

**The circadian system of African mole-rats:
Behavioural activity rhythms and early gene
expression (*c-fos*) in the suprachiasmatic nucleus.**

by

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ABSTRACT

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Mole-rats from the family Bathyergidae are endemic to Africa, this family exhibits a continuum of sociality, comprising both solitary and social species. Sociality is related to the degree of aridity and the distribution of the underground food resource. All the members of the bathyergid family are strictly subterranean, and occur in a niche which is devoid of light cues and thermally buffered from ambient and surface extremes. Since vision is redundant in a lightless environment, mole-rats have subsequently undergone ocular regression over evolutionary time. As a consequence of the minute eyes, the visual system of mole rats is severely regressed and, in addition, the proportional retinal innervation to different structures is modified. The classical visual system is reduced while the circadian system is expanded.

Retinal projection studies on the giant Zambian mole-rat, *Cryptomys mehowi* and an albino highveld mole-rat, *Cryptomys hottentotus pretoriae*, confirmed sparse contralateral retinal projections to structures of the visual system, while the circadian system received relatively dense bilateral innervation. The innervation pattern of an albino Damaraland mole-rat, *Cryptomys damarensis* differed from the other animals.

Investigations of Fos expression in neurons over circadian time suggested that the phase response curve of the solitary mole rat, *Georychus capensis*, resembles that of aboveground mammals whereas the social *Cryptomys hottentotus pretoriae*, does not display differential sensitivity to light in the subjective day and night.

The influence of increasing light intensities showed that higher light intensities elicit a more pronounced Fos expression in SCN of all the species investigated. In addition, longer light pulses also increases the Fos induction in the SCN.

A preliminary investigation into the effect of temperature on the Fos induction in the SCN of three mole-rat species, demonstrated that a higher Fos response could be expected with higher ambient temperatures. However the sample size was very small, and could have influenced the outcome of the experiment.

Behavioural locomotor activity rhythms of the solitary species, *Georchus capensis*, and the social species *Cryptomys hottentotus pretoriae* and *Cryptomys damarensis*, confirmed that activity patterns correlate with trends displayed in Fos expression. The solitary species exhibited much more defined rhythmicity than the social species and a higher percentage of the animals displayed distinct endogenous rhythms.

African mole rats provide an interesting model to study not only the features of the circadian system in a group of animals with a naturally regressed visual system, but also the influence of sociality on the degree of regression.

Keywords: Activity, circadian rhythm, Fos expression, mole rat, retinal projection, SCN, subterranean.

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Fil. 4.13. Ek is tot alles in staat deur God wat my krag gee.



GLOSSARY

ABC: Avidin-biotin complex	26L/D: Light/dark cycle
ACh: Acetylcholine	LGN: Lateral geniculate nucleus
AOS: Accessory optic system	NAAG: N-acetylaspartylglutamate
AP-1: Activator protein 1	NADA: N-acetyl-D-aspartate
PKA: Protein kinase A	NMDA: N-methyl-D-aspartate
BNST: Bed nucleus stria terminalis	NO: Nitric oxide
cAMP: cyclic Adenosine 3',5'-monophosphate	NPY: Neuropeptide Y
c-fos: fos gene	PBS: Phosphate buffer, saline (0.1M, pH7.6, saline 0.9%)
CaM: Calmodulin	PBST: Phosphate buffer, saline, triton (0.1M, pH 7.6, saline 0.9%, triton 0.4%)
CaMK: Calmodulin kinase	PBSTA: Phosphate buffer, saline, triton, azide (0.1M, pH 7.6, saline 0.9%, triton 0.4%, azide 0.1%)
CRE: cAMP response element	PFA: Paraformaldehyde fixative
CREB: cAMP response element binding protein	PRT: Pretectum
CT: Circadian time	RGC: Retinal ganglion cells
CTS: Circadian timing system	RHT: Retinohypothalamic tract
DAB: 3,3'-diaminobenzidine tetrachlorohydrate	SC: Superior colliculus
DD: Constant darkness	SCN: Suprachiasmatic nucleus
Fos: Protein Fos	SOM: Somatostatin
GABA: γ -amino butyric acid	TRIS: Tris-hydroxymethyl aminomethane (0.05M, pH 7.6)
GAD: Glutamic acid decarboxylase	VIP: Vasoactive intestinal polypeptide
GHT: Geniculohypothalamic tract	VP: Vasopressin
GLU: Glutamate	ZTS: Zeitgeber timing system
GRP: Gastrin releasing peptide	
IEG: Immediate-early gene	
IGL: Intergeniculate leaflet	
Jun-b: Gene jun-b	
Jun: Protein Jun	

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CHAPTER 1: GENERAL INTRODUCTION

GENERAL INTRODUCTION

Our sensory perception of the daily world is so dominated by complex visual processes, that it is easy to overlook the minute part of the visual system that is devoted to another function, the synchronization of biological rhythms by light. Over the course of millions of years, organisms have evolved to optimize survival in time and space. The circadian system allows organisms to adjust their activity patterns as well as physiological and biochemical processes temporally through responding and more importantly, to anticipate environmental changes. Periodic environmental changes therefore act as synchronizing agents to which organisms react (Roenneberg & Foster 1997).

The different temporal niches that animals utilize influence the amount of light they are exposed to. Mole-rats are an extreme example of animals that are nearly never exposed to external light, as they are subterranean and rarely emerge above ground.

Over the course of evolution, the visual system of mole-rats has undergone both degenerative and progressive adaptations. The eye and all the brain structures associated with vision are severely regressed or absent, whereas the bilateral projection from the retina to the suprachiasmatic nucleus is expanded. Since the image forming part of the visual system is naturally eliminated, mole-rats provide a suitable model to study the circadian system. Hence, this thesis aims to investigate the properties of the circadian system in these unusual rodents.

Biological rhythms

Biological rhythms are a fundamental characteristic of all eukaryotic organisms under natural conditions and are expressed at every level of biological organization. Behavioural rhythms are frequently encountered in terms of diurnal and nocturnal activity patterns, body temperature rhythms, and also more complex behaviour including solar orientation and navigation of birds (Gwinner 1996) and time sense in bees (Moore *et al.* 1989). Rhythms have been demonstrated at the organ and tissue level, well known examples being the SCN in mammals (Inouye & Kawamura 1979), the pineal gland in birds (Deguchi 1979) and the eyes of several marine gastropods (Rothman & Strumwasser 1976, Roberts & Block 1983). Rhythms are expressed in various isolated unicellular microorganisms. Autonomous biological clocks have

been identified in *Paramecium* and *Gonyaulax* cells (Sulzman *et al.* 1982, Miwa *et al.* 1987), and mammalian cell cultures are known to display daily rhythms (Murakami *et al.* 1991). There is also evidence of rhythmic expression even at the sub-cellular level in *Acetabularia* (Schweiger *et al.* 1986).

Biological rhythms occur in multiple frequencies from milliseconds to years, and interact with each other (Haus *et al.* 1998). Over time, a complex structure is formed with rhythms of different frequencies superimposed upon each other. Ultradian rhythms have frequencies higher than a day, and are expressed as neuronal responses, heart beat rate, breathing rate and hormonal changes. Circadian rhythms, the focus of this study, have lengths of around 24 hours, the period length commonly ranging between 20 and 28 hours. Numerous circadian rhythms have been described, and they are encountered in many forms, from photosynthesis in plants to highly complex behavioural and endocrinological rhythms in mammals. Circadian rhythms are frequently superimposed on rhythms of lower frequencies (Haus *et al.* 1998). There are also various rhythms with periods longer than a day, for example lunar cycles. Circannual rhythms have lengths of approximately a year and are expressed in all biological attributes that exhibit seasonal changes like breeding and hibernation. Although biological rhythms vary greatly in period and nature, they have certain general characteristics in common.

Circadian rhythms

The existence of daily rhythms has been known for centuries, but the endogenous nature of the circadian rhythm has not been detected until fairly recently. Early experiments on rhythmicity were almost exclusively conducted on plants. De Mairan (1729) was the first to notice the persistence of a daily rhythm of leaf opening when external cues are removed, but he ascribed it to another unknown cyclic environmental factor (cited Meijer & Rietveld 1989). The endogenous nature of the rhythms was only recognized in 1922 by Richter. He showed the persistence of a daily rhythm in the rat in constant darkness and formulated the concept of an endogenous clock, and also reported drifting of the activity period during constant darkness (free-running) (cited in Rusak & Zucker 1979). This concept remained largely hypothetical for decades until the discovery of the retinohypothalamic tract (Moore *et al.* 1971) and the involvement of the suprachiasmatic nucleus (Moore & Eichler 1972, Stephan & Zucker 1972) approximately 30

years later. The site of the endogenous clock generating circadian rhythms is thus identified, but the precise mechanism and neurotransmitters involved are still not known.

