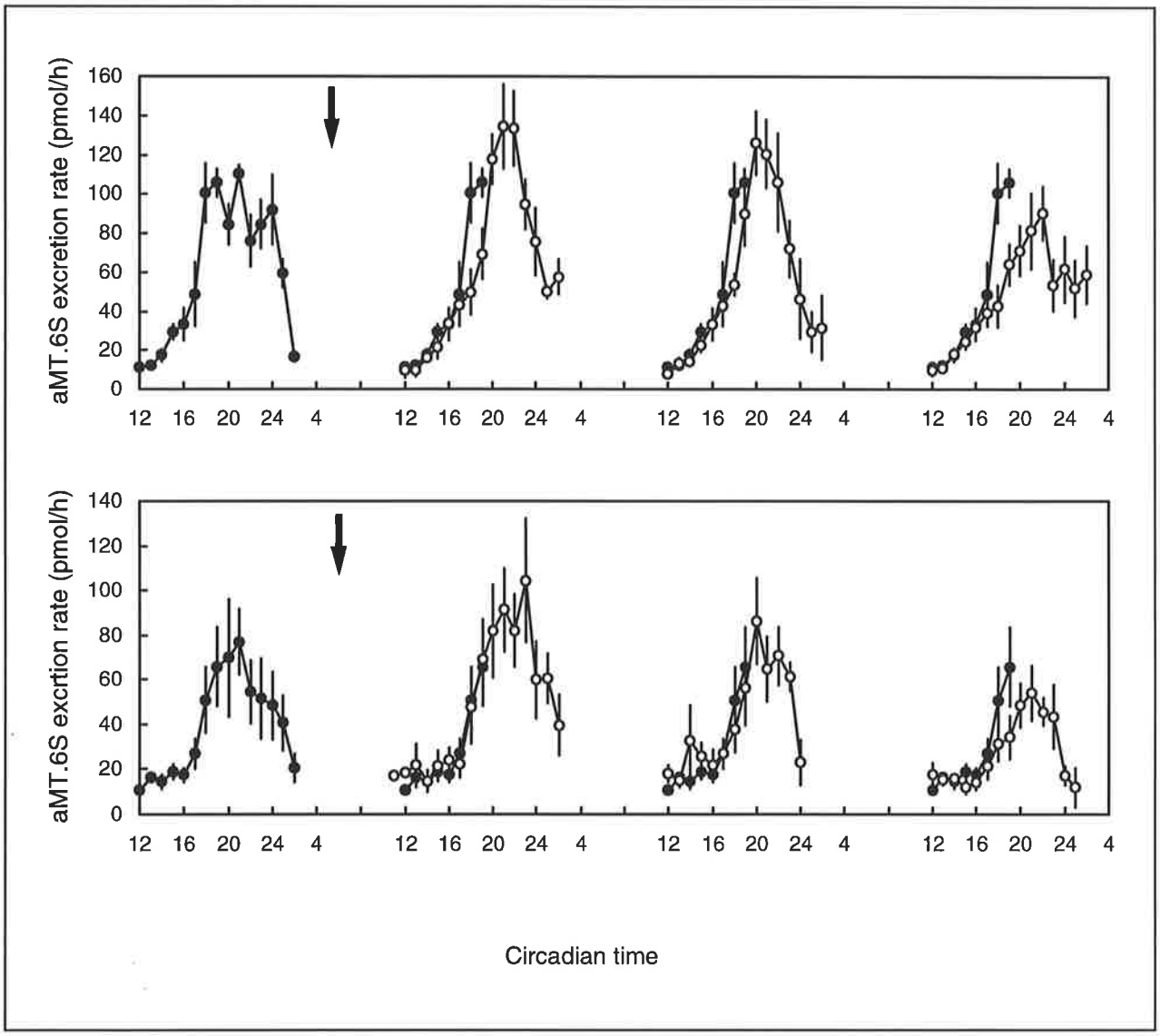

Figure 7.5
6-sulphatoxymelatonin excretion profiles for G10b SOX (upper panel) and ROX (lower panel) treated with a 1 minute/2 lux light pulse at CT6. Four consecutive nights are shown. In each panel data from night 1 represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for 5 animals.

Table 7.1
Effects of light administration on the timing of the onset of the 6-sulphatoxymelatonin excretion rate rhythm in rats. Delays on nights 3 and 4 represent the change in onset time compared to night 1.

Line	Gen	Time	Light pulse	Delay N3	Delay N4
SOX	4/5	CT16	1min/2lux	0.2 ± 0.3	0.0 ± 0.2
ROX	4/5	CT16	1min/2lux	4.6 ± 0.6	3.4 ± 0.8
SOX	10	CT16	1min/2lux	1.1 ± 0.1	1.2 ± 0.1
ROX	10	CT16	1min/2lux	3.0 ± 0.5	3.6 ± 0.3
SOX	10	CT18	1min/2lux	2.5 ± 0.3	1.8 ± 0.2
ROX	10	CT18	1min/2lux	4.1 ± 0.4	2.8 ± 0.5
SOX	10	CT16	15min/2lux	2.8 ± 0.6	2.7 ± 0.7
ROX	10	CT16	15min/2lux	2.4 ± 0.3	3.6 ± 0.5
RB		CT16	15min/2lux	4.0 ± 0.6	5.3 ± 0.6
SOX	11	CT6	1min/2lux	0.5 ± 0.4	0.5 ± 0.5
ROX	11	CT6	1min/2lux	0.0 ±0.6	0.6 ± 0.3

- The data show the mean ± SEM of the delays for 5-10 animals following the various treatments.

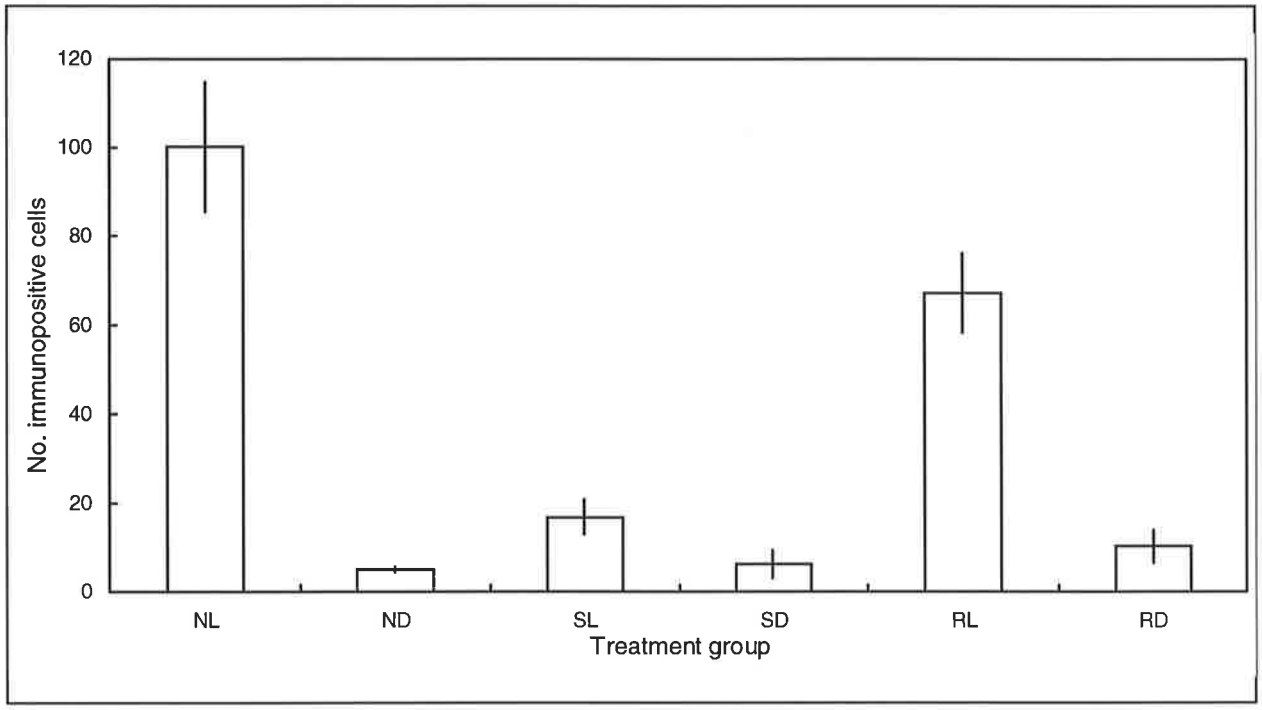

Figure 7.6
The number of immunopositive cells in the SCN of rats after exposure to a light pulse (1 minute/2 lux) at ZT16 or no treatment. Treatment groups on the x-axis are NL - normal RB animals exposed to light, ND - RB animals left untreated, SL - SOX animals exposed to light, SD - SOX left untreated, RL - ROX animals exposed to light, RD - ROX left untreated. Each data set represents the mean ± SEM for 10-23 animals.

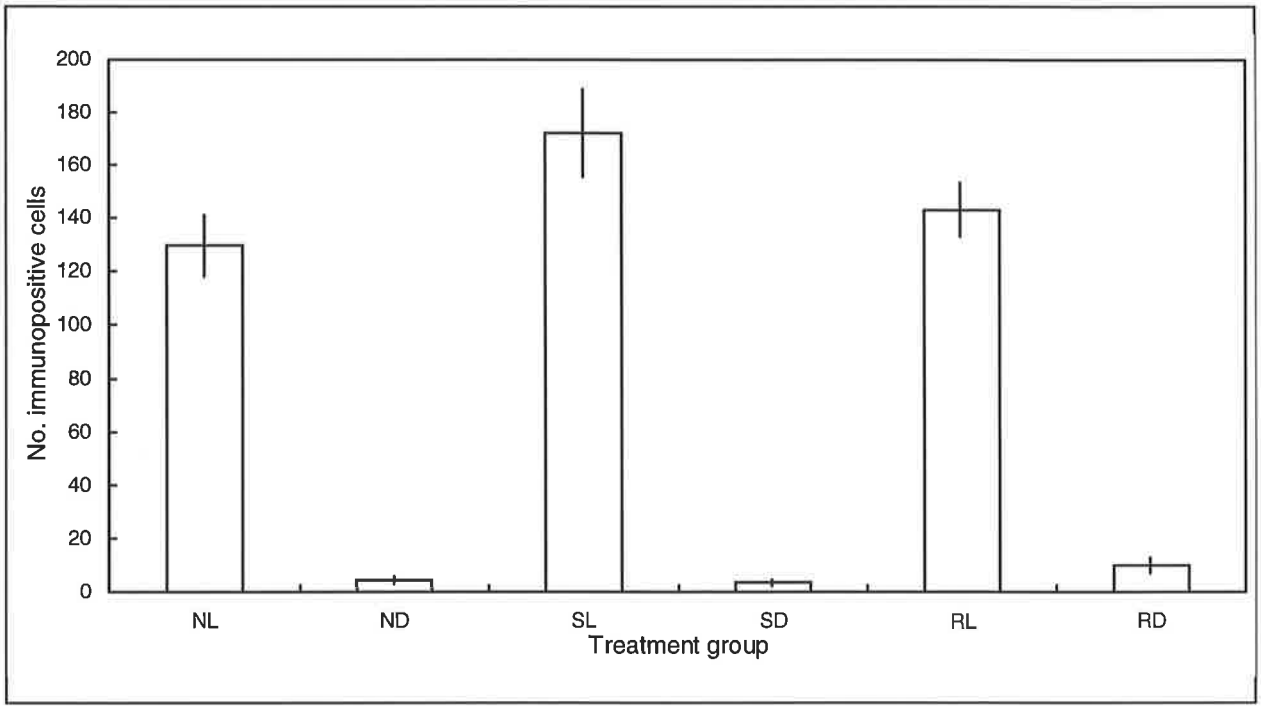

Figure 7.7
The number of immunopositive cells in the SCN of rats after exposure to a light pulse (1 minute/2 lux) at ZT18 or no treatment. Treatment groups on the x-axis are NL - normal RB animals exposed to light, ND - RB animals left untreated, SL - SOX animals exposed to light, SD - SOX left untreated, RL - ROX animals exposed to light, RD - ROX left untreated. Each data set represents the mean ± SEM for 4-12 animals.

Table 7.2
Effects of light treatment on the number of c-FOS-immunopositive cells in the SCN of SOX, ROX and RB animals.

Line	Generation	Time	Light pulse	No. cells
SOX	7/8	ZT16	1 min/2 lux	16.8 ± 4.0
ROX	7/8	ZT16	1 min/2 lux	67.2 ± 9.0
RB		ZT16	1 min/2 lux	100.2 ± 14.7
SOX	10/11	ZT18	1 min/2 lux	172.2 ± 16.6
ROX	10/11	ZT18	1 min/2 lux	143.1 ± 10.3
RB		ZT18	1 min/2 lux	129.6 ± 11.7
SOX	12	CT2	1 min/2 lux	2.0 ± 0.5
ROX	12	CT2	1 min/2 lux	0.0 ± 0.0

The data show the mean ± SEM number of c-FOS immunopositive cells in the SCN after treatment with a light pulse at various time points.

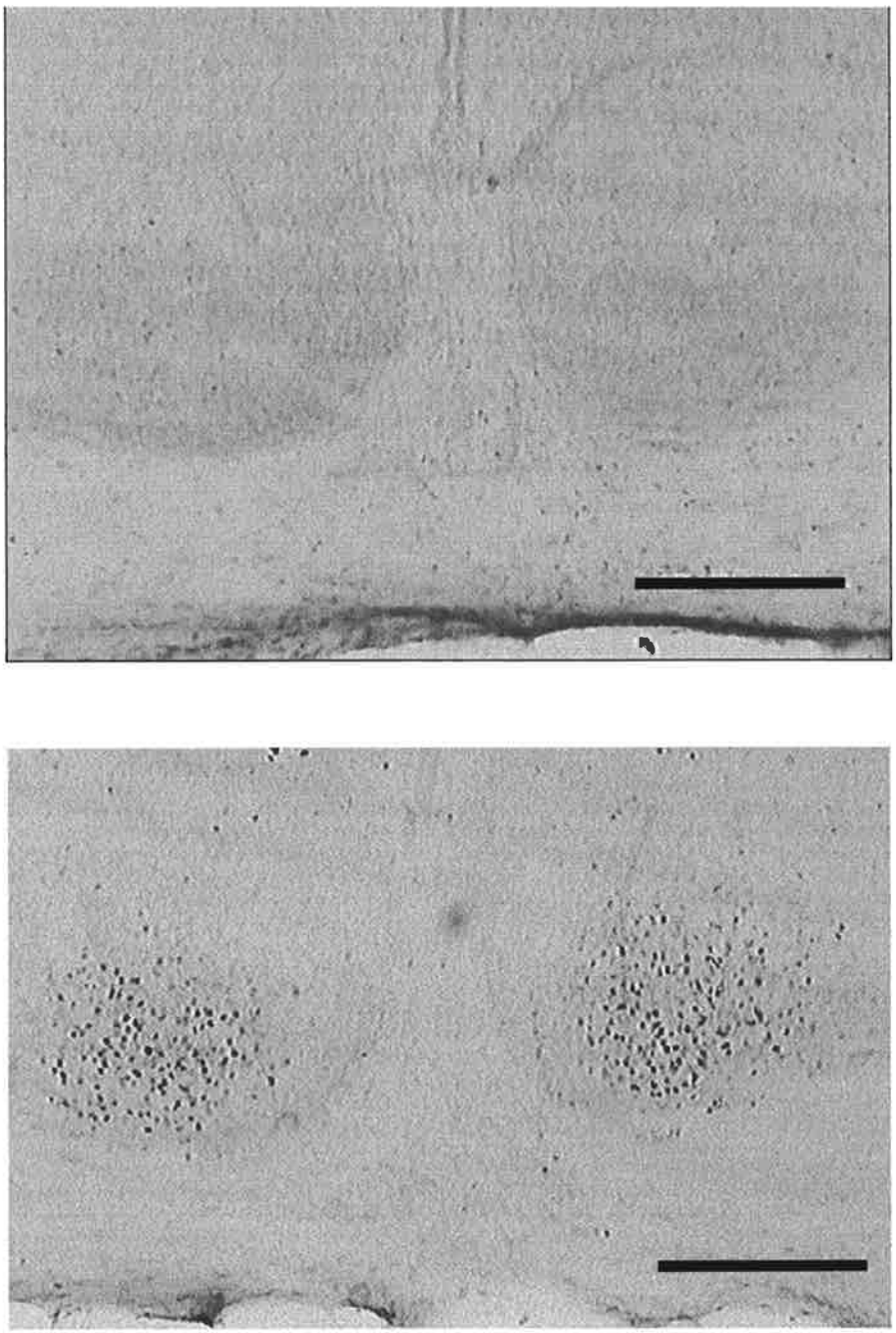

Photo 7.1
Representative micrographs of the SCN region of SOX (upper panel) and ROX (lower panel) males treated with a 1 minute/2 lux light pulse at ZT16. Horizontal bars = 500μm.