Characteristics of circadian rhythms:

- a) Circadian rhythms are determined genetically, their genetic nature best demonstrated by spontaneous gene mutations that alter the period of rhythms, for example the tau mutation in hamsters, and the clock mutation in mice (Ralph & Menaker 1988, Vitaterna *et al.* 1994).
- b) The rhythms are able to entrain to cyclical environmental stimuli. The period of the rhythm becomes equal to that of the entraining stimulus with a stable phase angle between the two oscillations. Light cycles are the primary zeitgeber that serve to entrain rhythms, but other stimuli are used in the absence of light.
- c) Circadian rhythms are endogenous and self-sustained. The endogenous, self-sustained nature of the circadian rhythm distinguishes it from all other biological rhythms. In the absence of external cues, they free-run with an intrinsic period of approximately a similar length to that of the environmental period. Since endogenous rhythms are not exactly 24 hours, they must be synchronized with the 24-hour daily cycle to be of functional use to the organism.
- d) Circadian oscillations are temperature compensated, the period remains relatively stable regardless of the ambient temperature (Ruby *et al.* 1999), although the amplitude of the oscillation may change. Normally physiological processes follow the Q_{10} rule whereby biochemical reactions double or half in rate with each 10°C change in temperature ($Q_{10} \leq 2$), while temperature compensated rhythms have a Q_{10} closer to 1 (Jacklet 1980). The amplitude of temperature compensated oscillations is reduced as the ambient temperature drops, until the rhythms are completely damped, but as soon as the temperature rises again, the rhythms are re-expressed.

Basic organization of the circadian system

The mammalian visual system is functionally and anatomically subdivided into an image forming system and a non-image forming or optic system (Sadun 1985, Fig. 1.1).

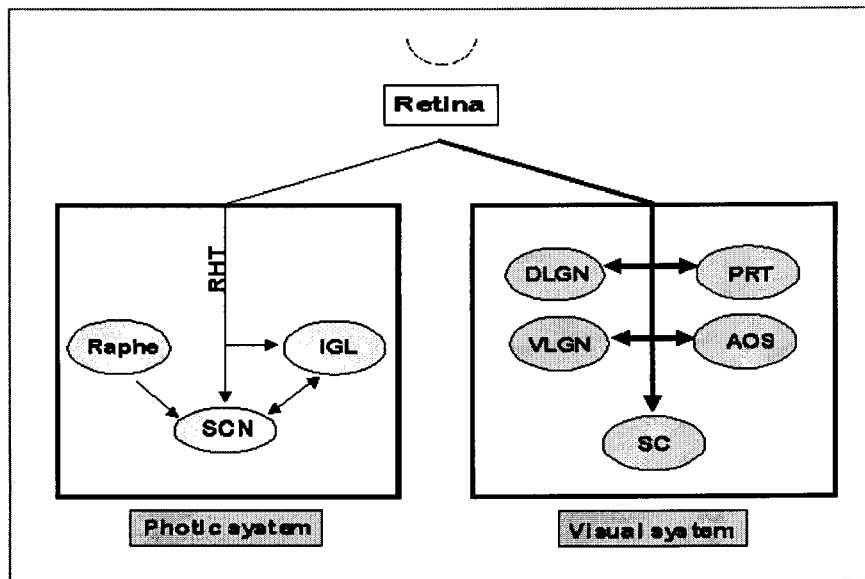


Figure 1.1: Schematic representation of the dichotomy of the visual system and circadian system in mammals (from Negroni, 1998, thesis).

AOS = Accessory optical system, GHT = geniculohypothalamic tract, IGL = Intergeniculate nucleus, dLGN = dorsal lateral geniculate nucleus, vLGN = ventral lateral geniculate nucleus, PRT = Pretectum, RHT = retinohypothalamic tract, SC = Superior colliculus, SCN = suprachiasmatic nucleus.

There are distinct differences between the circadian system and the image forming system. Several mutations and degenerations, natural and induced, cause an organism to be visually blind but still render a circadian response to photic stimulation (Foster *et al.* 1991, Cooper *et al.* 1993b, Foster *et al.* 1993, Czeisler *et al.* 1995).

The primary function of the circadian system is the integration of changes in irradiance levels, therefore, in comparison to the visual system it has a low resolution and sensitivity. The threshold light intensity required to invoke a response in the circadian system is much higher than that required in the visual system (Nelson & Takahashi 1991, Dkissi-Benhyahya *et al.* 2000). In addition, the circadian system requires a long integration time (>30 seconds) compared to the visual system where light integration takes place in less than 100 milliseconds (Nelson & Takahashi 1991). The two separate systems can be tracked by using viral tracing techniques.

The circadian timing system is comprised of certain elements essential for expressing biological rhythms. In order for the circadian system to provide an organism with local time information, the circadian oscillator has to maintain synchronization with the external environment.

External environmental light acts on photopigments in the retina. These photoreceptors capture the photons and transduce it into a neural signal. The signal is then transmitted to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT). In mammals, the SCN is the site of the circadian clock and is responsible for the generation of rhythms. The clock is connected to downstream effector pathways that manifest rhythms. These rhythms are visible as daily oscillations in numerous behavioural and physiological processes, such as locomotor activity rhythms, melatonin synthesis and oxygen consumption (Fig. 1.2).

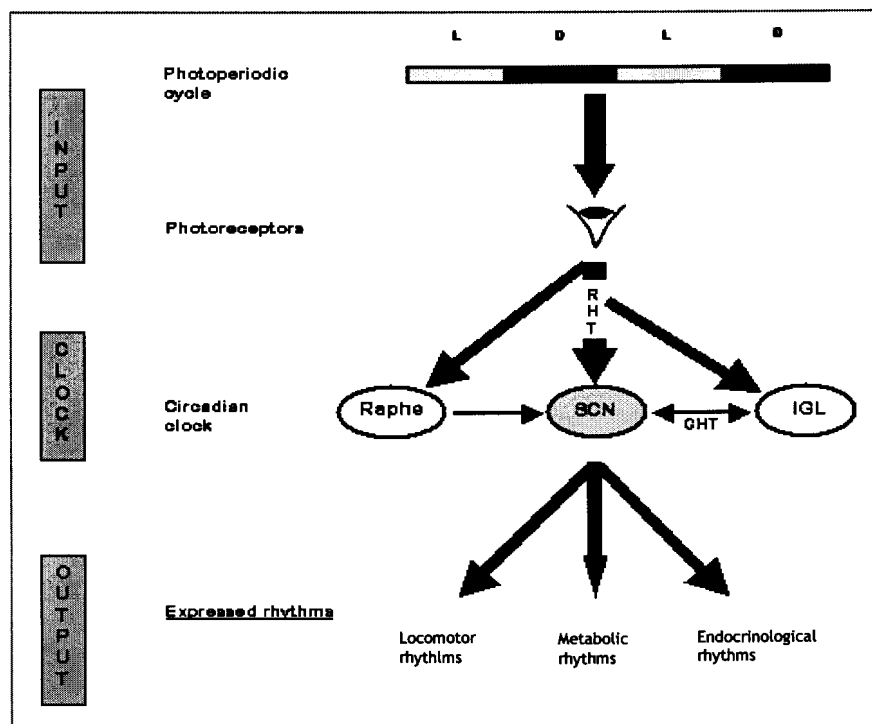


Figure 1.2: Schematic representation of the circadian system in mammals.

The SCN as the circadian clock

At the physiological level, both an oscillator and pacemaker function has been defined in mammals. An oscillator is a structure that expresses self-sustained oscillation under constant conditions, while a pacemaker imposes its rhythms on other processes. In vertebrates, three diencephalic structures have been identified to contain circadian oscillators. The mammalian SCN, the pineal of birds and reptilian retinas have shown persisting rhythmic oscillations by applying *in vitro* studies. The mammalian SCN houses the circadian clock that generates circadian rhythmicity, but the precise phase regulation is still dependent on the SCN afferent processes (Morin 1994).