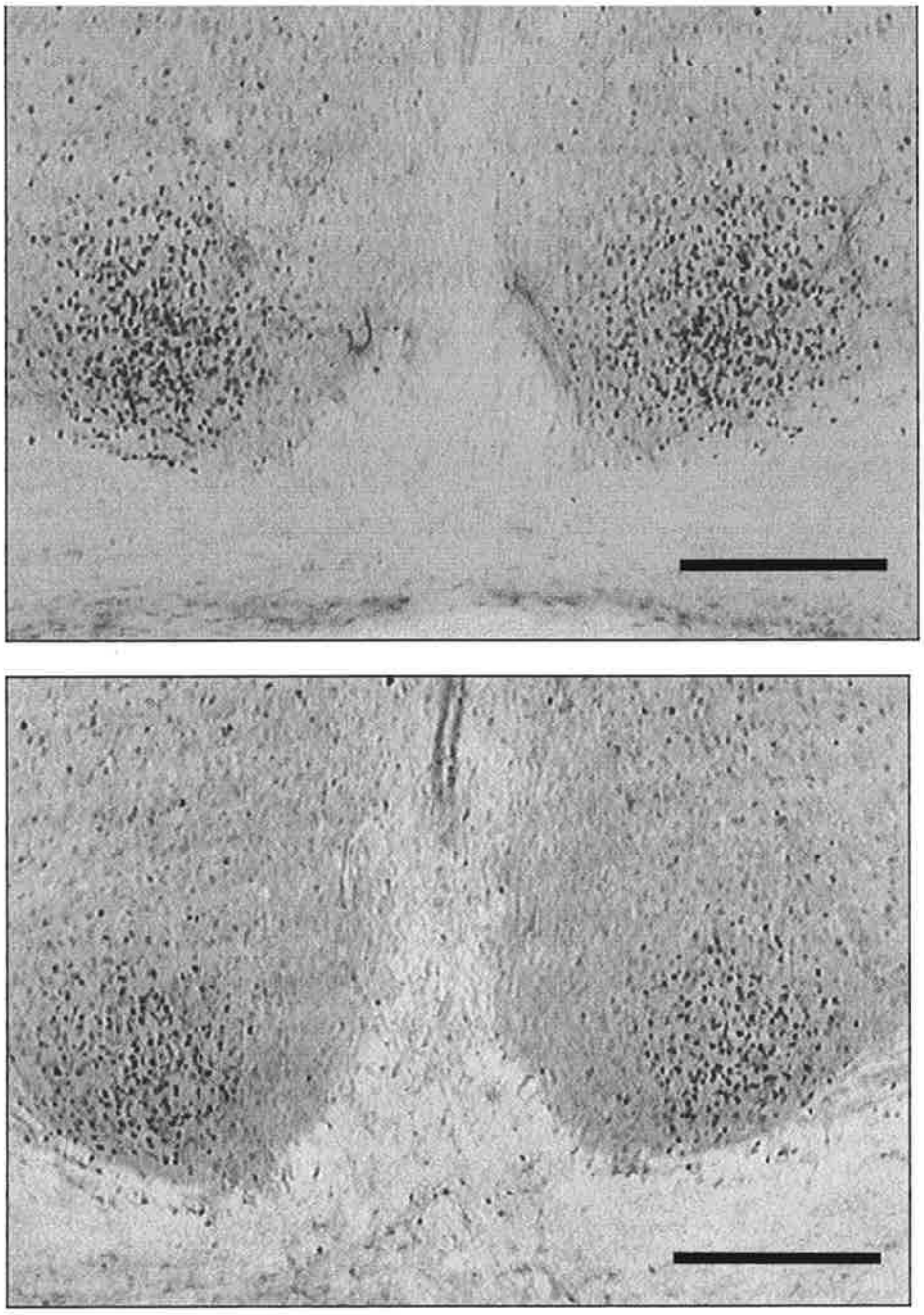

Photo 7.2
Representative micrographs of the SCN region of SOX (upper panel) and ROX (lower panel) males treated with a 1 minute/2 lux light pulse at ZT16. Horizontal bars = 500μm.

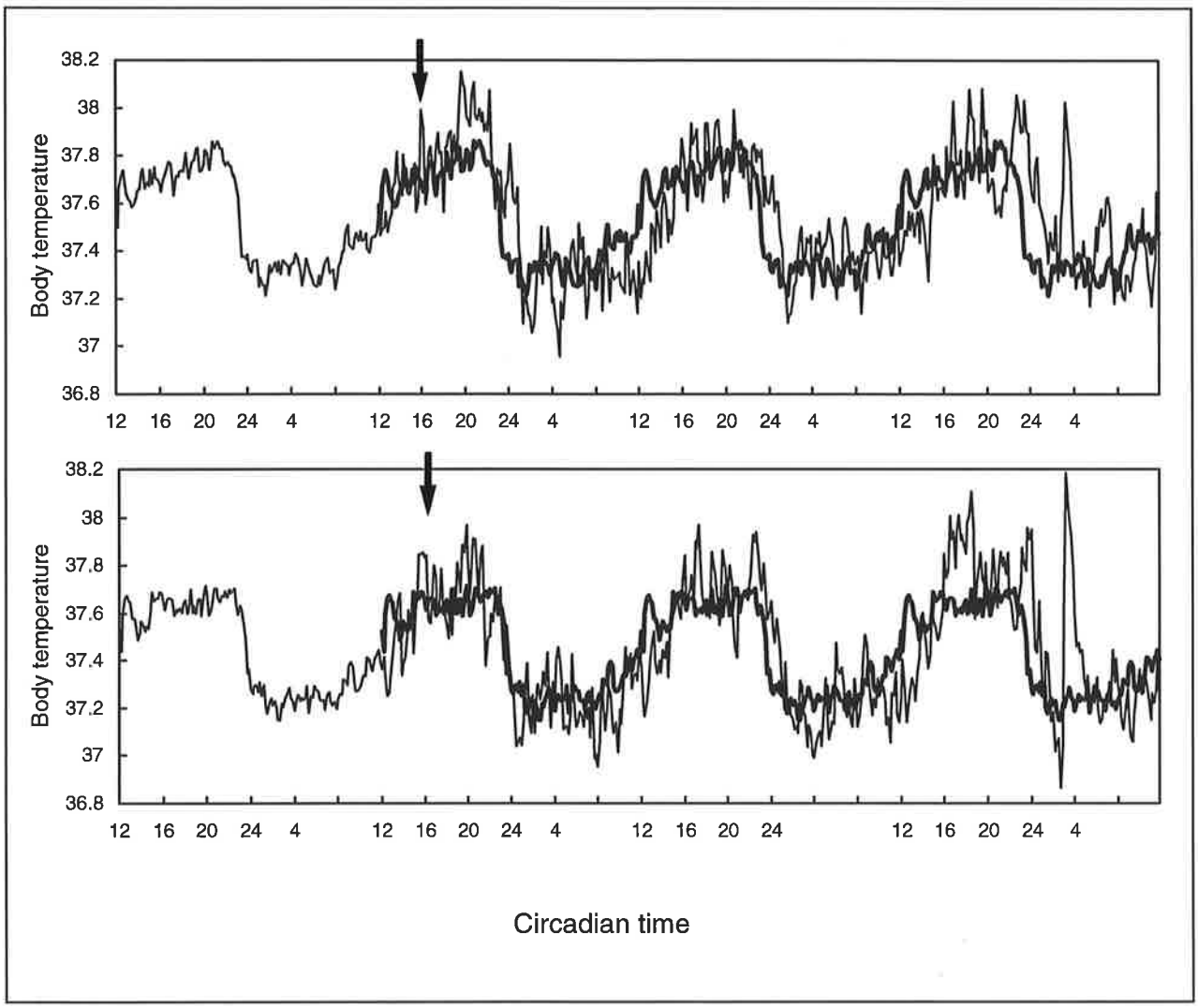

Figure 7.8
Core body temperature profiles for G8 SOX (upper panel) and ROX (lower panel) animals treated with a 1 minute/20 lux light pulse at CT16. Four consecutive nights are shown. In each panel night 1 data, represented by the heavy line, overlays nights 2, 3 and 4 (lighter line). Arrows indicate the time of treatment on night 2. Each data set represents the mean ± SEM for five animals.

7.4 DISCUSSION

Circadian rhythmicity in SOX and ROX animals was analysed by monitoring the response of the circadian timing system to light stimuli. A short light pulse (1 minute) 4 hours after (subjective) darkness caused very small phase changes in the rhythm of melatonin excretion rate, and minimal numbers of c-FOS-immunopositive cells in the SCN of SOX rats. The ROX animals however responded in an identical manner to RB rats to the one minute pulse with respect to both delays in aMT.6S rhythmicity and c-FOS expression in the SCN. After treatment at CT18 the SOX and ROX animals exhibited significant numbers of c-FOS-positive cells in the SCN as well as phase delays in the aMT.6S rhythm, similar to the responses seen in RB animals. The temperature rhythms of both groups were also phase delayed after treatment with a light pulse at CT18. A longer stimulus of 15 minutes of light at CT16 caused similar phase delays of the aMT.6S excretion rhythm in SOX animals as ROX and RB animals. Thus, the SCN of the SOX animals responded normally to a light pulse at CT18, in an identical manner to that of ROX and RB rats, however, a longer stimulus was required at CT16 to elicit a comparable response from the SOX animals.

Altered timing of the aMT.6S and temperature rhythms of SOX animals in both light/dark and constant dark conditions has already been demonstrated. The data suggested that the SCN was functioning differently in the SOX animals under both photoperiods, and the smaller phase angle difference in LD and shorter *tau* in DD were expected according to Aschoff's discussion of phase angle differences (Aschoff, 1965b). It would seem logical then that the SCN of these animals would also respond differently to brief pulses of light dependent on the timing of the stimulus. Both the rhythm of melatonin excretion, as a marker of SCN timing, and the induction of *c-fos* in the SCN, a marker of SCN responsiveness to the light, are stimulated to a significantly lesser degree by a one minute light pulse at CT16 in SOX animals yet respond identically to normal animals to CT18 treatment. This would seem to be in contrast to the apparent phase of the SOX animals. It appears that while the rhythms of temperature and melatonin excretion run with a shorter *tau*, the rhythm of SCN responsiveness is somewhat delayed and this remains conceptually difficult to explain.

To examine the possibility of a delay in the rhythm of SCN responsiveness in the SOX rats, a light pulse was administered 2 hours after subjective lights on and c-FOS induction measured. Previously it has been shown that CT2 treatment does not induce the *c-fos* gene in normal

animals and thus immunopositive cells at this time would imply altered timing of SCN responsiveness. No positive cells were found in either group suggesting the rhythm of SCN responsiveness was not shifted in the SOX animals.

The reduced level of response from the SCN of SOX rats after CT16 treatment, as measured by *c-fos* induction, correlates with the minimal acute effect of light on the aMT.6S excretion rate. In ROX animals treated with a light pulse at CT16, melatonin production is suppressed for several hours after treatment, yet at CT18, the acute response in the SOX animals mirrors exactly that of the ROX rats. Thus, at CT16, the neurons of the SCN are either not receiving the signal in the same way or not responding to it in the same way. A 15 minute pulse caused identical acute and phase delaying effects in SOX, ROX and RB animals, suggesting the neurons are responsive at this time, and indeed data in chapter 8 shows the SOX animals respond to nicotine at CT16 again suggesting the SCN can be phase delayed at CT16. Therefore, it is more likely that the shorter signal at CT16 is supplying a lower level of stimulus to the SOX animals at this time.

This section of the study has demonstrated a difference in the degree of response to a 1 minute/2 lux light pulse at CT16 in the SOX animals as measured by melatonin rhythmicity and *c-fos* induction in the SCN. The responsiveness of the circadian timing systems of SOX animals to light stimuli may be phase-dependent.

Chapter 8

Circadian rhythms and acetylcholine in SOX and ROX rats

8.1 INTRODUCTION

To investigate the role of acetylcholine in SCN function we have developed a line of rat that exhibits a genetically upregulated cholinergic system. This increased cholinergic sensitivity was measured via the thermic response to a cholinergic drug (oxotremorine) and the Sensitive to OXotremorine (SOX) rats showed a dramatic reduction in the core body temperature, significantly larger than that of their co-selected Resistant to OXotremorine (ROX) line, in the third generation of breeding. The cholinergic super-sensitivity is inherited and the degree of response has increased with each new round of offspring (chapter 4). The SOX animals have been studied with respect to the timing of their circadian rhythms in light/dark conditions and free-running (constant dark) conditions (chapters 5 & 6). In addition, the response of the SCN of these animals to a light pulse has also been examined using both the phase markers of the melatonin and temperature rhythms and the induction of *c-fos* in the SCN as measures (chapter 7). With the results showing that nicotine caused phase shifts in the aMT.6S rhythm of normal randomly bred animals and also induced the *c-fos* gene in the SCN (chapter 3) it was considered important to determine the responsiveness of the SOX and ROX animals to this agent.

Studies in RB rats in this project found that the nicotinic agonist, nicotine, but not the muscarinic agonist oxotremorine, was capable of causing shifts in the phase of the melatonin excretion rhythm. Both agents however, resulted in the detection of a small but significant number of c-FOS-positive cells in the SCN. The FSL animals, on which the SOX line was based, were reported to have increased muscarinic cholinergic receptor levels in various brain regions including the hippocampus and striatum (Overstreet *et al,* 1984). No differences were reported in the hypothalamus of those animals, however specific nuclei within these larger brain regions, relevant to circadian control, were not examined (ie. SCN, raphe nucleus, geniculate body). The altered sensitivity of the FSL animals to the cholinergic agent DFP also extended to other neuroactive agents. Diazepam (a benzodiazepine agonist) and muscimol (a GABAa agonist) produced greater depressant effects as measured by behavioural factors in the FSL rats (Pepe *et al,* 1988). FSL rats also exhibited increased thermal sensitivity to nicotine (Overstreet, 1991), selective M1 and M2 agonists (Schiller *et al,* 1988) and some serotonin agonists (Wallis *et al,* 1988; Schiller *et al,* 1992). As the current breeding program was established on the basis of differing thermic responses to oxotremorine it has already been

established that the SOX animals exhibit an inherited increase in muscarinic sensitivity of the thermal control centres.

Although oxotremorine had no effect on circadian rhythmicity in RB animals the drug did cause induction of *c-fos* in the SCN. As discussed previously the induction of the *c-fos* gene does not necessarily precede a concurrent phase shift and it is possible that the muscarinic stimulus may not have been sufficient to induce phase changes in SCN timing. An increased sensitivity to cholinergic challenge as exhibited by the SOX animals may supply the circadian timing system with a larger stimulus than that received by RB animals from the same muscarinic treatment. Studies into the effects of oxotremorine on the SCN of SOX and ROX animals will provide further information about the importance of the muscarinic receptor in SCN function.

While the SOX animals exhibit increased muscarinic sensitivity, there is no indication that this extends to the nicotinic receptor system. FSL animals were reported to have increased sensitivity to nicotine as measured by a thermic response (Overstreet, 1991), however this is the only known study on these animals using nicotinic agonists. There was no work reported with respect to nicotinic receptor number or binding in the brains of the FSL and FRL rats. We have already shown that nicotine does have an effect on the circadian timing system of normal animals but the pathway and mechanism of action are not completely defined. The SOX animals provide a unique opportunity for the study of the importance of acetylcholine and specifically in this case, the nicotinic receptor in SCN function.