Evidence supporting the oscillating and pacemaker properties of the SCN is considerable; this hypothalamic structure possesses all the criteria to qualify as the major mammalian pacemaker:

- a) It is known that the RHT is required for entrainment to light cycles, it provides a direct projection of photic input from the retina to the SCN (Johnson *et al.* 1988a,b).
- b) When the SCN is surgically isolated, circadian rhythms persist in the SCN island while all rhythmicity in the rest of the brain, as well as behavioural rhythms, are abolished (Inouye & Kawamura 1979).
- c) Circadian rhythms of electrical activity and secretion of vasopressin persist *in vitro*. Diurnal firing rate of the SCN in the rat continues and phase shifts occur following phase shifting stimuli (Gillette 1986, Gillette & Reppert 1987).
- d) Surgical lesion of the SCN causes loss of rhythmicity (Moore & Eichler 1972, Stephan & Zucker 1972).
- e) Transplantation of the fetal SCN can restore rhythmicity in SCN lesioned animals. More significant, the period of activity rhythms can be transferred between different tau mutant genotypes (Ralph *et al.* 1990).
- f) Electric stimulation of the SCN causes phase dependent phase shifts of free-running activity. Phase shifts are also induced when the SCN is stimulated with different neurotransmitters or neuropeptides such as glutamate, NPY, and 5HT (Page 1987, Franken *et al.* 1999, Hall *et al.* 1999).

The mechanism of the circadian clock

Over the years, a wide variety of clock models have seen the light. These models include hourglass models based on unidirectional processes, molecular models relying on the periodicity in the structure of molecules as well as the periodicity of the molecules themselves, feedback models involving biochemical enzyme activity where the product feeds back to a starting point and transcriptional models based on gene transcription and translation.

The cell autonomy of the circadian clock has been demonstrated by the persistence of rhythms in isolated SCN cells *in vitro* (Welsh *et al.* 1995) as well as the fact that unicellular organisms possess biological clocks. Based on the autonomous nature of the clock, a molecular mechanism is favored for the circadian clock. Since it is known that the circadian clock is located in the SCN, it is believed that the SCN is an oscillator that either causes rhythmicity by itself, or is a master oscillator that governs a series of slave oscillators that are responsible for various physiological rhythms (Vaz Nunes & Saunders 1999, Stokkan *et al.* 2001).

In all organisms studied, circadian clocks seem to use the same basic principles where the period, amplitude and phase are determined by a specific set of interconnected proteins (Edery 2000).

Currently it is thought that the molecular mechanism of the clock is based on a transcription/translation autoregulatory negative feedback loop involving a set of clock-controlled genes that inhibit transcription of themselves. In mammals, individual positive and negative elements for this loop have been identified.

The genes *bmal1* and *clock* encodes for the proteins BMAL1 and CLOCK, which functions as positive transcription factors. These two proteins form a heterodimeric complex that binds to E-box enhancers that promotes the transcription of three period genes, *period 1, 2* and *3* and a timeless gene, *tim*. The protein products of these genes (PER 1,2,3 and TIM) represent the negative transcriptional component of the loop. As the levels of PER and TIM rises, they are phosphorylated and translocated back to the nucleus. Once in the nucleus, these proteins inhibit their own transcription by interfering with the CLOCK-BMAL1 activation of transcription (Jin *et al.* 1999, Fig 1.3).

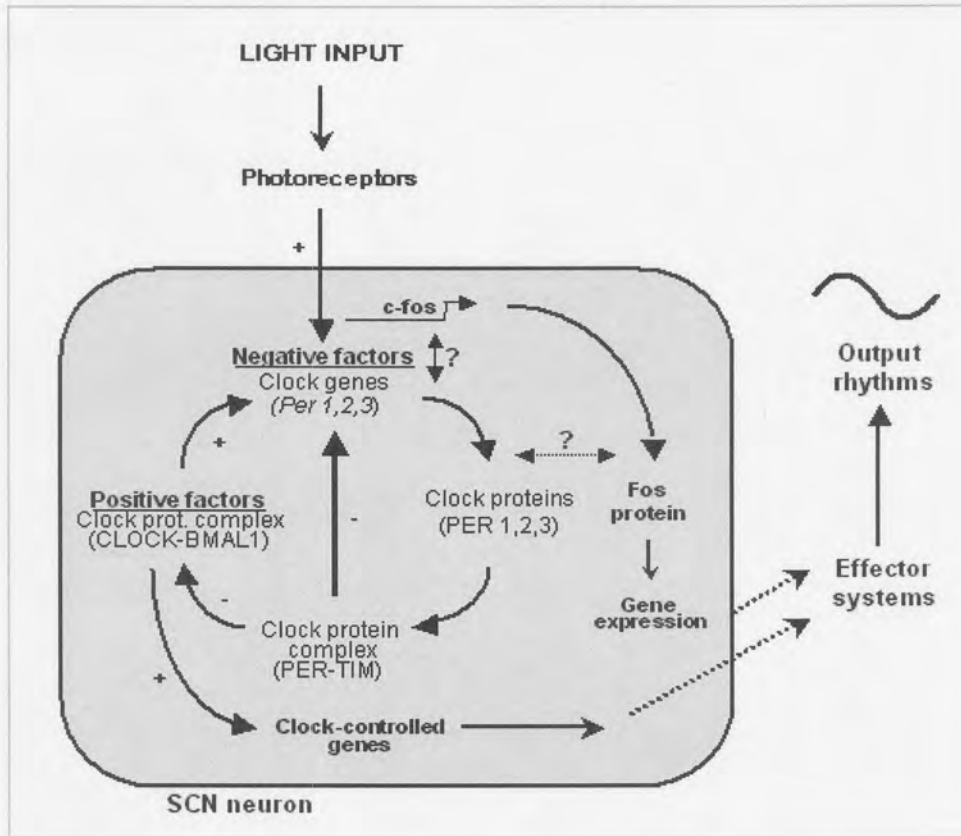


Figure 1.3: Schematic representation of the clock mechanism. (Modified from O. Dkhissi-Benhyahya)

It has also been suggested that the circadian clock of rodents consists of two oscillators, a morning component (M) and an evening component (E). This model is largely based on the phenomenon of splitting where the main activity bout divides into two distinct parts under constant light conditions (Pittendrigh & Daan 1976, Daan *et al.* 2001).

Properties of circadian entrainment

As endogenously generated rhythms are not exactly 24 hours long, rhythms have to be entrained to keep physiological and behavioural processes synchronized with the external environment.

The response of the circadian clock to external environmental changes involves the adjustment of the period of the circadian system and is called entrainment. During entrainment

the circadian system adopts a specific phase relationship with the entraining stimulus (Daan & Aschoff 1996). A rhythm is entrained when its period is equal to the period of the entraining stimulus with a stable phase angle between the two oscillations. If the entraining factor is removed, the rhythm will freerun from exactly the same phase angle.

Entrainment takes place by means of shifts in the phase of circadian rhythms in response to external cues. Phase shifting can take place via an array of photic and non-photoc stimuli. Of the three major projections that reach the SCN, one is of photic origin whereas the remaining two are non-photoc.

Usually, light is the most important environmental stimulus entraining the circadian clock, photic entrainment occurs through daily phase shifting of the clock period. In addition to photic cues, the clock is also able to entrain to non-photoc cues, for example social and olfactory cues, hormonal changes involving melatonin, activity, food availability and temperature. Temperature entrainment concerns the clock's ability to respond to a rhythmic change in temperature, and has to be distinguished from temperature compensation, which affects only the ability of the clock to remain stable relative to constant temperature levels. In normal circumstances non-photoc cues are thought to play a supporting role to photic stimuli. Photic and non-photoc cues cause a different response in the SCN relative to the phase of the period, implying that different neural or cellular mechanisms are used by these two groups cues to reset the circadian clock.

Photic entrainment

Light is an extremely potent stimulus and has powerful effects on the circadian clock. To date, all intact animals examined have been found to be responsive to light, even the so-called 'blind' animals. During the natural light/dark period, the dawn and dusk transitions are probably the times that provide the most reliable indication of the daily phase, and the changes in light quality is most significant. The quantitative level of light alters drastically over several log units; the spectral composition of light changes from longer wavelengths (500-650nm) to predominantly short wavelengths (<500nm) and the sun's position relative to the horizon also undergoes a significant change (Roenneberg & Foster 1997).

Light has a specific and quantitative effect on the clock. Responses of the clock are phase dependent and the illumination conditions such as the wavelength, intensity and duration of light

exposure, determine the magnitude of the phase shifts. (Takahashi *et al.* 1984, Nelson & Takahashi 1991, Boulos 1994, Dkissi-Benhyahya *et al.* 2000).