8.2 METHODS

8.2.1 Temperature studies

Fourteen SOX animals (7M, 7F) and 14 ROX animals (7M, 7F) from G12 were selected for this experiment which was carried out over 2 consecutive days. On day 1 animals were weighed and a baseline core body temperature was recorded using a rectal thermistor. On day 2 each animal received a 0.3mg/kg injection of nicotine subcutaneously and temperature was recorded 40 minutes later, at the same time as the baseline recording. This dose was used as it was in a similar range as the dose of oxotremorine used for phenotyping in addition to being

the minimum effective dose of nicotine in the induction of c-FOS immunopositive cells in the SCN of RB rats. Change in temperature was calculated for each animal and differences between groups were analysed using a student's t-test.

8.2.2 Melatonin studies

Part 1.

Ten SOX and 10 ROX males from generation 6a in the top 10% of their respective screens were selected for this experiment. Animals were placed in metabolism cages in a light-controlled environment chamber and allowed 4 days to acclimatise. Protocol for this study followed the 4-day test as outlined in section 2.1. On the treatment night animals received either a peripheral injection of nicotine (3mg/kg sc.) or saline at CT16 (4 hours after subjective lights off). Urine was collected hourly over each subjective night and vials were weighed and stored frozen for RIA (section 2.2).

Part 2.

Ten SOX and 10 ROX males from generation 9 in the top 25% of their respective screens were selected for this experiment. Animals were placed in metabolism cages in a light-controlled environment chamber and allowed 4 days to acclimatise. Protocol for this study followed the 4-day test as outlined in section 2.1. On the treatment night animals received either a peripheral injection of nicotine (1mg/kg sc.) or saline at CT16 (4 hours after subjective lights off). Urine was collected hourly over each subjective night and vials were weighed and stored frozen for RIA (section 2.2).

Part 3.

Fourteen SOX males and 14 ROX males from generations 4a and 5a and in the top 20% of their respective screens were selected for this experiment. Animals were placed in metabolism cages in a light-controlled environment chamber in a 12L:12D photoperiod for 4 days to acclimatise. Protocol for this study followed the 4-day test as outlined in section 2.1, however only one post-treatment night was used. On the treatment night the animals received either a peripheral injection of oxotremorine sesquifumarate (2mg/kg sc. a dose selected on the basis of experiments in RB animals) or saline at CT16 (4 hours after subjective lights off). Urine was collected over each subjective night and vials were weighed and stored frozen for RIA (see section 2.2).

8.3 RESULTS.

8.3.1 Temperature studies

Nicotine had no significant effect on the core body temperature of either line. The average change in SOX animals was an increase of 0.3 ± 0.1°C and in ROX an increase of 0.1 ± 0.1°C.

8.3.2 Melatonin studies.

Part 1.

Nicotine (3mg/kg) caused an acute suppression of melatonin excretion rate in both SOX and ROX animals that lasted twice as long in the SOX group. The drug caused a significant delay in the timing of onset of aMT.6S excretion in both groups evident on both post-treatment nights and SOX animals exhibited phase delays of twice the magnitude of ROX animals (Figure 8.1) (Table 8.1).

Part 2.

Nicotine (1mg/kg) caused a small transient reduction in aMT.6S excretion of SOX animals which was not evident in the ROX animals. At a dose of 1mg/kg nicotine caused a significant delay in the timing of onset of aMT.6S excretion in SOX animals evident on both night 3 and 4 when compared to night one (Table 8.1). There was no significant effect of nicotine treatment on the timing of aMT.6S onset in ROX animals (Figure 8.1) (Table 8.2).

Part 3.

Oxotremorine (2mg/kg) treatment caused a small reduction of melatonin excretion acutely in both groups (Figure 8.3). The oxotremorine treatment caused a significant delay in the timing of aMT.6S onset on night 3 in the SOX animals when compared to the control night (Table 8.1) (Figure 8.3). There was no significant delay in the timing of aMT.6S excretion onset on night 3 in the ROX group treated with oxotremorine when compared to the control night (Table 8.1).

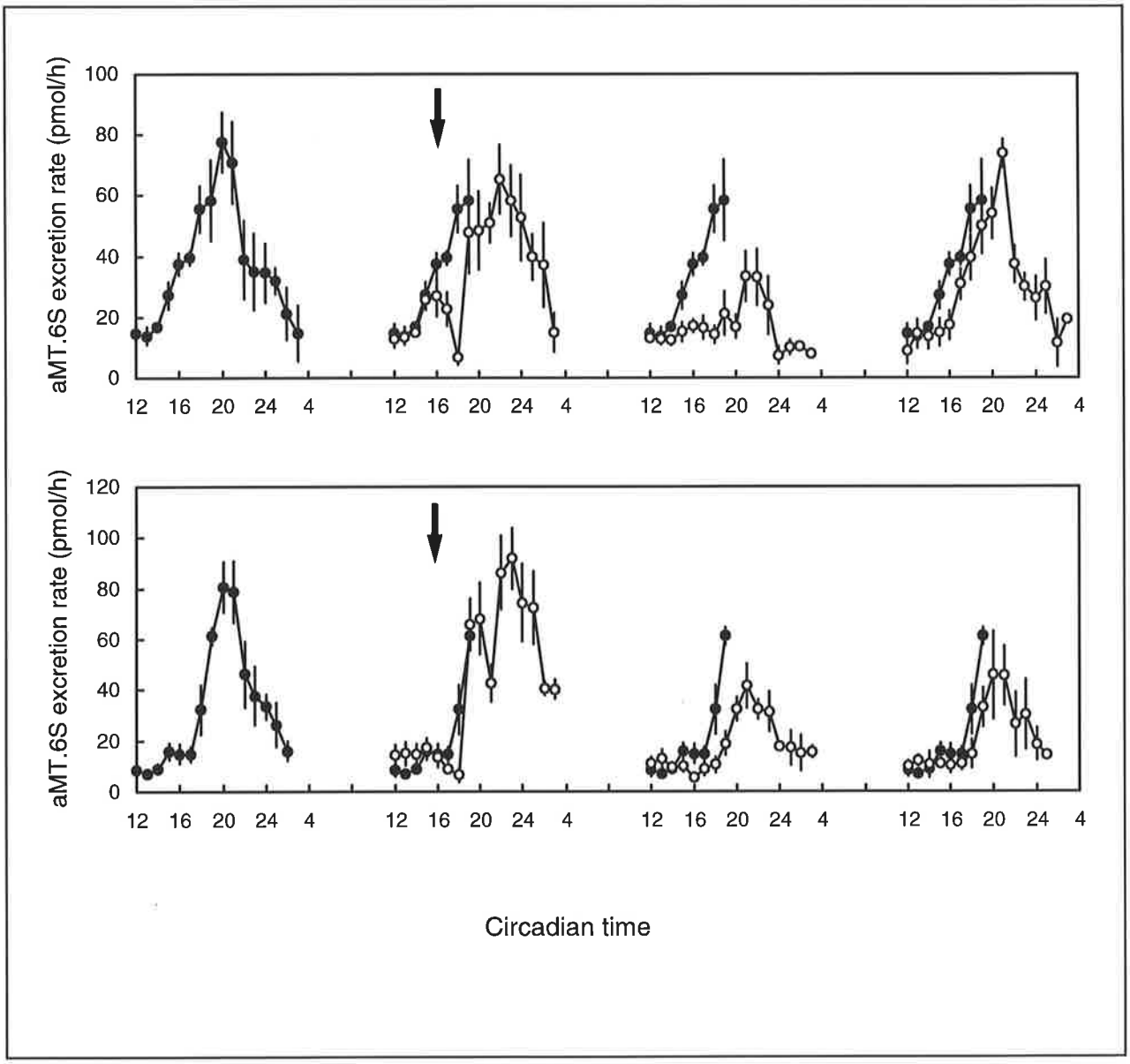

Figure 8.1.
6-sulphatoxymelatonin profiles for SOX (upper panel) or ROX (lower panel) animals treated with nicotine (3mg/kg) at CT16. Four consecutive nights are shown. In each panel data from the control night (represented by closed symbols) overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for ten animals.

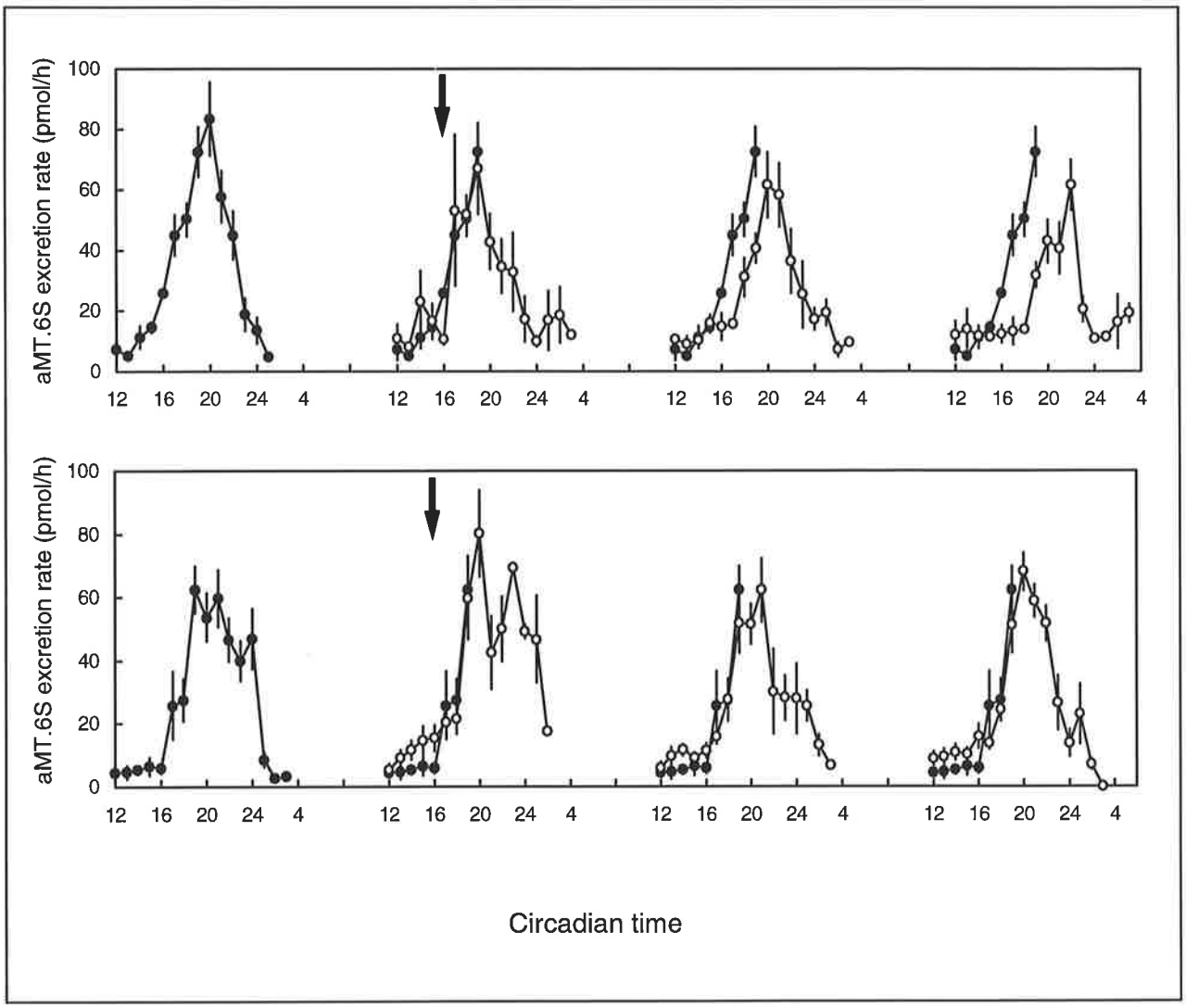

Figure 8.2.
6-sulphatoxymelatonin profiles for SOX (upper panel) or ROX (lower panel) animals treated with nicotine (1mg/kg) at CT16. Four consecutive nights are shown. In each panel data from the control night (represented by closed symbols) overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for ten animals.

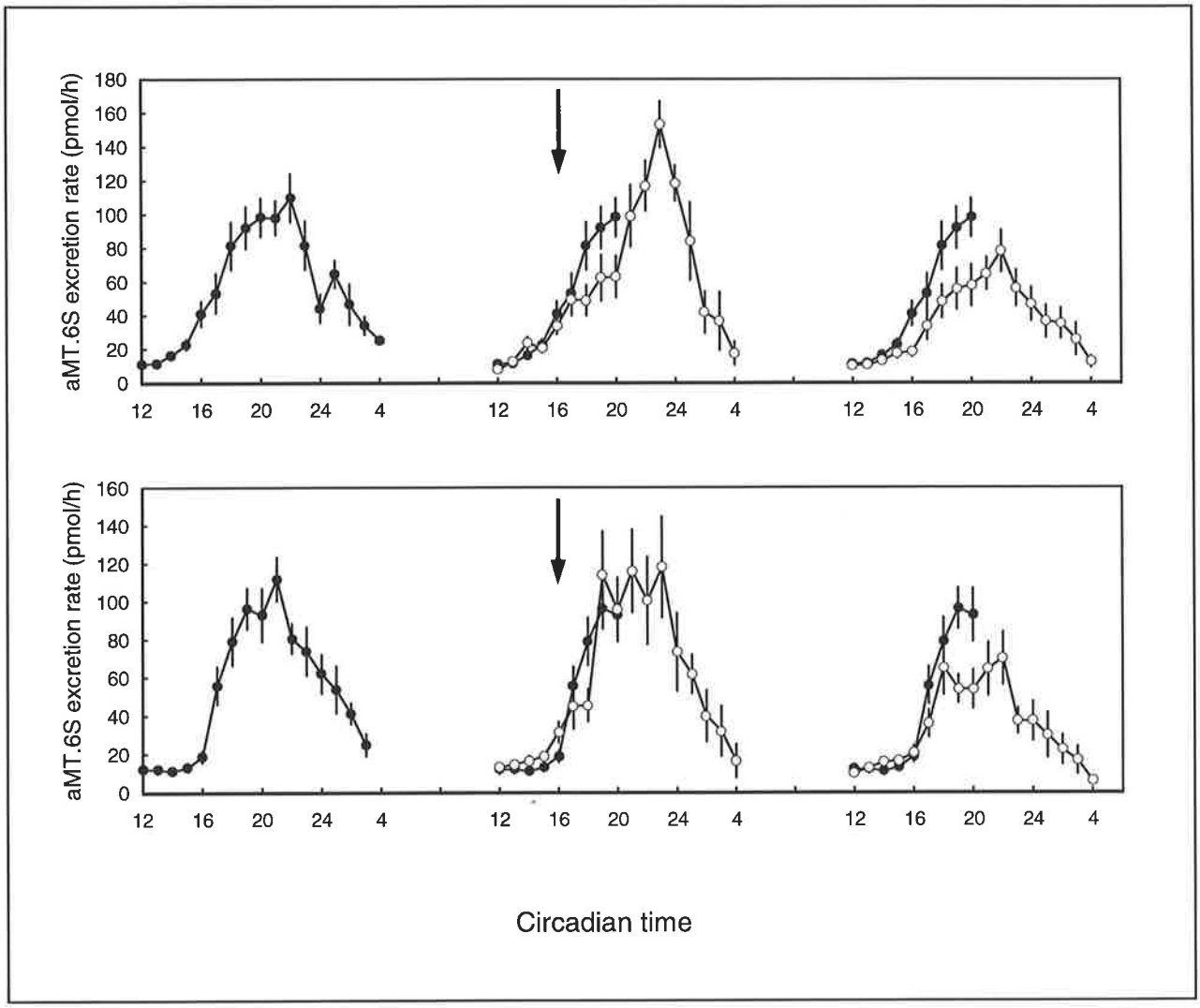

Figure 8.3.
6-sulphatoxymelatonin profiles for SOX (upper panel) or ROX (lower panel) animals treated with oxotremorine (2mg/kg) at CT16. Three consecutive nights are shown. In each panel data from the control night (represented by closed symbols) overlays data from nights 2 and 3 (open symbols). Arrows indicate the time of treatment on night 2. Each data point represents the mean ± SEM for fourteen animals.