Light exerts both a parametric (continuous) and a non-parametric (discrete) effect on the circadian system (Aschoff 1978). Parametric effects of light have a continuous influence on the clock and are characterized by phase dependent accelerations and decelerations in the angular velocity of the pacemaker (Daan & Aschoff 1996). The magnitude of the alterations in the period of the clock is dependent upon the light intensity.

In nocturnal rodents, the phase response of the circadian system can usually be explained by nonparametric entrainment, where the period of the free-running rhythm changes slightly, but the phase shows an obvious shift. The circadian clock can be entrained by short light pulses at discrete times in the photoperiod; this process is called a skeleton photoperiod. However, the continuous effect of light cannot be totally disregarded, since it is known that the circadian period length changes in continuous light conditions, and this change is correlated with the light intensity (Daan & Pittendrigh 1976).

Photic entrainment is also influenced by the afferent fibers originating from the IGL and raphe nuclei. Although these photic and non-photic innervations are not essential for photic entrainment, the IGL affects the rate of entrainment and the raphe nuclei modulate the phase angle of entrainment.

Phase response curves

By experimentally altering the phase of the photoperiod, the effects of the circadian pacemaker can be determined. Phase response curves (PRC) are determined by the behavioural response of an organism to external stimulation of the circadian pacemaker. Phase shifts in response to single light pulses over the course of the 24 hour day are plotted against time to obtain a phase response curve. This phase response curve demonstrates the phase dependence of the clock response to light. In nocturnal rodents, light pulses do not have a phase shifting effect on the circadian pacemaker during a large part of the subjective day (CT0-12), this is called an insensitive period. During the early subjective night (CT12-24), light pulses evoke phase delays and during the late subjective night, phase advances. The direction of the phase shifts is thus dependent on circadian time (Servière & Lavalie 1996, Fig. 1.4).

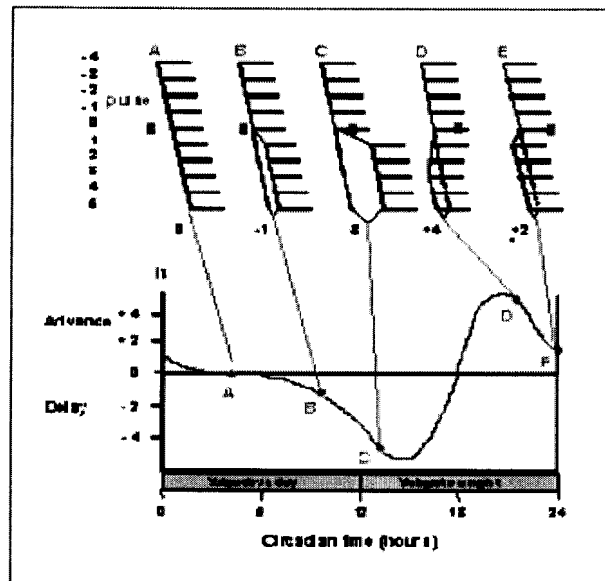


Figure 1.4: Schematic representation of a phase response curve by light. (Servière & Lavalie 1996)

The magnitude of the phase shift is dependent on the circadian phase of the rhythm, as well as the intensity and duration of the light pulse (Nelson & Takahashi 1991). Phase shifts in response to weak pulses are classified as Type 1 (average slope of 1), and those in response to strong pulses as Type 0 (average slope of 0) (Daan & Aschoff 1996).

The general curve of the light pulse phase response curve (PRC) is the same for all circadian systems although there may be large differences in the amplitudes. Usually, animals with a freerunning period longer than 24h have larger phase advances than delays, whereas those with freerunning periods shorter than 24h shows larger phase delays than advances (Rea 1998).

Following light stimuli, there are essentially two types of PRC's, light and dark PRC's. To obtain a light PRC, animals must be maintained in total darkness while light pulses are presented to an animal at different circadian times in order to record phase shifts in response to light at a specific time. To acquire a dark PRC, animals are presented with dark pulses on a background of light. These behavioural phase shifts are correlated with Fos expression in the SCN (Kornhauser *et al.* 1990).

Light pulse PRC's

All known light PRC's share similar features, with phase shifts occurring during the subjective night. The phase delays taking place in the early subjective night and phase advances in late subjective night.

The magnitude of a phase shift is dependent on three characteristics of light namely: the intensity, the duration and eventually the wavelength of the light. Light has a certain threshold value after which the clock starts to show a response, this value is relatively high in comparison with the threshold for a visual response (Nelson & Takahashi 1991) and varies between species. The magnitude of phase shifts increases with the light intensity until it reaches a saturation level after which no further increasing effect will take place. Intermediate durations of light pulses render the largest phase shifts when the total number of photons is kept constant; the clock is more sensitive to 300 seconds of light than 30s or 3000s. A reciprocal relationship exists between the intensity and duration of light pulses for up to 45 minutes (Nelson & Takahashi 1991, Dkissi-Benhyahya *et al.* 2000), indicating that the total number of photons received by the SCN determines the response of the circadian system. In rodents, maximal spectral responses are observed around 500nm (Takahashi *et al.* 1984, Provencio & Foster 1995, See Fig. 1.4).

Dark pulse PRC's

Dark PRC's are not as well studied as light PRC's. Dark pulses are presented on a background of LL and the resulting PRC is different from the light PRC. Dark pulses result in phase advances in the mid subjective day and early subjective night, and variable degrees of phase delays in late subjective night (Dwyer & Rosenwasser 2000).

Similar to the effect of higher intensities of the light pulse in light PRC's, the magnitude of the phase shifts are dependent on the background light intensity. The higher the background intensity, the larger the phase shifts. Dark PRC's seem to be more related to non-photoc phase shifting than photic phase shifting, as locomotor activity appears to be an important factor in determining the magnitude of phase shifts. The restraining of hamsters during a dark pulse reduces the magnitude of phase advances (Dwyer & Rosenwasser 2000).

Non-photic entrainment

Non-photic stimuli are thought to be of importance where photic cues are absent. A number of studies provide evidence of entrainment to non-photic stimuli. Animals are known to entrain to temperature rhythms (Goldman *et al.* 1997, Liu *et al.* 1998, Pohl 1998). Locomotor activity has also been shown to cause phase shifts. Thus, stimuli that induce locomotor activity are important in non-photic phase shifting as these phase shifts are blocked by the prevention of activity. A classical example of non-photic entrainment occurs where fetuses can entrain to physiological processes (e.g. melatonin synthesis) from their mothers (Honma *et al.* 1984, Davis 1997).

The phase response curve for non-photic stimulation differs from curves in response to light stimulation. PRC's recorded from non-photic cues vary considerably, but a consistent difference from photic PRC's is that while generally very little phase shifting takes place during the day following photic stimulation, phase advances are encountered during the middle of the subjective day in response to non-photic stimuli.

There appears to be a relationship between photic and non-photic phase shifting. The intergeniculate leaflet (IGL) is involved in both photic and non-photic entrainment, but it is not essential for photic entrainment. Lesions of the IGL slow the rate of re-entrainment (Meijer & Rietveld 1989) and block the lengthening effect of constant light on the period of rhythms (Goel *et al.* 2000). Serotonin is also not necessary for photic entrainment, but it is likely to modulate the phase angle of entrainment (Meyer-Bernstein *et al.* 1997). Therefore, it appears that photic stimulation is the dominant cue for entrainment while non-photic entrainment modulates the phase angle and rate of entrainment.

Neuroanatomy of the circadian system

a) The mammalian retina

In mammals, the retina is the only location for photoreceptive pigments. Although it has been suggested that extra-retinal illumination could shift rhythms in humans (Campbell & Murphy 1998), these results remain highly controversial and could not be confirmed (Lockley *et al.* 1998, Yamakazi *et al.* 1999, Eastman *et al.* 2000).

The retinal component of the circadian system comprises 3 components - photoreceptors,

the intrinsic retinal pathways and the retinal ganglion cells (Miller *et al.* 1996). Photoreceptors capture photic information, translate it to a neural signal and transmit it to the retinal ganglion cells (RGC's) via the intrinsic pathways. The RGC axons form the retinohypothalamic tract that project into the brain for further processing.

It is still unclear which photopigments in the retina are responsible for mediating photic entrainment. Initially it was thought that classical photopigments (found in rods and cones) were involved, since the peak spectral sensitivity of the circadian timing system is around 500nm, which closely matches the action spectrums of rhodopsin and L/M cones (Bowmaker & Dartnall 1980, Argamaso *et al.* 1995). However, circadian sensitivity to light of retinally degenerated mice (rd/rd) proved not to be significantly different from the wild types (Foster *et al.* 1991), despite the extensive loss of cones and total loss of rods. In addition, transgenic rodless, coneless mice also exhibit normal phase responses (Freedman *et al.* 1999). The site of photoreception however, remains in the retina, since ocular enucleation abolishes rhythms. The possibility exists that the small population of surviving cones without outer segments is sufficient for circadian entrainment. Alternatively, another unidentified, non-visual photoreceptor in the retina mediates entrainment, and the dichotomy of the visual system starts at the photoreceptor level. A few putative opsin-based photopigments have been identified in the retina, VA opsin in some amacrine and horizontal cells of fish (Soni & Foster 1997), peropsin and retinal-binding G protein-coupled receptor in the retinal pigment epithelium (Sun *et al.* 1997) and melanopsin in the amacrine and ganglion cells (Provencio *et al.* 1998). Cryptochromes have also been mentioned in association with photic entrainment.

From the photoreceptors, light is transmitted to the ganglion layer in the inner retina. Unlike the photoreceptors, the ganglion cells that mediate entrainment have been identified. A discrete, but distinct subset of retinal ganglion cells project to the SCN (Moore *et al.* 1995). These RGC's have been identified as Type 3 or W ganglion cells, which are small neurons with sparse branches (Meijer & Rietveld 1989). These neurons represent less than 1% of the total amount of ganglion cells, and are evenly distributed throughout the retina.

b) Input pathways to the SCN

In mammals, the SCN receives photic information strictly from the retina, unlike some reptiles and birds that utilize extra retinal photic input to the hypothalamus. Retinal projections to

the SCN are mainly bilateral, although some mammals may show more projections to the ipsilateral or the contralateral side of the SCN (Murakami & Fuller 1990, Smale & Boverhof 1999). There are three distinct transduction pathways whereby temporal information can reach the SCN. Photic information can be transmitted directly via the retinohypothalamic tract (RHT), and indirect pathways that are represented by both the geniculohypothalamic tract (GHT) and a 5HT projection from the midbrain raphe (See Fig.1.2). All these projections have an overlapping termination area in the ventro-lateral part of the SCN (Rusak & Zucker 1979).

I. RHT projection

Axons of the type III RGC's form the RHT, these fibers are unmyelinated, and are characterized by slow responses (Moore 1973, cited by Meijer & Rietveld 1989). The RHT is a specialized pathway that does not project to classical visual structures, but sends bilateral projections to two main sites of axon termination, the SCN and IGL (Miller *et al.* 1996). A single neuron can project to both the SCN and IGL (Picard 1985).

The RHT is embedded in the optic nerve among axons projecting to the geniculate and tectum, and leaves the optic tract through the optic chiasma where it enters the SCN. The main site of termination for this projection is the ventro-lateral SCN; there is also a sparse projection to the dorso-medial SCN and a few projections to areas outside the SCN, with unknown function (Johnson *et al.* 1988a).

The RHT is both necessary and sufficient for maintaining entrainment of the circadian clock as lesions of the RHT disrupt photic entrainment (Johnson *et al.* 1988b), and enucleation or complete bilateral section of the optic nerves will cause blindness and prevent any entrainment from taking place (Inouye & Kawamura 1979).

Neurotransmitters of the RHT

It is still unknown which neurotransmitters mediate photic input to the SCN. Since the mechanism of entrainment relies on neurotransmitters, it is important to identify the neurotransmitters that are involved in the transduction of light from the retina to the SCN. A large number of neuroactive substances have been identified within the SCN (Van den Pol & Tsujimoto 1985), many of them putative neurotransmitters of the RHT.

Both acetylcholine and glutamate are present in the RGC's projecting to the SCN as well

as in RHT innervated SCN neurons (Castel *et al.* 1993). In addition, light pulses and stimulation of the SCN result in an increase in the concentration of these substances in the SCN neurons (Liou *et al.* 1986). Agonists of these neurotransmitters cause small phase shifts while antagonists prevent phase shifting (Daan & Pittendrigh 1976, Colwell *et al.* 1991, Colwell & Menaker 1992). Currently glutamate is favoured as the principal neurotransmitter of the RHT, the effect of glutamate is clock controlled, and direct application of glutamate to the SCN *in vitro* produces a light pulse type PRC (Ding *et al.* 1994).

Substance P is also present in some RHT fibers terminating in the SCN (Takatsuji *et al.* 1991). The *in vitro* application of substance P to the SCN results in a dose dependent response of SCN neurons, and it facilitates glutamate responses (Shirakawa & Moore 1994). The PRC of Substance P also resembles the light PRC (Shibata *et al.* 1992). It is likely that substance P colocalizes with glutamate, but it does not serve as the principal neurotransmitter in the RHT.

II. GHT projection

The geniculohypothalamic projection is an indirect pathway from the retina to the SCN. The geniculohypothalamic tract projects to the IGL, and from the IGL to the ventral SCN. There are also IGL projections to the contra-lateral IGL of unknown function.

IGL lesions do not significantly affect behavioural or cellular responses to light (Maywood *et al.* 1997), indicating that the GHT projection is not essential for activation of SCN neurons or circadian entrainment by light. Although not essential for photic entrainment, the IGL does appear to affect the speed of re-entrainment to new cycles, and the magnitude of phase shifts induced by short light pulses (Meijer & Rietveld 1989). IGL lesions also block the lengthening effect of LL on the period (Harrington & Rusak 1986).

The neurotransmitters involved with transmitting information from the IGL to the SCN have not yet been identified, though neurosubstances implicated in this regard are different from that of the RHT. IGL neurons are characterized by the production of neuropeptide Y (NPY) and neurons projecting to the SCN are immunoreactive for NPY and GABA, the two neuropeptides appear to be colocalized in the GHT cells (Meijer & Rietveld 1989). The NPY and GABA phase response curves mimic dark pulse phase response curves (Albers & Ferris 1984). Their expression is phase dependent, suggesting that these neurotransmitters are under circadian control (Shinohara *et al.* 1993).

There are indications that the GHT is important for non-photic entrainment as non-photic phase shifting stimuli result in IEG expression in the IGL, this IEG immunoreactivity colocalize with NPY immunoreactivity and blocking of NPY activity attenuates non-photic phase shifting (Mrosovsky 1995).

III. Raphe projection

The midbrain raphe nucleus innervates the SCN and the IGL from its medial and dorsal parts respectively (Meyer-Bernstein & Morin 1996). This projection represents the third pathway to the SCN. The raphe itself is responsive to light but it is unknown whether the responsive cells in the raphe are connected to neurons that project to the SCN (Meijer & Rietveld 1989). Serotonin innervates the SCN from the raphe acting through various 5HT receptors.

The raphe is not necessary for photic entrainment, but it does alter the SCN's response to light. Only light induces Fos expression in the SCN, stimulation of the raphe attenuates the response of the SCN to light, and the attenuation is proportional to the intensity of the stimulation (Meyer-Bernstein & Morin 1999). The serotonin output to the SCN as well as the SCN response to serotonin is rhythmic (Meyer & Quay 1976, Mason 1986, Dudley *et al.* 1998), indicating that serotonin is under circadian control. Lesions of the raphe do not prevent entrainment, but change the period length by advancing the onset and delaying the offset, creating an overall expansion of the activity phase (Meyer-Bernstein *et al.* 1997). Thus, serotonin appears to have a modulatory role in the photic regulation of circadian phase (Weber *et al.* 1998).

Neuroanatomy of the suprachiasmatic nucleus

Although there are species-specific differences, the general organization of the SCN is relatively similar in mammals. The SCN is a small paired nucleus comprising an estimated 8-10 000 neurons per nucleus (Van den Pol 1980) and is located in the anterior hypothalamus of the brain, at the base of the third ventricle, dorsal to the optic chiasm. Based on morphological characteristics, the SCN can be distinguished from the surrounding hypothalamic tissue. It has extremely small, densely packed neurons, the lateral neurons slightly larger than the dorso-medial ones. In the dorso-medial part of the SCN, perikaryal contacts form chains between opposed cells (Meijer & Rietveld 1989).