Table 8.1
Effect of light treatment on the timing of the onset of the 6-sulphatoxymelatonin excretion rate rhythm in rats. Delays on nights 3 and 4 represent change from onset on night 1.

Treatment	Line	Time	Delay night 3	Delay night 4
Nicotine (3mg/kg)	SOX	CT16	4.3 ± 0.1	2.4 ± 0.2
Nicotine (3mg/kg)	ROX	CT16	1.5 ± 0.4	1.3 ± 0.3
Nicotine (1mg/kg)	SOX	CT16	1.7 ± 0.4	3.6 ± 0.5
Nicotine (1mg/kg)	ROX	CT16	0.2 ±0.5	0.2 ±0.4
Oxotremorine (2mg/kg)	SOX	CT16	1.9 ± 0.5	
Oxotremorine (2mg/kg)	ROX	CT16	0.2 ±0.2	

Data represent the mean ± SEM of the delays for 5-10 animals following cholinergic treatment at CT16.

8.3 DISCUSSION.

Nicotine at the higher dose (3mg/kg) caused phase delays in the timing of aMT.6S onset in SOX animals twice the magnitude seen in RB animals. ROX animals also exhibited a phase delay after treatment with 3mg/kg which was of smaller magnitude than that reported in RB animals (refer chapter 3). At the lower dose of nicotine (1mg/kg), only the SOX animals showed any phase delay in the timing of aMT.6S onset and it was of a similar magnitude to that recorded in RB animals. Oxotremorine (2mg/kg) caused a significant delay in the timing of aMT.6S onset in SOX animals but had no effect in ROX animals. These results indicate that the increased cholinergic sensitivity of the SOX animals demonstrated by a larger response from the thermoregulatory system may also extend to specific components of the circadian timing system. The SCN of the SOX animals appears more susceptible to the phase shifting effects of the nicotinic agonist nicotine and the muscarinic agonist oxotremorine than both normal and ROX animals.

The mechanisms through which acetylcholine effects the timing of circadian rhythms remain undefined. The SCN contains both muscarinic and nicotinic cholinergic receptors (Brownstein *et al,* 1975; Block and Billiar, 1981; van der Zee *et al,* 1991) in addition to the synthetic enzyme of ACh, choline acetyltransferase (Ichikawa and Hirata, 1986). A light pulse given at CT15 was reported to increase ACh levels three-fold within the SCN intimating a role for the transmitter in light effects on the SCN (Murakami *et al,* 1984). This early study was followed by reports that carbachol, a non-specific cholinergic agonist, could produce phase shifts in various species (Zatz and Herkenham, 1981; Furukawa *et al,* 1987; Wee *et al,* 1992). In this project (chapter 3) it was reported that nicotine had significant phase shifting effects on the melatonin excretion rhythm and the induction of *c-fos* in the SCN of normal rats. Taken together these studies provide strong evidence for a fundamental role of ACh in SCN function. The quandary remaining is that acetylcholine is not found in any of the classical entrainment pathways from the retina to the SCN, begging the question, 'through which pathway is acetylcholine having these effects'?

As reported in chapter 3 the rate of melatonin production was partially and briefly suppressed acutely by the nicotine treatment. In this section of the project, both SOX and ROX animals exhibit a similar brief suppression of aMT.6S excretion rate after treatment with 3mg/kg but only SOX after 1mg/kg of nicotine. In contrast, a light pulse given at CT16 results in a

transient suppression of the aMT.6S excretion rate which lasts for approximately 2-3 hours. The lower magnitude acute effect of nicotine may be an indication that the agonist is not acting through major neural pathways that are involved in light information transfer in the rat. Alternatively, if acetylcholine is acting through a light transfer pathway albeit at a lower level of stimulus than light, the resultant suppression of aMT.6S excretion rate may be antagonised by the effect of the cholinergic stimulation at the level of the pineal gland. There are conflicting reports with regard to the effect of cholinergic stimuli on melatonin secretion. Drifhout *et al* (1996) reported no direct pineal effect of nicotine on the secretion of melatonin in the rat (Furukawa *et al,* 1987) however, carbachol was reported to stimulate melatonin secretion from the pineal in rats (Finocchiaro *et al,* 1989; Finocchiaro *et al,* 1990; Laitinen *et al,* 1992). As the treatment in this study was applied around the time of increasing melatonin levels it may not be possible to stimulate production further. Kennaway and Rowe (unpublished) failed to increase melatonin production above normal with isoproteronol. It would seem probable then that nicotine (and oxotremorine in SOX animals) is acting through a minor pathway(s) perhaps not as important in the direct effects of light on the SCN of the rat.

The studies thus far on the rhythmicity of the SOX and ROX animals in both entrained and free-running conditions suggest that the cholinergic sensitivity may be correlated to changes in period and phase angle difference. The SOX animals display both a decreased phase angle difference in a LD photoperiod and a shorter free-running period in constant dark. This suggests that acetylcholine may be more important in rhythm maintenance than in the mediation of phase changes as an immediate response to photic or chemical challenge. The smaller magnitude delays and smaller number of c-FOS-positive cells following nicotine treatment as compared to light may be a result of the activation of different target genes controlling long-term entrainment and therefore gradual or smaller phase shifts as opposed to short-term immediate phase changes.

As reported in chapter 7 SOX animals do not respond in the same way as ROX or RB animals to a 1 minute/2 lux light pulse at CT16. SOX animals exhibit smaller delays in the timing of aMT.6S onset at this time when the SCN is clearly responsive to a light stimulus, and both RB and ROX rats exhibited similar, larger delays. In contrast, the SOX animals do respond with larger phase shifts to nicotine and oxotremorine at this time. This result suggests that the cholinergic agonists may not act through the same pathways as light. However, it is difficult to explain why the SCN of these animals is receptive to light and cholinergic stimuli to

differing degrees. Nicotine is only effective in RB animals at CT16 and CT18 and not at CT6 or CT14. This indicates a time-gated responsiveness of the SCN of these animals to the drug identical to the pattern seen with light pulses. Thus, nicotine is acting on the SCN in a similar manner to light in RB animals yet appears to supply a more effective stimulus in SOX animals than light at CT16. As mentioned earlier this may be a result of the cholinergic agent supplying a stronger stimulus to the sensitive animals which results in a larger response from the entrainment 'mechanisms', similar to that elicited by the 15 minute light pulse. It is apparent therefore that at CT16 a 'stronger' stimulus is required to produce a 'normal' phase shift in the timing of the melatonin rhythm in SOX animals.

The SOX animals were selected on the basis of the degree of hypothermia exhibited after oxotremorine challenge. The dose of oxotremorine used in the present experiment was five-fold larger than that used in the screening process suggesting the SOX animals would have undergone a dramatic drop in temperature at the time of treatment. While there are no studies reporting a phase shifting effect of changing core body temperature alone in mammals, the possibility remains that the decrease in core body temperature may have had an effect on the timing of circadian rhythmicity. There are thermosensitive neurons in the SCN of rats that exhibit a circadian rhythm in their sensitivity and are thought to play a role in the circadian regulation of core body temperature (Derambure and Boulant, 1994). The majority of these neurons are distributed throughout the ventrolateral region of the nuclei which is also the retinorecipient region. It was reported that the peak of sensitivity of these neurons occurred at CT16, the time at which oxotremorine was administered in the present experiment. One possible mechanism for the phase shift of the aMT.6S excretion rhythm in SOX animals caused by oxotremorine is that the thermic load placed on the animals was a large enough stimulus to produce a response both from the temperature regulation system which may include the SCN, and the circadian timing system. It is known that the circadian rhythms of reptiles can be phase shifted with temperature alone (Underwood and Calaban, 1987; Firth *et al,* 1991) and this capacity for adaptation by the circadian timing system to environmental factors other than light may be conserved in higher mammals. However, such adaptation may not be required other than in situations of extreme body temperature changes such as those experienced by the SOX animals after oxotremorine treatment. This may be further investigated through treatment with other agents which cause thermoregulatory stress but not phase shifts of SCN-controlled rhythms.

The phase shift of aMT.6S excretion seen in SOX animals after treatment with oxotremorine and the larger shifts precipitated by nicotine may be a reflection of an increased cholinergic receptor sensitivity in the SCN or in brain regions with cholinergic projections to the SCN. As mentioned acetylcholine is not a transmitter of any of the three main SCN afferents, the RHT, R-GHT or raphe projection, thought to be primarily responsible for entrainment and light mediated effects on SCN function. Cholinergic effects must therefore be mediated through one of two possible scenarios. The first is indirectly through stimulation of one of the three main pathways. The most probable mechanism in this scenario is cholinergic interaction with the excitatory amino acids of the RHT. This is somewhat unlikely however due to the evidence presented by this laboratory indicating that the RHT and EAA have a limited role in SCN function in the rat (Rowe and Kennaway, 1996). The second possible mechanism is that ACh is acting through a separate pathway(s) not recognised as a major component of the circadian timing system.

As there are no cholinergic cell bodies in the SCN it is hypothesised that the cholinergic neurons whose terminals are located in the SCN originate in other brain regions. These include the brainstem and forebrains areas (Bina *et al,* 1993), basal forebrain and mesopontine tegmentum (Kiss and Halasz, 1996) and other hypothalamic areas (Rao *et al,* 1987; Tago *et al,* 1987). Kiss and Halasz also reported the existence of separate contacts between choline acetyltransferase-immunopositive elements and SCN neurons (Kiss and Halasz, 1996). It is possible therefore that these minor pathways are responsible for mediating the action of acetylcholine on SCN function. Whether these regions also receive retinal projections is not yet defined although Bina *et al* (1993) do report the basal forebrain receiving retinal projection. The upregulated cholinergic system of the SOX animals may therefore result in a larger stimulus at the level of the SCN as compared to ROX and RB animals as a result of the same cholinergic treatment.

This data indicates an increased sensitivity of the circadian timing system of SOX animals to both nicotinic and muscarinic stimuli. In addition, these results suggest that the phase shifting effects of nicotine and oxotremorine on rhythmicity in SOX animals are not mediated through the same classical pathways as light. This needs to be examined further using these animals.

Chapter 9

Circadian rhythms and serotonin in SOX and ROX rats

9.1 INTRODUCTION

Extensive research into the neurochemistry of the mammalian circadian timing system has defined three major neural pathways that connect the retina to the SCN (see section 1.5 for details). These tracts each play some role in the timing of circadian rhythms, however there is still much to learn about the precise mechanisms by which light controls rhythmicity. For a long time it was widely accepted that the excitatory amino acids, as the major transmitter in the retino-hypothalamic tract, were also the main mediators of light effects on SCN function in all species (Liou *et al,* 1986; Colwell *et al,* 1990; Colwell *et al,* 1991b; Colwell *et al,* 1993b). Using a rat model this idea was questioned by Rowe and Kennaway (1996) who demonstrated no effect of the EAA agonist NMDA on the timing of either the 6-sulphatoxymelatonin excretion rhythm or the rhythm of locomotor activity. In addition, the antagonist MK-801 was unable to block the phase-shifting effects of a light pulse on these rhythms. This provided preliminary evidence that the EAA do not have a dominant or exclusive role to play in the mediation of light effects on SCN function in the rat.

The serotonergic projection from the raphe nuclei is one of the three main SCN afferents. While the specific function of this tract is not well defined it has not previously been considered to have a major role in the mediation of light effects on rhythmicity. Serotonin receptors have been identified in the SCN (for review see section 1.5.2) and serotonergic agonists were reported to induce phase shifts both *in vivo* and *in vitro* (Prosser *et al,* 1990; Prosser *et al,* 1992; Edgar *et al,* 1993; Prosser *et al,* 1993). Subsequently, the non-specific serotonergic agonist quipazine was shown to induce phase shifts in the rhythm of 6-sulphatoxymelatonin excretion (Kennaway *et al,* 1996) and to induce *c-fos* in the SCN of the rat (Moyer *et al,* 1997). Further examination by our group of the specific receptor subtype mediating these effects demonstrated that the $5HT_{2a/2c}$ receptor agonist (±)-1-(4-Iodo-2,5dimethyoxyphenyl)-2-aminopropane hydrochloride (DOI) was the most effective agent at both eliciting phase shifts and inducing *c-fos* in the SCN (Kennaway and Moyer in press). These studies have provided compelling evidence that in the rat, the serotonergic projection from the raphe nuclei to the SCN may be more important in the mediation of light effects than previously thought.

The SOX and ROX animals were developed with the specific aim of examining the interaction between acetylcholine and the circadian timing system. Based on the Flinders Sensitive Line

(FSL) which demonstrated altered cholinergic sensitivity, the SOX animals exhibit several traits identical to those seen in the FSL rats. The SOX animals show increased sensitivity to muscarinic agents, lower body weight and altered timing of circadian rhythms under both entrained and free-running conditions. Another trait seen in the FSL animals was an increased sensitivity to serotonergic stimulation (Wallis *et al,* 1988) which we hypothesise may also be evident in the SOX animals. The SCN of the SOX animals appears to respond differently to light and as serotonin appears to have a dominant role in the mediation of light effects on circadian rhythmicity in the rat, this animal model could also be useful in further research into the specific role of 5-HT in SCN function. Thus, the specific aim of this section of the project was to examine the sensitivity of both the thermoregulatory and circadian timing systems of the SOX animals to serotonin agonists.

9.2 METHODS

9.2.1 Temperature studies

SOX and ROX animals from G9 in the top 50% of their respective screens were selected for this experiment. The experiment was carried out over 2 consecutive days. On the first day each animal was weighed and a baseline core body temperature was recorded using a rectal thermistor inserted 5-6 cm. On the second day animals were assigned to one of the two treatment groups.

1) 16 SOX, 16 ROX and 5 RB animals received an injection of DOI (0.5mg/kg) and body temperature was recorded 60 minutes later at the same time as the baseline recording.

2) 15 SOX, 15 SOX and 5 RB rats received an injection of 8-OH-DPAT (5mg/kg) and body temperature was recorded 30 minutes later at the same time as the baseline recording.

The differences in average change of core body temperature between groups after each treatment were analysed using a 1-way ANOVA.

9.2.2 Melatonin studies

Part 1

Ten SOX and 10 ROX males from G6 and in the top 20% of their respective screens were selected for this experiment. Animals were placed in metabolism cages in a light-controlled environment chamber and allowed 4 days to acclimatise. Protocol followed the 4-day test

outlined in section 2.1. On the treatment night animals received an injection of quipazine (3mg/kg) at CT18. Urine was collected hourly over each subjective night and vials were weighed and stored frozen for RIA (section 2.2).

Part 2.

Five SOX and 5 ROX males from G11 and in the top 20% of their respective screens were selected for this experiment. Animals were placed in metabolism cages in a light-controlled environment chamber and allowed 4 days to acclimatise. Protocol followed the 4-day test outlined in section 2.1. On the treatment night animals received an injection of 8-OH-DPAT (5mg/kg) at CT18. Urine was collected hourly over each subjective night and vials were weighed and stored frozen for RIA (section 2.2).

Part 3.

Ten SOX and 10 ROX males from G10a and in the top 20% of their respective screens were selected for this experiment. Animals were placed in metabolism cages in a light-controlled environment chamber and allowed 4 days to acclimatise. Protocol followed the 4-day test outlined in section 2.1. On the treatment night animals received an injection of DOI [(±)-1-(4-Iodo-2,5dimethyoxyphenyl)-2-aminopropane hydrochloride] (0.5mg/kg) at CT18. Urine was collected hourly over each subjective night and vials were weighed and stored frozen for RIA (section 2.2).