The SCN itself is divided into two subdivisions. The ventro-lateral part of the SCN is called the core and receives both direct and indirect innervation, and shows intrinsic connections with the dorsal part of the SCN (Leak & Moore 2001). It is characterized by large, spherical neurons of low density and organelle rich cytoplasm. Neuronal somata are separated by glial cells, which completely enclose synaptic junctions within ventro-lateral SCN, communicate with each other via gap junctions.

The dorso-medial part of the SCN is referred to as the shell and receives no direct photic input. This part is distinguished by very small, elongated neurons with large nuclei and few cell organelles, neurons are tightly packed and interconnected via somato-somatic appositions resulting in chains of neurons arranged in antero-posterior direction (Meijer & Rietveld 1989). The dorso-medial SCN is thought to act as a relay station, integrating various inputs to the SCN as the majority of afferent pathways from the SCN originate from the dorso-medial SCN (Moore 1983, Van den Pol & Tsujimoto 1985).

Neuropeptide and neurotransmitter content of SCN

Coinciding with the morphological differences, the two neural populations of the caudal SCN are also neurochemically distinct. Vasopressin (VP) producing neurons are located in the dorso-medial SCN, while the ventro-lateral part is characterized by vasoactive intestinal polypeptide (VIP). VIPergic neurons in vISCN receive glutamatergic input from retina (RHT), NPY input from the IGL (GHT), and serotonergic (5HT) input from raphe. Between these two neuronal populations, a small population of somatostatin (SOM) producing neurons is found. SOM does not colocalize with either VIP or VP, marking it as a distinct cell group (Meijer & Rietveld 1989). In addition, the great majority of SCN cells are GABAergic, and colocalize with all the above-mentioned neuropeptides.

Some of these neurotransmitters are inhibitory (GABA and SOM), whereas others are excitatory (VP and VIP). All of them display rhythmicity when subjected to LD cycles and most exhibit circadian variation under DD conditions. Thus far, no direct evidence has been acquired to implicate any specific neurotransmitter in the generation of rhythms.

The problem of circadian rhythms in subterranean mammals

A large number of mammalian species exploit the subterranean niche for shelter. Although most of them still spend the majority of their time above ground, there are some species that have become so well adapted to underground living that they rarely, if ever, emerge on the surface. Mole-rats in the families Bathyergidae and Spalacidae are classified as strictly subterranean, having developed specialized physical, sensory and behavioural adaptations to aid them in their world of darkness.

Despite the minute size of the mole-rat eye, anatomically it resembles the eye of optically normal rodents. Structurally, the retina too, consists of the same layers as other rodents, however, the number of retinal ganglion cells are reduced.

According to Cooper *et al.* (1993a), the optic tract of the blind mole-rat *Spalax ehrenbergi* sends projections to both the visual system and the circadian system. All the structures that receive input are reduced in size, and the absolute and relative size of the projections that the structures receive, is different. In this mole-rat, the circadian system receives approximately 20% of the total retinal input whereas in other rodents, less than 1% of the projection is allocated to the circadian system. Thus proportionally, the circadian system in mole-rats receives a much larger input compared to other rodents.

In addition to the anatomical reduction of the eye, mole-rats have been reported to be behaviourally insensitive to light (Eloff 1958, Zuri 1998). Behavioural and electrophysiological studies have confirmed that the visual cortex of the blind mole-rat lacks image formation abilities (Bronchti *et al.* 1989, Heil *et al.* 1991). In contrast to the visual blindness of mole-rats, several species have shown evidence of displaying circadian activity rhythms (Lovegrove & Papenfus 1995, Goldman *et al.* 1997, Tobler *et al.* 1998, Riccio & Goldman 2000a). Fos expression in the SCN also shows a selective response to light, depending upon whether light pulses are administered during the subjective day or night.

There is thus a selective reduction of image forming visual components and an expansion of the photic components. Thus the visual system of mole-rats appears to display a mosaic of regressive and progressive features.

These subterranean animals provide an unique opportunity to study the circadian system under tight ecological constraints. They also provide a suitable model for the study of the

circadian system in the absence of the image forming system that normally overshadows it.

Experimental animals

African mole-rats belong to the family Bathyergidae, which comprises 5 genera and 18 species endemic to sub-Saharan Africa, occurring in habitats ranging from mesic to xeric (Bennett & Faulkes 2000). Three of these genera are solitary and two social. Solitary species are highly xenophobic towards conspecifics outside the breeding season, and social species are xenophobic towards animals outside of their colony. The social genera live in colonies comprising of tight knit family groups with a reproductive pair and their offspring that represent non-reproductive workers. The non-reproductive offspring delay their dispersal and stay in the family group to help dig tunnels and defend the system. The reproductive animals are usually the largest animals in the colony and retain their dominance by social and physiological suppression of the fertility of the remaining colony members (See Bennett *et al.* 1999 for overview).

Physiologically, mole-rats are well adapted to life in conditions of high humidity (up to 90%), hypoxia (6-21% O₂) and hypercapnia (0.5-4.8% CO₂) (Widmer *et al.* 1997). Because of their habitat, mole-rats spend nearly all of their time in total darkness, and are not naturally exposed to the daily light-dark cycle of the environment. Most of the species, however, do exhibit locomotor activity, body temperature and metabolic rate rhythms (Lovegrove & Papenfus 1995, Lovegrove & Muir 1996, Goldman *et al.* 1997, Riccio & Goldman 2000a,b). These results imply that despite the lack of image formation, there is still some light perception to render the circadian system functional.

Georychus capensis

Georychus capensis is a solitary species that occurs in mesic areas along the southern coasts of the western Cape Province. This mole-rat is a seasonal breeder producing offspring in spring (August-September). Since it is highly xenophobic towards other individuals outside the breeding season, it requires some external cue to inform it of the time of the year in order for procreation to take place. It is therefore predicted that this species would exhibit locomotor activity and body temperature rhythms, and would be capable of entraining to external stimuli. Previous studies have shown that this species exhibits daily locomotor rhythmicity, as well as

body temperature rhythms (Lovegrove & Papenfus 1995, Lovegrove & Muir 1996, Plate 1.1a).

Cryptomys hottentotus

All members in the polytypic genus *Cryptomys* are social. *Cryptomys hottentotus*, the common mole-rat, is distributed over most parts of South Africa. This species comprises a number of subspecies, two of which are used as study models in this thesis. *Cryptomys hottentotus hottentotus* occurs in the winter rainfall area in the south and western parts of South Africa. This animal is colonial (mean body mass 70g), colony sizes ranging from 2-14 individuals (Spinks *et al.* 1999). Reproduction is confined to a single reproductive pair within the colony. It is a seasonal breeder reproducing in spring and can produce two litters per annum (Spinks 1998).

A subspecies, *Cryptomys hottentotus pretoriae*, the Highveld mole-rat, is distributed in the north east of South Africa. It is also colonial, (mean body mass 90g) and colony sizes ranges from 2-11 animals (Moolman *et al.* 1998). Similar to the common mole-rat, this species has a reproductive division of labour in which a single female is responsible for reproduction. Again, it is a seasonal breeder and can produce 2 litters per annum, but in contrast with the other subspecies, it reproduces in summer (Janse van Rensburg 2000, thesis).

Observations show that these species do not exhibit a clear circadian rhythmicity either in single animals or as entire functional colonies (Bennett 1992, Plate 1.1b).

Cryptomys darlingi

The Mashona mole-rat is a social species found in the mesic Miombo woodlands of Zimbabwe and Mozambique. It was formerly regarded as a subspecies of *C. hottentotus*, but is now regarded as a full species (Aguilar 1993). It is a small (mean body mass 64g) social animal, and occurs in small colonies of 5-9 individuals (Bennett *et al.* 1994). The colonies consist of a founding breeding pair and their offspring of several litters. It breeds aseasonally and can produce up to 4 litters per annum. No information is available concerning their circadian biology (Plate 1.1c).

Cryptomys damarensis

The Damaraland mole-rat is widely distributed in arid areas of northern South Africa and central and northern Namibia. This species is a medium sized mole-rat, with a mean body size of 131g. It is one of two known eusocial mole-rat species, and colony sizes can reach up to 41 individuals (Jarvis & Bennett 1993). Each colony comprises a reproductive female with one of two male consorts and colonies exhibit a work related secondary division of labour. It breeds throughout the year and has the potential to produce 4 litters per annum (Bennett & Faulkes 2000).