9.3 RESULTS

9.3.1 Temperature studies.

There was no difference in the thermic response to DOI between the SOX, ROX and RB groups although all groups did experience significant hyperthermia (Figure 9.1). All three groups exhibited a significant decrease in core body temperature after treatment with the 5-HT_{1a} agonist 8-OH-DPAT. The response to 8-OH-DPAT was significantly greater in the SOX group than both the ROX and RB animals (Figure 9.1).

9.3.2 Melatonin studies.

Part 1.

On the control night SOX animals had a significantly earlier onset than ROX (14.6 ± 0.3h vs. 16.1 ± 0.4h). Quipazine caused a significant suppression of the aMT.6S excretion rate at the time of treatment in both SOX and ROX animals (Figure 9.2). The drug also caused a significant phase delay on the post-treatment nights in both groups as compared to the onset recorded on the control night. The delays were significantly larger in the SOX animals than the ROX (Table 9.1), however the ROX animals displayed greatly reduced melatonin production on both post-treatment nights (Figure 9.2).

Part 2.

The G11 SOX animals had a significant earlier (1.9h) time of onset of aMT.6S excretion rate on the control night than the ROX animals. There was no significant or obvious acute effect of 8-OH-DPAT although the total melatonin production was reduced in both groups as compared to the control night (Figure 9.3). There were significant delays recorded on both post-treatment nights in both groups which were significantly larger in the SOX group than the ROX which had small but significant phase delays (Figure 9.3)(Table 9.1).

Part 3.

The time of onset on the control night for SOX animals was 15.4 ± 0.2h, significantly earlier than the time of onset for ROX animals which was 17.1 ± 0.3h. The melatonin production of the ROX animals was not affected acutely by DOI administration, however in the SOX group the levels reached on night 2 were approximately half the peak levels on night 1 (Figure 9.4). Further, there appears to be a delayed transient suppression of the excretion rate 2 hours after treatment. On the post-treatment nights both groups exhibited significant delays and the degree of change was not different between the groups (Figure 9.4) (Table 9.1).

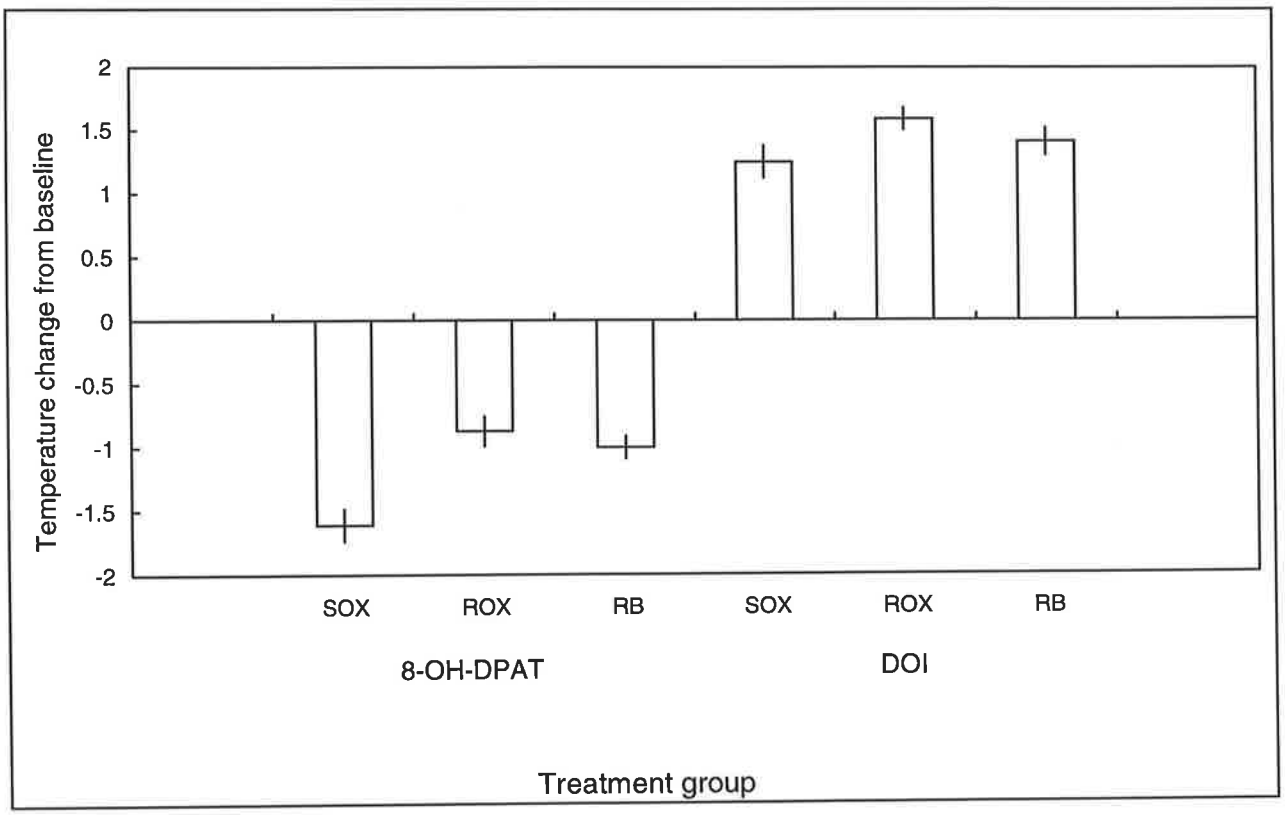

Figure 9.1
The change in core body temperature of SOX, ROX and RB animals as a response to the serotonergic agonists 8-OH-DPAT and DOI. Each data set represents the mean ± SEM for 15 -16 animals.

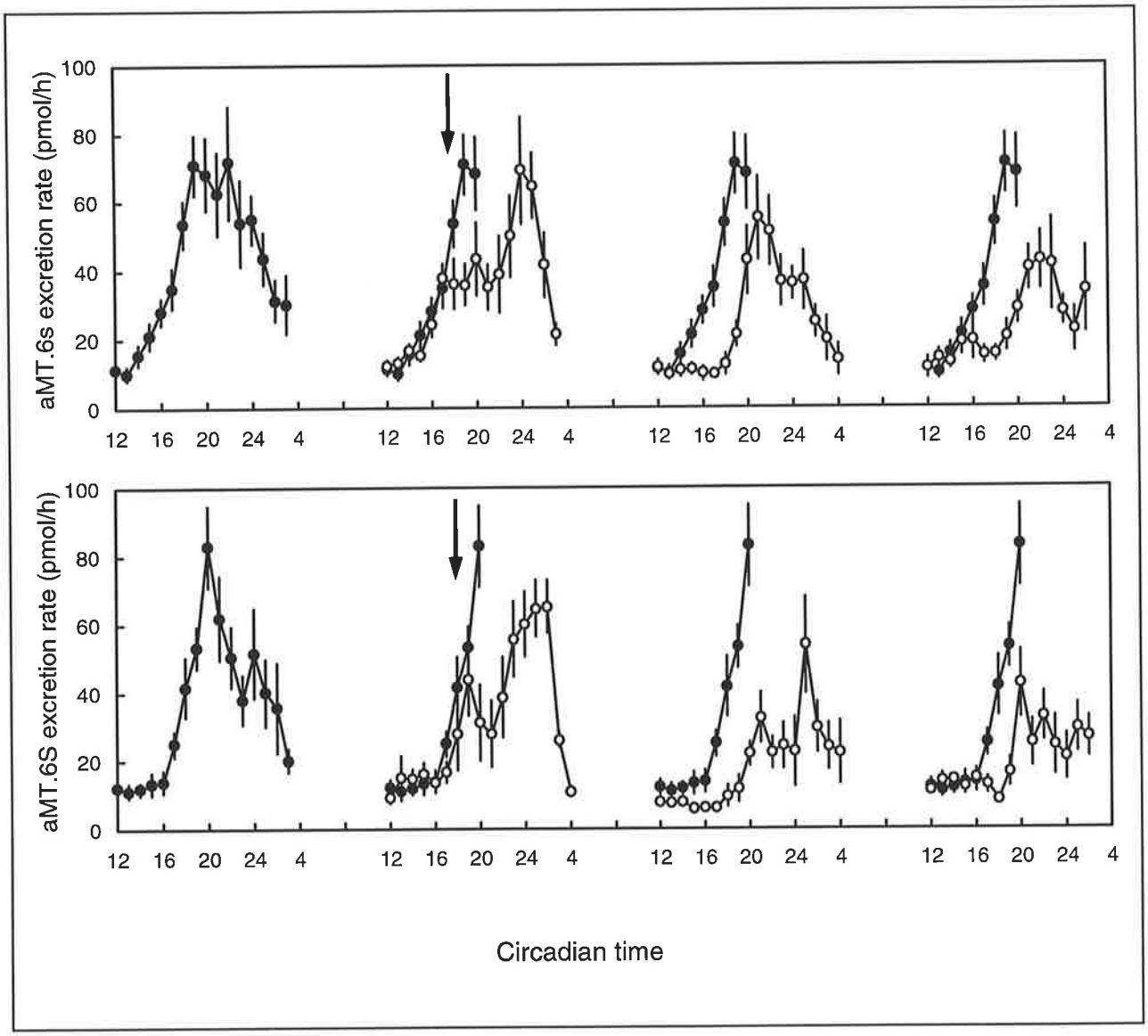

Figure 9.2
6-sulphatoxymelatonin excretion profiles for G10 SOX (upper panel) and ROX (lower panel) animals treated with quipazine (3mg/kg) at CT18. Four consecutive nights are shown. In each panel data from night 1, represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate time of treatment. Each data point represents the mean ± SEM for ten animals.

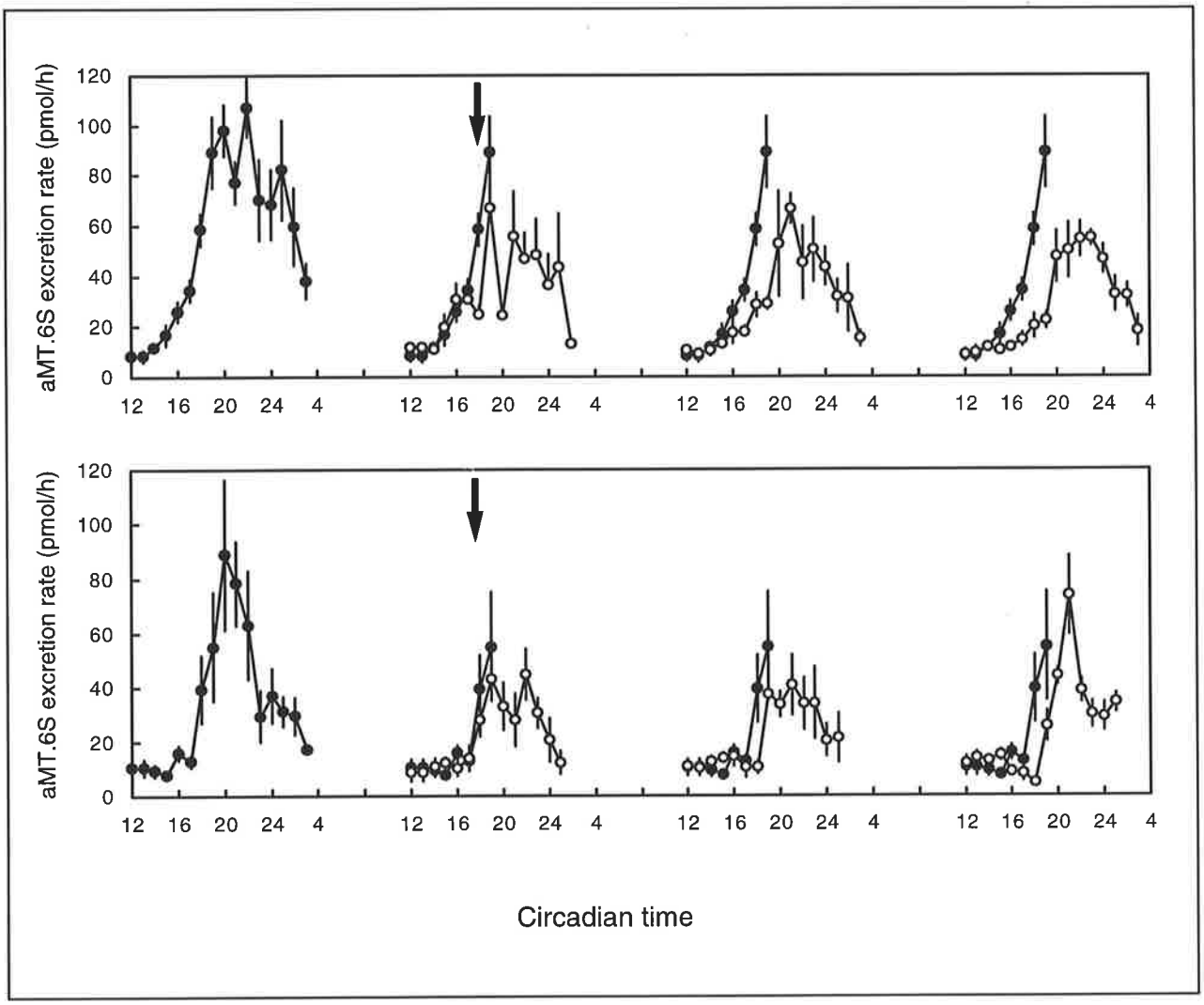

Figure 9.3
6-sulphatoxymelatonin excretion profiles for SOX (upper panel) and ROX (lower panel) animals from G11 treated with 8-OH-DPAT (5mg/kg) at CT18. Four consecutive nights are shown. In each panel data from night 1, represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate time of treatment. Each data point represents the mean ± SEM for five animals.

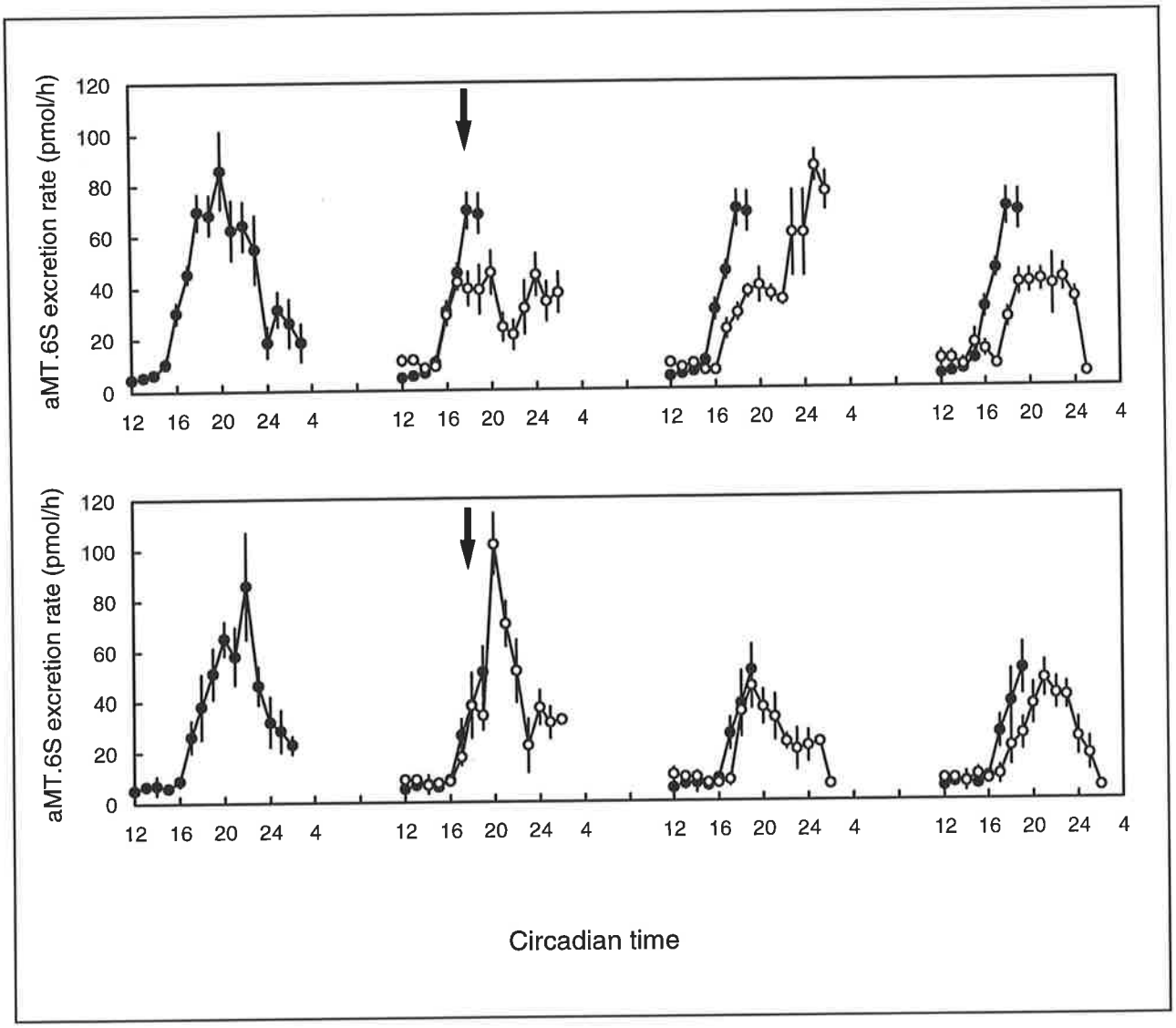

Figure 9.4
6-sulphatoxymelatonin excretion profiles for SOX (upper panel) and ROX (lower panel) animals from G10 treated with DOI (0.5mg/kg) at CT18. Four consecutive nights are shown. In each panel data from night 1, represented by solid symbols, overlays data from nights 2, 3 and 4 (open symbols). Arrows indicate time of treatment. Each data point represents the mean ± SEM for ten animals.

Table 9.1
The effect of serotonergic agonists on the timing of the 6-sulphatoxymelatonin excretion rate rhythm.

Treatment	Line	Time	Delay N3	Delay N4
Quipazine	SOX	CT18	2.9 ± 0.5	3.5 ± 0.4
Quipazine	ROX	CT18	2.1 ± 0.3	2.4 ± 0.4
8-OH-DPAT	SOX	CT18	2.0 ± 0.3	3.2 ± 0.4
8-OH-DPAT	ROX	CT18	0.9 ± 0.2	1.4 ± 0.2
DOI	SOX	CT18	1.5 ± 0.2	1.9 ± 0.3
DOI	ROX	CT18	1.2 ± 0.2	1.3 ± 0.3

Data show the mean ± SEM delays in onset time of the aMT.6S excretion rhythm on night 3 and 4 recorded after treatment with serotonergic agonists at CT18 as compared to the onset on night 1.

9.4 DISCUSSION.

The sensitivity of the SOX and ROX animals to serotonergic agents was assessed using the thermoregulatory responses and circadian phase changes. The 5-HT_{1a} agonist 8-OH-DPAT caused significant decreases in the body temperature of both lines which were of a larger magnitude in the SOX animals, whereas the 5-HT_{2c} agonist DOI caused similar an increase in core body temperature in SOX, ROX and RB rats, of a similar magnitude. These results were mirrored in the phase delays recorded in 6-sulphatoxymelatonin excretion rate rhythm after serotonergic treatment. DOI caused similar, significant phase changes in both groups. However, both the non-selective agonist quipazine and the 5-HT_{1a} agonist 8-OH-DPAT caused significant delays in the melatonin rhythms of both SOX and ROX animals which were larger in the sensitive group. This data suggest that the SOX animals which were bred for increased cholinergic sensitivity may also exhibit an increased sensitivity to serotonergic stimuli, specifically mediated by the 5-HT_{1a} receptor.

The Flinders Sensitive Line (FSL) of rat were reported to be more sensitive to muscarinic and nicotinic agents in addition to having a lower body weight and displaying altered circadian timing under various conditions (Overstreet, 1991). Thus far, the SOX animals have mirrored the development of the FSL in all of these factors. Further, the FSL animals were reported to have an increased sensitivity to serotonergic stimulation (Wallis *et al*, 1988). The antagonist cyproheptadine and the 5-HT_{2c} agonist mCPP both induced significantly larger hypothermia in the FSL animals. While the present study indicated that there was no difference between the SOX and ROX lines with respect to thermic sensitivity to the 5-HT_{2c} agonist DOI, the SOX animals did exhibit a significantly larger temperature decrease after 8-OH-DPAT treatment. This suggests that the 5-HT_{1a} receptor may be more important in the apparent increased sensitivity seen in SOX animals although antagonist studies would need to be conducted before definitive conclusions could be made.

Using the system of constant urine collection from freely moving rats we have previously been very successful in studying the effect of various neurotransmitter agents on the timing of the melatonin rhythm, an accurate marker of SCN function. An extensive analysis of the role of serotonin in the circadian timing system has demonstrated that the 5-HT_{2c} agonist DOI is the most potent agent with respect to the induction of both phase shifts of the melatonin rhythm and c-FOS positive cells in the SCN of rats (Kennaway and Moyer unpublished). The

serotonergic agents quipazine, 8-OH-DPAT and buspirone, were also administered to rats and caused significant phase changes in the aMT.6S rhythm of a smaller magnitude. The non-selective agonist quipazine caused phase delays of around 2 hours on the nights following treatment and 8-OH-DPAT caused one hour delays in randomly bred animals (Kennaway *et al,* 1996). These shifts are comparable to those recorded in ROX animals suggesting no altered sensitivity to these agents in the resistant line of rat. In contrast, the SOX animals exhibited much greater delays after treatment with both quipazine and 8-OH-DPAT. This increased sensitivity to serotonergic agents may have emerged as a result of one of two possible mechanisms (1) co-selection with the cholinergic sensitivity or (2) as an interaction directly with the cholinergic system.

Serotonin agents can induce responses from the thermoregulatory system, differing according to the receptor subtype being activated. As temperature change was the sole selection criteria for the SOX model, it is possible that animals most sensitive to serotonergic as well as cholinergic stimuli were preferentially selected. If this model were applicable there would by definition have been some interaction between the cholinergic and serotonergic systems at the time of cholinergic challenge such that the serotonergic system was stimulated. Thus, the animals would have been predisposed to increased serotonergic sensitivity which may have contributed to the large change in core body temperature following oxotremorine treatment. However, the results from this section of the project would tend to negate this possibility. The SOX animals did not respond differently to the hyperthermia-inducing effects of DOI, whereas they did show a larger decrease in temperature following 8-OH-DPAT. Having noted that however, we did not expose the animals to the non-specific agonist quipazine and record temperature, nor has temperature been monitored after direct exposure to serotonin itself. Such studies would need to be conducted to further clarify the interaction.

The second possible mechanism is that serotonergic sensitivity is altered in the SOX animals as a result of the altered cholinergic sensitivity. This implies a post-selection change and is the more likely mechanism. The changes in the sensitivity of the FSL animals with regard to serotonin were hypothesised to be 'the consequence of a primary change in the cholinergic system during the selective breeding program' (Overstreet, 1991). Again further studies will need to be conducted to determine the extent of the altered serotonergic sensitivity, however, these results do suggest that the SOX and ROX model may also be very useful in the study of other neurotransmitters in the control of the circadian timing system.

The SOX animals have a lower body weight at 28 days of age than the ROX animals, a similar characteristic to the FSL animals (Overstreet, 1991). The possible mechanisms for this change were not discussed by the Flinders group, only to suggest that this trait is also reported in human depressives. The altered serotonergic sensitivity of both SOX and FSL animals may provide an answer to this question. As mentioned in chapter 4, serotonin plays a role in the satiety/hunger regulation system (Kitchener and Dourish, 1994; Dryden *et al,* 1996) and via a possible interaction with neuropeptide Y affects the amount of food intake of the animal (Myers *et al,* 1996). The decreased body weight of the SOX animals may therefore be an indirect result of the altered serotonergic tone as acetylcholine is not reported to have any role in feeding behaviour.

Taken together these results suggest that the SOX animals do indeed have an altered serotonergic system that impinges on both the control of thermoregulation and circadian rhythmicity. This may also extend to the satiety/hunger control centre however, further experiments are required to verify that hypothesis. As a model for the study of the role of acetylcholine in the circadian timing system, the SOX animals have become invaluable and it now appears that both lines will be useful for further work with the serotonergic system.

Chapter 10

Receptor binding studies

10.1 INTRODUCTION

The SOX animals have developed a number of similar characteristics to the Flinders Sensitive Line (FSL). Most importantly the line has continued to show increasing hypothermic responses to the muscarinic agent oxotremorine as the selective breeding program has progressed. This alone suggests that the animals are becoming increasingly sensitive to cholinergic challenge, as did the FSL animals (Overstreet, 1991). Further to this, the SOX line have a lower body weight, altered circadian timing under both LD and DD photoperiods, and display altered sensitivity to a second cholinergic drug, nicotine, as well as some serotonergic agonists. The physiological basis for the upregulated cholinergic systems in these animals is unknown.

Studies were conducted in the FSL animals to determine the molecular mechanisms underlying the increased cholinergic sensitivity. They reported no difference in the sensitivity of acetylcholinesterase to DFP between the lines subsequently going on to examine the degree of synthesis and turnover of ACh as well as the numbers of muscarinic acetylcholine receptor (mAChR) sites in the brains of the two lines. Rats were injected with deuterium-labelled choline via a tail vein and 60 seconds later were killed and assessed for labelled acetylcholine in the cortex. FSL animals were reported to have higher concentrations of labelled acetylcholine that the control FRL animals (Overstreet *et al,* 1984). Further to this, ligand binding studies demonstrated higher levels of mAChR in the striatum and hippocampus of the FSL animals compared to the FRL animals (Overstreet *et al,* 1984). The authors suggested that both pre- and post-synaptic cholinergic mechanisms contributed to the altered sensitivity observed in the FSL animals, however the relevance of this depended on the brain region involved.

As the SOX animals appear to have developed along a similar vein as the Flinders line it is hypothesised that the mechanisms underlying the altered sensitivity may also be of a similar nature. While the selection criteria did differ between the two models the outcomes have been the same. Hence, this section of the project examined the nicotinic and muscarinic receptor binding levels in specific brain regions of the SOX and ROX animals with the aim of determining the neurochemical basis for the altered sensitivity.

10.2 METHODS

SOX and ROX animals used in the studies outlined below were selected from generations 10-12 and were in the top 50% of their respective screens. All animals including RB rats were 200-250g at the time of tissue collection (section 2.6). SOX and ROX animals used in these studies had not been exposed to any cholinergic agent prior to the study other than the 0.25 μmol/kg injection of oxotremorine at the time of phenotyping. This occurred at least three weeks prior to the collection of brains for analysis in this study. The RB rats were not previously exposed to any cholinergic stimuli.

10.2.1 Nicotinic studies.

The animals were decapitated and brains collected as outlined in section 2.6. For the nicotinic studies only the hypothalamus was collected. Sections were homogenised according to specifications in section 2.6 and stored at -20°C until assay. Preliminary studies to optimise assay conditions are detailed in section 2.6.1 of the general methods chapter.

Ten SOX, 10 ROX and 5 RB males were used for the ligand binding analysis. The binding assay was set up using the conditions described in section 2.6.1 and homogenates were incubated in the presence of 0.2nM [^{125}I]α-BTX (~½K_D of 0.33nM). The difference in binding levels between the three groups was assessed using a one-way ANOVA.

10.2.2 Muscarinic studies.

The animals were decapitated and brains collected as outlined in section 2.6. For the muscarinic studies hypothalamus, striatum, hippocampus and cortex were collected. Sections were homogenised according to specifications in section 2.6 and stored at -20°C until assay. Preliminary studies to optimise assay conditions are detailed in section 2.6.2 of the methods chapter.

Eight SOX, 8 ROX and 5 RB males were used in these analyses. Hypothalamic homogenates were incubated with 40pM [^{3}H]QNB, striatal homogenates with 80pM [^{3}H]QNB and hippocampal and cortex homogenates with 20pM [^{3}H]QNB according to the K_D's calculated for each brain region. The difference in binding levels in each region between the three groups was assessed using a one-way ANOVA.

10.3 RESULTS.

10.3.1 Nicotinic studies.

The binding assay results for nicotinic receptors in the hypothalamus are shown in Figure 10.1 and indicate no significant difference in the number of receptors between the three groups of animals.

10.3.2 Muscarinic studies.

The binding assay for muscarinic receptors in the hypothalamus showed no difference in the amount of [^{3}H]QNB bound between the 3 groups (Figure 10.2). In the striatum however, there were significantly higher levels of binding in SOX animals than both ROX and RB, which were not different from each other (Figure 10.2). Similarly levels of binding were significantly higher in the hippocampus of SOX animals than both ROX and RB rats (Figure 10.2). Samples from the cortex of the three groups showed no significant differences in binding levels.

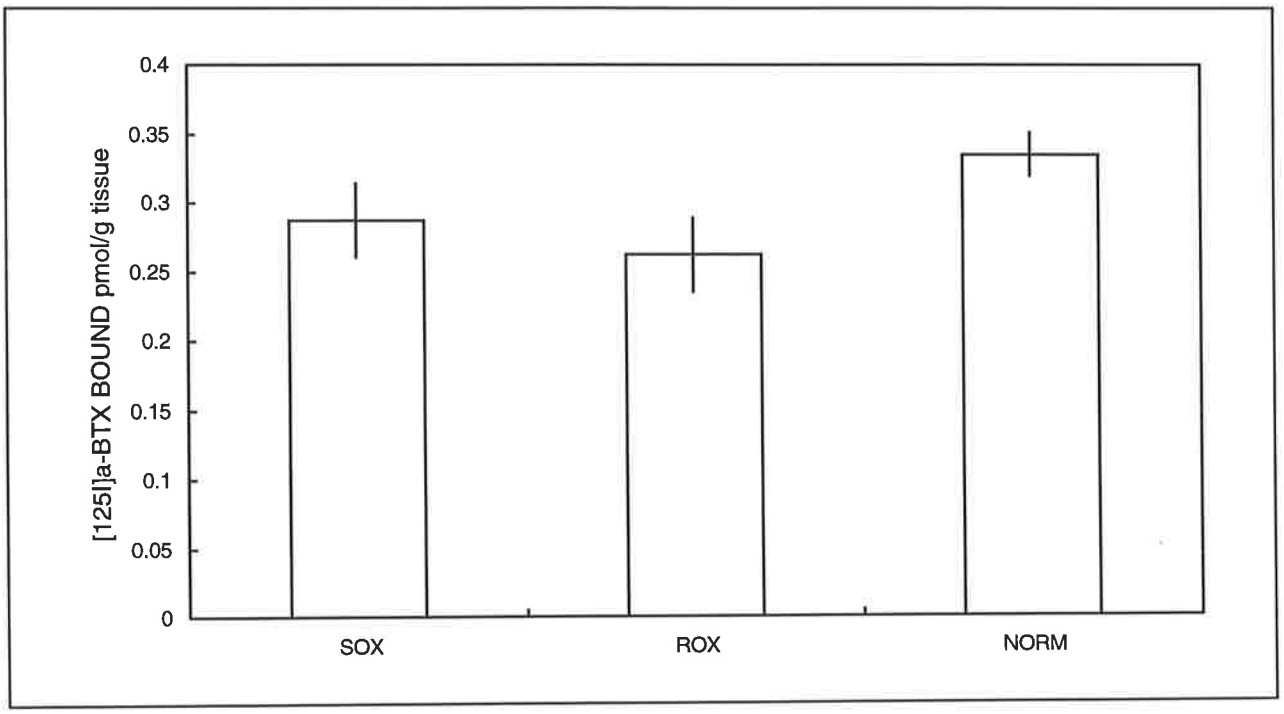

Figure 10.1
Numbers of nicotinic binding sites in the hypothalamus of SOX (n=10), ROX (n=10) and RB (n=10) rats as determined by ligand binding assay using [^{125}I]α-BTX. Values represent the mean ± SEM.