A previous study has shown that entire colonies of *C. damarensis* exhibited circadian rhythmicity and displayed a freerunning period different from 24 hours in constant conditions (Lovegrove *et al.* 1993). According Lovegrove (1993), it could however, not be determined whether this species is nocturnal or diurnal (Plate 1.1d).

Cryptomys mechowii

The giant Zambian mole-rat occurs in the Miombo tropical woodlands and savanna of central Africa. This mole-rat is the largest of the social species (mean body mass 252g) and occurs in colonies of between 8-11 individuals, although rural Zambians claim that colony sizes can be as large as 41 animals (Burda & Kawalika 1993, Bennett & Aguilar 1995). The colonies consist of a reproductive pair and non-reproductive offspring from several litters. The Zambian mole-rat breeds aseasonally and has the potential of producing 3 litters per annum. Nothing is known about the circadian biology (Plate 1.1e).

Heterocephalus glaber

The naked mole-rat is found in the arid regions of East Africa. This species is the smallest of all the African mole-rats with a mean body mass of 34g (Jarvis & Bennett 1991). The naked mole-rat is at the extreme end of social organization and colonies can attain up to 300 animals. Similar to the other social species, a single breeding female and one to three breeding males are present in a colony, and there is a division of labour based on burrow maintenance activities (Jarvis 1991). This mole-rat breeds throughout the year and has the capacity to produce up to 3 litters per annum. Litter sizes are relatively large compared to other social species, with a mean of 11 animals per litter.

Recent studies have revealed that this species does exhibit circadian locomotor activity patterns as well as temperature rhythms (Riccio & Goldman 2000a,b, Plate 1.1f).

AIMS

In mammals, there is an anatomical and functional dichotomy in the visual system, dividing it in an image forming and a non-image forming or photic system. The circadian system is often overlooked simply because of the small size of the projection. By using a model with a naturally regressed visual system effectively eliminating the image forming part, it is easier to study the function and mechanism of the photic system. In contrast to previous suggestions, recent studies provide evidence of circadian behavioural patterns in all bathyergid species investigated (Bennett 1992, Lovegrove *et al.* 1993, Lovegrove & Papenfus 1995, Tobler *et al.* 1998, Riccio & Goldman 2000a). This thesis investigates the locomotor activity patterns of several mole-rat species and a number of aspects of gene expression in the SCN.

Chapter 2

The first chapter investigates the retinal projections of *Cryptomys mechowii*, a social species of African mole-rat. It has been established that the solitary *Spalax ehrenbergi* has retinal projections to most of the brain structures similar to aboveground rodents. This study aimed to elucidate whether the optic tract of *C. mechowii* projects to the same structures that receive retinal innervation as in aboveground rodents, and to determine whether the projections are the same relative size to that of other rodents.

Chapter 3

This chapter aims to explore the early gene expression in the SCN of several species of mole-rats under a range of different lighting conditions. Since Fos proved to be a reliable marker of light activated neurons in the SCN, immunohistochemical techniques were used to stain for its presence in the SCN. The main objective of these experiments was to determine whether light is effectively transmitted to the SCN and if so, whether there is any circadian variation in Fos expression. The effects of intensity and duration of the illumination on Fos induction in the SCN

were investigated in several species. Finally, a pilot study was initiated to determine whether these animals are capable of entraining to temperature cycles.

Chapter 4

Since locomotor activity provides a good indication of the phase of the circadian clock, the third chapter explores circadian rhythmicity within these animals. The key questions to be asked here are whether entrainment to light cycles can take place, and if so, whether they phase shift according to shifts in the light cycle and the approximate time it takes to accomplish re-entrainment. Lastly endogenous rhythms were investigated under constant conditions.

Chapter 5

This chapter attempts to relate the immunohistochemical results to that of the activity studies. The sensitivity of the circadian system of mole-rats is compared and contrasted with aboveground rodents. The different species of mole-rats are compared with each other and an attempt is made to find a general trend in photic sensitivity. The main results of the thesis are summarized.

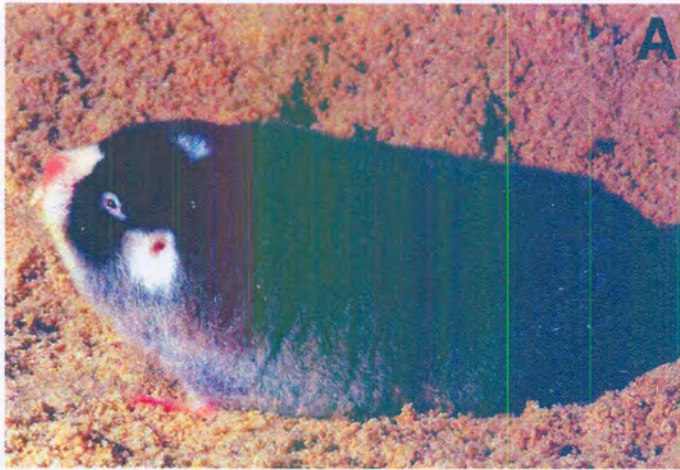


Plate 1.1: Photographs of the mole-rat species used in this thesis. A - *Georychus capensis*, B - *Cryptomys hottentotus*, C - *Cryptomys darlingi*, D - *Cryptomys damarensis*, E - *Cryptomys mechowi*, F - *Heterocephalus glaber*. Photographs with courtesy of Dr. T.P. Jackson.

CHAPTER 2: RETINAL PROJECTIONS IN SELECTED AFRICAN MOLE-RAT SPECIES

RETINAL PROJECTIONS IN SELECTED AFRICAN MOLE-RAT SPECIES

African mole-rats are strictly subterranean rodents that rarely emerge aboveground. The underground habitat results in the animals being in total darkness for most of their lives, consequently they are very infrequently exposed to the daily environmental light/dark cycles. As an adaptation to their underground habitat, they exhibit a number of distinct morphological and physiological adaptations, which includes a reduction in eye size, and a severely regressed visual system.

Associated with the minute eyes, mole-rats appear to be visually blind. However, despite the lack of vision, the circadian system is intact and functional. Thus mole-rats still possess a degree of light perception that involves the eye.

Anatomy of the eye

A normal functional eye consists of three different layers: the outer, middle and inner tunics. The sclera and the cornea form the external layer (outer tunic), the anterior part of the intermediate layer (middle tunic) consists of the iris and the ciliary body, while the choroid represents the posterior part. The lens is located behind the iris and is attached to the ciliary body via ligaments. Changes in the shape of the lens allow light to be projected onto the retina, the internal layer or inner tunic of the eye (Fig. 2.1).

The retina is important as it contains the sensory components of the eye. The retina is several cell layers thick, it consists of three layers of nerve cell bodies and two layers of synapses, called plexiform layers (Fig. 2.2). The outer nuclear layer contains the photoreceptor cell bodies, the rods and cones. The inner layer consists of cell bodies of bipolar, horizontal and amacrine cells. The third nuclear layer is the ganglion layer, containing cell bodies of ganglion cells and displaced amacrine cells. The outer plexiform layer is found between the inner and outer nuclear layer, forming contacts between the two layers. The inner plexiform layer connects the bipolar and horizontal cells with the ganglion cells. Axons from the retinal ganglion cells form the optic tract that projects into the brain.

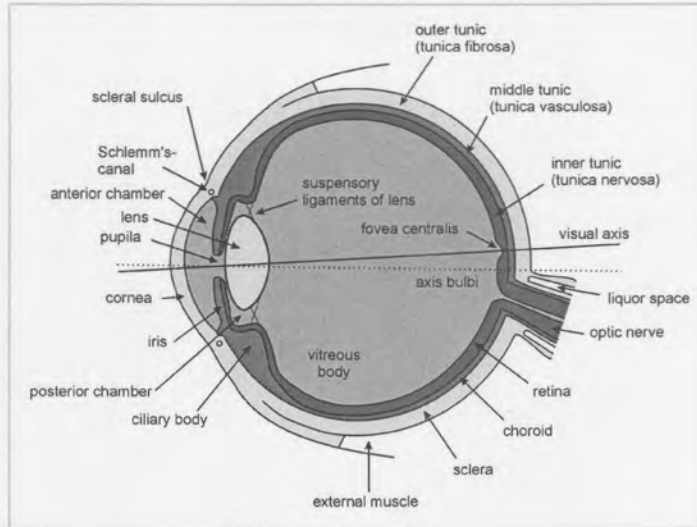


Figure 2.1: Schematic representation of the anatomy of the eye. (with permission of Dr. A.Szel, Budapest, Hungary)