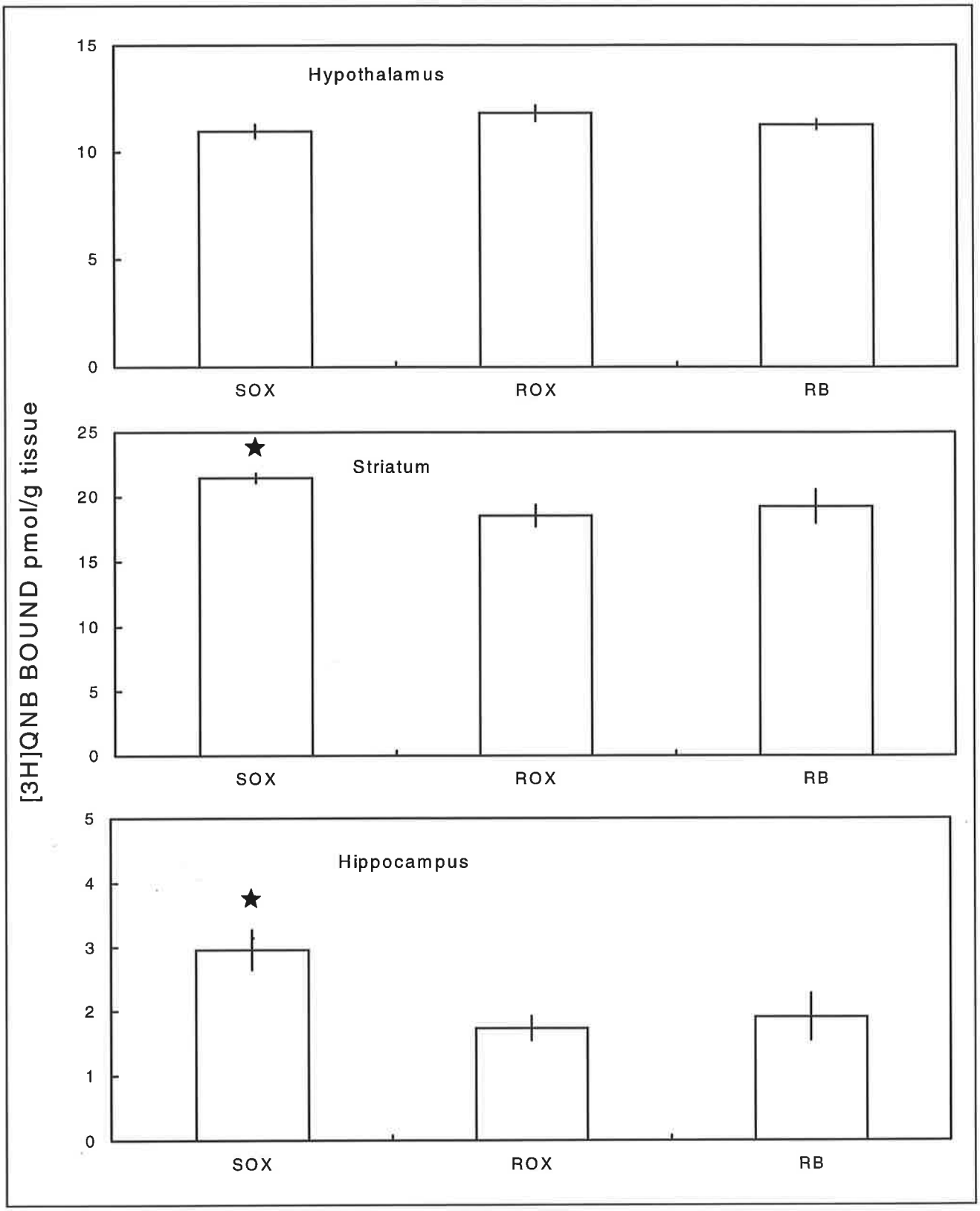

Figure 10.2
Numbers of muscarinic binding sites in three discrete brain regions of SOX (n=8), ROX (n=8) and RB (n=5) rats as determined by ligand binding assay using [^{3}H]QNB. The upper panel contains data from the hypothalamus, the centre panel contains data from the striatum and the lower panel contains data from the hippocampus. Values represent the mean ± SEM. Note that the scales on the y-axis are not identical in each graph. Stars indicate significant differences from RB.

10.4 DISCUSSION.

The SOX animals had higher levels of muscarinic receptors in the striatum and hippocampus than both ROX and RB rats. In the hypothalamus and cortex however the levels of muscarinic receptors were identical in all three groups, as were the levels of nicotinic receptors in the hypothalamus. The altered receptor density in these specific brain regions may underlie the cholinergic sensitivity of the SOX animals.

Although the development of the SOX line was based partly on the breeding program of the FSL (Overstreet *et al,* 1979) the specific aim was to investigate the role of acetylcholine in the regulation of circadian timing. This aim was developed after studies on the Flinders lines showed that the cholinergically sensitive line displayed altered timing of circadian rhythms under both constant and cyclic photoperiods. The authors suggested that the increased sensitivity to cholinergic challenge was correlated with changes in the functioning of the SCN of those animals. Thus, the SOX line was developed using a similar protocol of assessment of thermic sensitivity to cholinergic agents and several physiological parameters were monitored from the outset of the breeding program, with emphasis on the emergence of any differences in circadian function between the sensitive and resistant lines.

Previous chapters have described apparent differences between the functioning of the circadian timing systems of the SOX and ROX lines, suggesting similarities between the newly developed lines and the Flinders animals. Further, the SOX animals have similar physiological characteristics as the FSL animals including lower body weight and increased sensitivity to serotonergic agents. From the results presented in the current chapter it appears that the mechanisms underlying the increased cholinergic sensitivity may also be similar to the FSL animals. Although the current investigation was limited only to ligand binding studies assessing the receptor numbers in each brain region, the results mirror exactly that found in the Flinders animals. Higher levels of muscarinic receptors in both the striatum and hippocampus of SOX animals, and no differences in the hypothalamus or cortex parallel the results reported in FSL animals.

The hypothalamus reportedly contains the centre for control of thermoregulation however, there were no differences in the cholinergic receptor numbers between the SOX and ROX lines with respect to this brain region. This result was similar to that reported in the Flinders lines,

however, as temperature was not the sole parameter used to assess sensitivity in that program changes in the thermoregulatory region may not have been of particular significance. In the current breeding program thermic response was the sole measure of cholinergic sensitivity and thus changes in hypothalamic muscarinic receptor number were expected between the lines. It is possible that the changes in other cholinergic rich regions such as the striatum and hippocampus directly or indirectly impinge upon the function of the thermoregulatory systems contained in the hypothalamus. Alternatively, the ligand binding assay used may not have been sensitive enough to accurately assess any differences between the lines with respect to receptor number. To further investigate this possibility *in vitro* autoradiographical studies would need to be carried out.

This section of the project has showed that receptor numbers in the striatum and hippocampus may be the underlying mechanism of the increased cholinergic sensitivity in the SOX animals. While the molecular investigations are somewhat limited at this stage the results mirror those reported in the FSL, and *in vitro* studies are required to further clarify the specific mechanisms of the altered cholinergic sensitivity.

Chapter 11

Behavioural testing

11.1 INTRODUCTION.

The development of the Sensitive to OXotremorine (SOX) line of rat has closely followed that of the Flinders Sensitive Line. The SOX animals exhibit similar large temperature responses to cholinergic challenge, have a lower baseline body weight and altered timing of circadian rhythmicity. While the FSL animals were not originally developed as such, the line was subsequently reported as an animal model of human depression based on several physiological, pharmacological and behavioural parameters.

Human depressive disorder can affect up to 1 in 5 people at some stage in their lifetime. The illness is characterised by a persistently lowered mood and sustained bouts of melancholia (Caldecatt-Hazard *et al,* 1991). Symptoms most obvious include anhedonia or the inability to experience pleasure and a lack of emotional response to stimuli (Hamilton, 1982; Caldecatt-Hazard *et al,* 1991). Such mood swings may result in weeping, loss of interest in normal activities and loss of appetite leading to weight reduction (Hamilton, 1982). Depression can cause feelings of such worthlessness and helplessness that many suicides are directly attributable to the disease state. The hypotheses regarding the underlying neurochemical alterations in the brain giving rise to clinical symptoms of depression are varied, however the cholinergic-adrenergic balance hypothesis proposed by Janowsky *et al* (1972) suggested that the cholinergic transmitter system had an important role to play in the development of the disease state. On the basis of this the FSL rat was promoted as an animal model for the study of human depressive disorder.

One of the symptoms commonly reported, not only in depressive illnesses but also other psychiatric illnesses such as schizophrenia and mania, is that of disruption of normal circadian patterns. The FSL animals underwent a series of studies to assess the timing of their circadian rhythms under both entrained and constant conditions. These studies showed that the timing systems of the FSL animals functioned differently under both lighting conditions than those of FRL and RB rats, a similar situation as is seen in human depressives. Further the FSL animals showed reduced activity after footshock experiments and greater immobility in a forced swim test, commonly used for the assessment of new antidepressants. Thus, this section of the project aimed to determine the depth of the similarities between the FSL animals and the newly developed SOX rats using a test commonly applied to assess behavioural despair, the forced swim test. Further, an analysis was carried out on the general activity profiles of the animals

in entrained and free-running conditions to determine any difference in the baseline levels of activity.

11.2 METHODS.

11.2.1 Forced swim test.

Fourteen SOX animals (10M, 4F) and 14 ROX animals (10M, 4F) from generation 10 and in the top 50% of their respective screens and 10 RB males were chosen for this experiment. Animals weighed approximately 130g at the time of experiment and were housed in home cages 4-5 to a cage under a 12L:12D photoperiod with lights off at 1900h.

Each test was conducted over a five minute period according to the protocol set out in section 2.7. The animal was placed gently in the tank and monitored closely for five minutes to ensure it stayed afloat and was not unduly stressed. All tests were videotaped for assessment at a later date. The experimenter was blind to the line of each rat (tail markings were used to code the animals).

Assessment of immobility was done by two investigators blind to the line of each animal. The time spent immobile was recorded for each animal and was defined as the time the rat spent moving only minimally (1 of the 4 paws) to keep the nose out of the water as opposed to movement using all limbs in an attempt to swim or scramble out of the tank. Data are expressed as seconds spent immobile. Differences between the groups were assessed using a 1-way ANOVA.

11.2.2 General activity.

Five SOX males and 5 ROX males from generation 12 and in the top 20% of their respective screens were selected for this experiment. Animals were implanted with transmitters according to section 2.3 and placed in individual home cages in a light-controlled environment chamber in a 12L:12D photoperiod with lights off at 1900h. General activity was monitored continuously in each individual animal. Animals remained in LD conditions for 5 days and were then transferred to constant dark for a further 14 days. No circadian analysis was done

on the data, only assessment of the levels of activity of the two lines and determination of any differences in levels between the lighting conditions.

The data was analysed using the Tau computer program and the mean waveform was calculated for the DD period. In addition the total activity during the LD and DD conditions was calculated along with the total activity in the light and dark phases of the LD period.

11.3 RESULTS

11.3.1 Forced swim test

Male and female data were analysed together as there was no differences found in the time immobile between the sexes of each line. A one-way ANOVA showed no significant differences between the three groups of animals. However, Figure 11.1 indicates a trend towards the ROX animals spending less time immobile than the SOX and RB groups, which both have immense variation as indicated by the standard error bars.

11.3.2 General activity

The mean activity as calculated using the TAU program for the DD period was significantly less in the SOX group (131.8 ± 6.8) than the ROX group (170.1 ± 10.5). In concordance, the total activity over the DD period was significantly lower in SOX animals. There was no difference between the groups with respect to the total activity across the entire LD period, nor were any differences found when the light and dark phases were analysed separately in this condition.

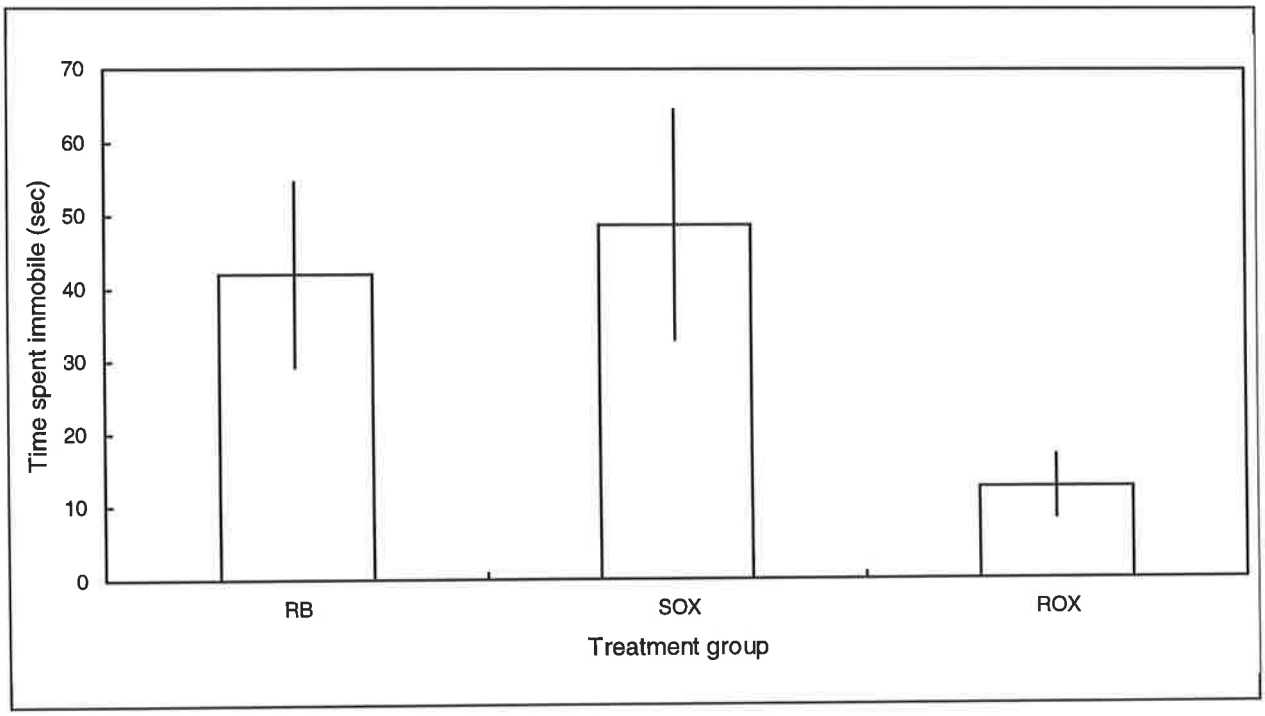

Figure 11.1
The time spent immobile during a five minute forced swim test by SOX (n=14), ROX (n=14) and RB (n=10) rats. Male and female data are combined. Each data set represents the mean ± SEM.

11.4 DISCUSSION.

The forced swim test used for assessing depressive tendencies in animals showed no significant differences between the SOX, ROX and RB animals. There was a significant difference however with respect to the level of general activity between the lines under constant dark conditions with the SOX animals displaying a lower level of activity than ROX. A direct comparison between the behavioural characteristics of the SOX line and the FSL animals is difficult due to differences in protocol between studies, however at this stage of the current breeding program it is suggested that the SOX may not be displaying the typical depressive traits as were reported in the Flinders Sensitive Line.

Behavioural despair in animals is measured by a variety of tests however most widely used is the forced swim test. This test is also commonly used for the assessment of efficacy of newly developed antidepressant agents in animal models. There is much discussion in the literature as to the validity of the measure of 'immobility' in the test as an indicator of behavioural despair. However, since it was first described by Porsolt *et al* (1978), the test has received widespread use (Overstreet *et al,* 1986; Borsini and Meli, 1988; Nishimura *et al,* 1988). A number of factors contribute to the degree of immobility displayed by animals in the test including water temperature, depth and freshness, the length of the test period, the number of test periods and the manner of housing prior to the test (ie. individual or group)(Porsolt *et al,* 1978; Overstreet *et al,* 1986; Borsini and Meli, 1988; Nishimura *et al,* 1988). As the aim of the current study was to assess similarities between the SOX and FSL animals the protocol followed closely that of Overstreet *et al* (1986) with some modifications imposed by our institutional Animal Ethics Committee.

The use of an initial swim period followed by a second test period which examined the time spent immobile in 5 minute bins of a 15 minute test was not employed in the current study and may account for the lack of significant difference between response times in the three groups. Alternatively, we used only a five minute period for the test swim and the shorter length test may have precluded differentiation between the groups. It may be necessary therefore to follow this line of investigation using changes to the protocol to employ longer and successive swim periods in addition to studying more animals. Further, the temperature of the water was held at 37°C in our study compared to 25°C in the Overstreet protocol which may also have contributed to the lack of significant effect.

The SOX and ROX animals used in this study were selected from generation 12 and the Flinders animals came from the 26th generation. This may account for the lack of differences between the lines and will be addressed by the examination of more animals of a later generation. Alternatively, the differences in selection criteria in the two programs may have produced behaviourally divergent lines. Whereas the current program used only temperature as a measure of cholinergic sensitivity, the Flinders group used a behavioural measure in drinking activity as one of the selection criteria. This factor may have contributed to the development of depressive-like behaviours in those animals. Repeating the study in a later generation with an altered protocol may supply further clarification of the behavioural characteristics in the lines.

The FSL animals were reported to have lower levels of general activity as measured by line crossings in an open field chamber during a one minute test period (Overstreet *et al,* 1986). This test was carried out as part of a drug withdrawal experiment and the baseline recordings were done prior to any stressor in saline treated FSL, FRL and RB animals. The data showed that FSL animals crossed significantly less lines than both FRL and RB in the baseline measure. It is presumed that this test was conducted in the light portion of the LD cycle, however this is not specified. Data gathered from a single short test such as the open field line crossings cannot be compared with long-term continuous measurements of general activity. The results of the current study showed significant differences between the SOX and ROX groups during the DD portion of the experiment, however no differences were evident across the whole LD period nor the light or dark phases of the LD period. The relevance therefore of the result found in SOX animals to that reported in the FSL animals is limited. However, the results in the SOX are intriguing in that a photoperiod change appears to have altered the level of general activity in the SOX rats. The possible mechanisms for this action are not understood.

This section of the project has studied some behavioural parameters of the SOX and ROX animals with the aim of comparing the depressive tendencies of the SOX and FSL animals. The changes made to the forced swim test protocol may have precluded differentiation between the groups and will require further investigation with a larger number of animals possibly under altered conditions. The activity measurements in the SOX animals indicate changes in the activity function with a change in the light regime. Whether this is a circadian timing

system-based change or a result of cholinergic changes affecting other brain regions in as yet unknown.

Chapter 12

Summary and Conclusions

12.1 INTRODUCTION

The suprachiasmatic nucleus (SCN) controls the timing of circadian rhythms in mammals. The SCN maintain a spontaneous, endogenously generated oscillation of neural activity that directly controls the timing of biological rhythms such as temperature, sleep/wake and hormonal levels. The underlying mechanism producing the rhythm of activity in the SCN is the cycle of a number of clock genes and their products within individual cells. These genetically derived rhythms are synchronised within the SCN to produce the robust circadian rhythm although the specific genes responsible for the oscillation are not described in mammals.

The endogenous rhythm of the SCN is synchronised to the external environment by light information received at the level of the retina. The circadian timing system encompasses the eyes, various neural pathways connecting the retina and SCN, the SCN itself and efferents from the SCN to effector systems within the brain. Of major interest is the neurochemistry of the pathways involved in light transfer to the SCN and their respective roles in the light-mediated effects on SCN function. A complete definition of the mechanisms governing light effects on circadian timing would facilitate precise control of biological rhythms using neurochemical agents to manipulate the timing of the body clock.

The neurochemistry of the SCN afferents is extremely complex and is not yet fully defined. Three major pathways are currently thought to be responsible for light control and entrainment of SCN function in mammals. The sole direct neural afferent from the retina to the SCN termed the retino-hypothalamic tract (RHT) utilises the excitatory amino acids as the primary neurotransmitter (Moore, 1973; Millhouse, 1977). Two other indirect tracts are involved, one projects from the retina, via the ventral lateral geniculate nucleus and intergeniculate leaflet to the SCN and has GABA and neuropeptide Y as the major transmitters and is called the retino-geniculo-hypothalamic tract (R-GHT) (Hickey and Spear, 1976; Moore and Speh, 1993). The other projecting from the retina via the raphe nucleus to the SCN and having serotonin as its primary transmitter is called the retino-raphe-hypothalamic tract (Van der Kar and Lorens, 1979; Shen and Semba, 1994; Kawano *et al,* 1996). Together these 3 afferents are thought to be primarily responsible for the entrainment of the SCN to environmental photoperiod. However, in addition to these tracts and their major neurotransmitters several other minor pathways project to the SCN and many other neuroactive substances have been found in the SCN or have been reported to affect SCN function (van den Pol and Tsujimoto, 1985). The

widely held belief that the 3 major afferents and their transmitters are the sole mediators of SCN function may therefore be somewhat limiting.

Acetylcholine is one of the transmitters found in the SCN although not in the three main afferents. The neurons which contribute the fibres shown to be immunoreactive for choline-acetyltransferase (the biosynthetic enzyme for ACh) project from other brain regions as no cholinergic somata are found in the SCN (Ichikawa and Hirata, 1986). A recent study has identified separate contacts between choline-acetyltransferase-immunopositive elements and SCN neurons (Kiss and Halasz, 1996). Other cholinergic neurons have been traced from the SCN to various brain regions including the basal forebrain and brainstem and stimulation of these cells caused the release of ACh in the SCN (Bina *et al,* 1993). One or all of these minor projections to the SCN may mediate the cholinergic effects on the circadian timing system.

Several avenues of investigation have led to the hypothesis that acetylcholine plays a role in the functioning of the SCN. Levels of ACh in the SCN were reported to rise 3-fold after a light pulse at night suggesting a role for the transmitter in light effects on the SCN. Further, the ACh agonist carbachol administered intraventricularly caused phase changes in the timing of the circadian rhythm of locomotor activity of hamsters (Meijer *et al,* 1988; Wee *et al,* 1992), and mecamylamine, a nicotinic antagonist, was shown to block light-induced phase changes (Keefe *et al,* 1987). Carbachol caused similar time-dependant phase changes in the rhythm of locomotor activity in mice (Zatz and Herkenham, 1981) and altered the timing of the free-running period of drinking behaviour in rats (Murakami *et al,* 1986). Using SCN slices isolated from the brain researchers have also shown that the cholinergic agents nicotine, carbachol and acetylcholine as well as other specific M1 agonists can affect the timing of the SCN neural firing rate rhythm *in vitro* (Trachsel *et al,* 1995; Liu and Gillette, 1996). Together, this information indicates that acetylcholine may play a significant role in the functioning of the SCN in mammals.

12.2 Cholinergic effects on SCN function

Two cholinergic agents were administered to randomly bred animals to assess (1) the phase-shifting effects on the melatonin excretion rate rhythm and (2) the induction of the immediate-

early gene *c-fos* in the SCN. A nicotinic agonist, nicotine and a muscarinic agonist oxotremorine were used to determine more specifically the receptor subtype mediating the reported cholinergic effects on SCN timing.

Nicotine caused dose-dependent and time-gated phase delays in the timing of the onset of the 6-sulphatoxymelatonin excretion rate rhythm in a similar manner to light. The minimum effective dose of nicotine (1mg/kg) caused significant phase delays when administered at 4 or 6 hours (CT16 or CT18) after subjective darkness but not at CT14. These results mirror those reported in animals treated with a light pulse at these times (in the present project and in previous studies in this laboratory (Kennaway and Rowe, 1994)). Oxotremorine treatment did not produce phase changes in aMT.6S excretion onset at any dose used, suggesting that the nicotinic receptor mediates the cholinergic-induced phase delays.

To show that the agonist was having its effect at the level of the SCN, c-FOS protein induction by the cholinergic agonists was used as a marker of action in the nuclei. Nicotine treatment resulted in the staining of a significant number of c-FOS-immunopositive cells in the SCN in a dose-dependant and time-gated manner. The highest dose of nicotine resulted in approximately a third the number of c-FOS-immunopositive cells as were recorded after a light pulse. Oxotremorine at the highest dose caused the induction of a significant number of immunopositive cells however the number was similar to that recorded after the lowest dose of nicotine. Thus, the nicotinic agonist produced a larger response from the SCN which was partially blocked by pre-treatment with the nicotinic antagonist mecamylamine. This data reinforces the idea that nicotinic receptors mediate the cholinergic effects on the SCN and that nicotine and oxotremorine can have effects (direct or indirect) at the level of the SCN.

12.3 A newly developed cholinergically sensitive rat.

Two unique lines of rat were developed at the Flinders University of South Australia using a selective breeding program based on sensitivity to cholinergic challenge and a hypersensitive line (Flinders Sensitive Line, FSL) and a hyposensitive line (Flinders Resistant Line, FRL) were produced (Overstreet *et al,* 1979). Displaying increased thermic sensitivity to nicotinic, muscarinic and serotonergic agents in addition to higher levels of brain muscarinic receptors, lower levels of activity and higher scores on measures of behavioural despair, the FSL animals

were reported to be a unique animal model for the study of human depression (Overstreet, 1991).

A proposed link between the cholinergic system, depressive disorders and altered circadian timing precipitated the analysis of circadian function in the FSL rats. A series of studies examined the timing of the rhythms of core body temperature, general activity and drinking behaviour under both entrained and free-running conditions. The FSL animals exhibited a shorter phase angle difference of the rhythm in LD conditions as well as a shorter free-running period (Shiromani *et al,* 1991; Shiromani and Overstreet, 1994). These results suggested a link between the upregulated brain cholinergic system and altered circadian timing and provided a unique avenue of research into the interaction of acetylcholine and SCN function.

The current project developed a new cholinergically sensitive line under stringent lighting conditions with the specific aim of investigating the role of acetylcholine in SCN function. The selective breeding program used the thermic response to oxotremorine as the measure of cholinergic sensitivity. By generation 4 the response of SOX and ROX animals had diverged significantly and continued to diverge to generation 12. Physiological measurements recorded during the breeding program showed that the SOX animals had a lower body weight than ROX at 28 days of age from generation 6. These parameters were in line with the results reported in the FSL animals (Overstreet, 1991) and thus, the project was successful in developing a new cholinergically sensitive animal.

From the outset of the program the SOX and ROX animals that were selected as breeders (ie the most sensitive and resistant animals from each round of phenotyping) were phase-typed using the onset of the melatonin excretion rate rhythm as a marker. Under light/dark conditions melatonin profiles from the 2 lines were found to be significantly different from generation 3 with SOX having an earlier onset under the cyclic photoperiod than ROX and RB rats. The ROX animals selected from generation 8 and 9 exhibited a significantly later onset than RB rats indicating that both lines had diverged from the normal in opposite directions. The shorter phase angle of the SOX animals was correlated with increased cholinergic sensitivity and the longer phase angle in ROX animals with decreased cholinergic sensitivity (or cholinergic resistance). It is interesting to note that the divergence between the lines in temperature response to the muscarinic challenge was significant in the fourth generation and the melatonin onset diverged significantly at generation three. Thus, the animals that first

showed significant differences in the timing of the melatonin rhythm subsequently gave rise to the animals that first showed significantly different temperature responses. It is also important to note that the thermic response of the breeders was significantly different from the first selection but that the response of the general populations only diverged significantly in the fourth generation. Thus, the changes in cholinergic sensitivity that produced alterations in the thermoregulatory system in all animals were preceded by changes that correlated with alterations in the circadian timing system in the most sensitive animals. This result suggests that significant cholinergic changes in the brain caused altered functioning of the thermoregulatory and circadian timing systems in a similar timeframe.

Differences in SCN function between the lines were assessed using the free-running period of temperature, running activity and general locomotor activity in SOX and ROX animals of various generations. This group of studies showed that the SOX animals had a shorter free-running period than both ROX and RB rats in generations 8-12 but not in generation 2. The mechanisms underlying the altered circadian function in the SOX rats most likely involve the increased muscarinic receptor levels identified in different brain regions. Although the breeding program used a heightened response to cholinergic agents from the thermoregulatory control centres as a measure of sensitivity, differences in receptor levels were higher in the hippocampus and striatal areas. Thus, the SOX animals likely have differences in the cholinergic system that extend globally throughout the brain, which may in turn have effects on many control centres including those involved in the circadian timing system.

The altered cholinergic 'tone' may affect SCN function directly via minor afferents to the SCN from regions such as the brainstem, basal forebrain and mesopontine tegmentum. Alternatively, the effects could be indirect via interaction with any of the major SCN afferents or by alteration of the tonic control exerted by other neurotransmitters such as serotonin or excitatory amino acids. It seems at this stage that the most likely mechanism is that of direct effects of minor cholinergic afferents. This hypothesis is supported by the results in this project using RB animals, showing effects of nicotine on *c-fos* induction and the timing of the melatonin rhythm. However, studies with cholinergic and serotonergic agents in SOX animals indicate the possibility of more than one of the aforementioned mechanisms being involved.

Nicotine administered peripherally caused larger magnitude phase delays in SOX animals than both ROX and RB animals. Thus, the higher degree of cholinergic 'tone' hypothesised in SOX

rats, combined with direct nicotinic stimulation resulted in a larger response from the circadian timing system. This result fits with the minor afferent model. However, the SOX animals also showed greater thermic and phase changes to a serotonergic 5-HT_{1a} agonist, 8-OH-DPAT. This result indicates altered sensitivity of SOX animals to serotonin, a result also reported in the FSL animals. Thus, either the altered cholinergic tone has affected serotonergic tone indirectly, or serotonergic sensitivity was co-selected in the SOX animals. The former seems more probable however further investigation into the specifics of the serotonergic sensitivity in SOX is required. The data indicating altered serotonergic sensitivity in the SOX animals are important in light of the recent reports suggesting that serotonin may play a primary role in the control of circadian rhythmicity in the rat (Kennaway *et al,* 1996; Moyer *et al,* 1997). This makes the SOX model invaluable for future study of both the cholinergic and serotonergic systems and their interaction within the circadian timing system.

An assessment of the underlying mechanisms mediating the cholinergic hypersensitivity in the SOX animals was conducted using ligand binding studies for muscarinic and nicotinic receptors. This series of studies showed that the SOX animals had higher levels of muscarinic receptors in the hippocampus and striatal areas of the brain than both ROX and RB animals. Interestingly, there was no difference in nicotinic or muscarinic receptor number in the hypothalamus. The results are identical to those reported in the FSL animals (Overstreet *et al,* 1991) and further suggest that the selective breeding program has produced a very similar line of animal. Further molecular studies will need to be conducted using more specific techniques such as *in vitro* autoradiography or quantitative *in situ* hybridisation to identify differences in receptor makeup of discrete brain nuclei including the SCN of the SOX rats. The initial work however, has shown that the underlying mechanisms of the increased cholinergic sensitivity in the SOX animals involve upregulated receptor numbers in the brain.

12.4 Summary

The project has shown that cholinergic stimulation can cause phase shifts in the timing of the melatonin rhythm in rats and that this is most likely due to an effect (direct or indirect) at the level of the SCN. The number of nicotine-induced c-FOS positive cells recorded in the SCN was reduced by pretreatment with the nicotinic antagonist indicating action at the nicotinic

receptor. Further, the SOX line of animals was developed with the specific aim of investigating the role of acetylcholine in the timing of circadian rhythms. The SOX line showed increased sensitivity to muscarinic and nicotinic agents as well as a serotonergic 5-HT_{1a} agonist. Most importantly however were the circadian analyses conducted using these animals. The SOX animals exhibited a decreased phase angle difference of the melatonin excretion rate rhythm under entrained conditions and a shorter free-running period of the temperature and activity rhythms. This data suggests that the upregulated cholinergic system may be affecting the timing of the animals circadian rhythms either directly, through minor cholinergic afferents to the SCN, indirectly through interaction with major SCN afferents or their transmitters or indirectly by a general increase in tonic cholinergic stimulation to the SCN. Further work will ultimately define the specific mechanisms by which acetylcholine controls the functioning of the SCN.

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