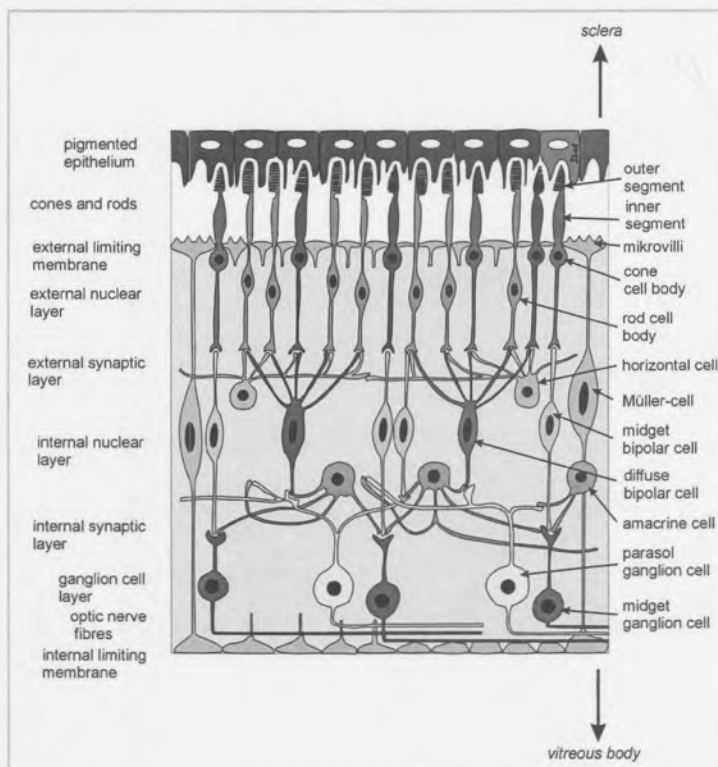


Figure 2.2: Schematic representation of the structure of the retina. (with permission of Dr. A.Szel, Budapest, Hungary)

Receptors in the retina relay information to the visual cortex for image formation, as well as temporal information of significance to the circadian system. Rods and cones are well established as photoreceptors for primary vision. Previously it was believed that these classical receptors were also involved with the circadian system, as the peak sensitivity of the circadian system coincides with the peak sensitivity of rod photoreceptors. However, differences in light threshold and integration time of the two systems (Nelson & Takahashi 1991) initiated a search for novel photoreceptors in the retina that may rather be implicated in photic entrainment. Other findings also pointed to the existence of an unidentified photoreceptor in the retina. A subset of retinal ganglion cells project only to structures involved with the circadian system (Meijer & Rietveld 1989). These retinal ganglion cells are evenly distributed over the retina, providing an increased sampling area and a reduced spatial resolution (Provencio *et al.* 1998). Transgenic mice lacking rods and cones were still able to entrain to photic cues and produced phase shifts comparable to that of normal animals (Foster *et al.* 1991). To date, the photoreceptors mediating circadian entrainment have not been identified, although a number of putative photopigments have been suggested. It is also possible that both classical and novel photoreceptors are involved with the circadian system.

Retinal projections

The large majority of the retinal ganglion cells that form the optic tract, project to the primary visual system. The circadian system receives a very small portion of the total projection. The relative proportions of the ipsilateral and contralateral projections vary between species, but normally the contralateral side receives a larger innervation compared with the ipsilateral side.

Retinal ganglion cells that project to the contralateral side are present throughout the retina, whereas ipsilateral projecting retinal ganglion cells occur only in a small part of the retina (Provis & Watson 1981).

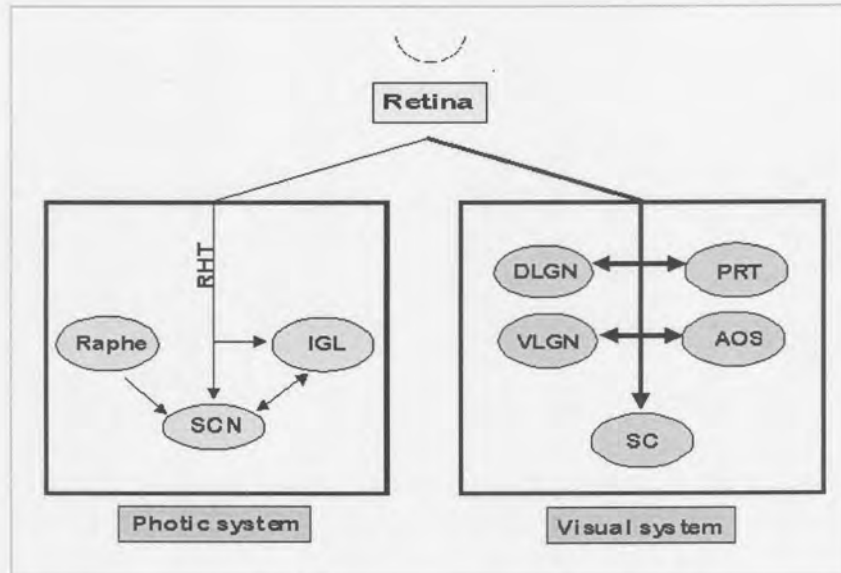


Figure 2.3: Schematic representation of the dichotomy of the visual system and circadian system in mammals (from Negroni, 1998, thesis).

AOS = Accessory optical system, GHT = geniculohypothalamic tract, IGL = intergeniculate nucleus, dLGN = dorsal lateral geniculate nucleus, vLGN = ventral lateral geniculate nucleus, PRT = Pretectum, RHT = retinohypothalamic tract, SC = Superior colliculus, SCN = suprachiasmatic nucleus.

Structures involved with the visual system

Pretectal nuclei (PRT)

In mammals, the pretectum is believed to be involved in the integration of visuomotor functions and pupillary light reflex as well as light/dark discrimination. This area could therefore be involved in the behavioural response to light (Cooper *et al.* 1993).

The pretectum is composed of several paired nuclei, including the nucleus of the optic tract (NOT), the olivary pretectal nucleus (OPN), the posterior pretectal nucleus and the anterior pretectal nucleus. Most of these nuclei receive a bilateral projection from the retina, although the contralateral projection is mostly denser than the ipsilateral projection (Argwala *et al.* 1989, Zhang & Hoffmann 1993, Telkes *et al.* 2000).

Accessory optic system (AOS)

The strongest response of the nuclei in this structure is to slow large-field visual motion, each nucleus responding to a particular direction. It has also been suggested that the AOS may be involved with transforming the visual motion signal from retinal coordinates to vestibular or oculomotor coordinates (McKenna & Wallman 1985).

These nuclei receive bilateral innervation via the optic tract, with an ipsilateral projection that is sparser than the contralateral projection (Argwala *et al.* 1989, Zhang & Hoffmann 1993, Uchiyama *et al.* 1995).

Superior colliculus (SC)

The superior colliculus (SC) responds to easily discriminable stimuli, it is responsible for reflex-like responses of the eye and movement detection (Johnson 1993).

The superior colliculus receives the vast majority of the retinal input, which in the rodent can exceed 90%. This structure receives both contralateral and ipsilateral innervation, but generally the contralateral side is more densely innervated (Zhang & Hoffman 1993).

Lateral geniculate nuclei (LGN)

According to Merigan and colleagues (1991), the dorsal LGN is primarily involved in chromatic vision, acuity and contrast detection of low temporal and high spatial frequencies. In contrast, the ventral LGN mediates contrast detection at high temporal and low spatial frequencies. In addition, the vLGN is believed to be related to structures of the visuo-vestibular and visuo-ocular systems (Oeschlager *et al.* 2000)

The LGN receives bilateral input from the retina with a predominant contralateral innervation that is related to the divergence of the optical axis of the eyes (Argwala *et al.* 1989, Dong *et al.* 1995, Higo & Kawamura 1999).

Structures involved with the circadian system

Suprachiasmatic nucleus (SCN)

The function of the SCN as the central pacemaker is well known. The SCN receives its innervation by the RHT, a minute part of the optic tract. In rodents such as mice and hamsters, the projection to the SCN constitutes about 1% of the total retinal projection (Cooper *et al.* 1993a).

However the SCN receives a bilateral innervation from the retina, the proportional size of the projections to the ipsilateral and contralateral sides differ in different species. (Kita & Oomura 1982, Argwala *et al.* 1989, Tessonneaud *et al.* 1994).

Intergeniculate nucleus (IGL)

The IGL is involved in both photic and non-photoc entrainment, although not essential for photic entrainment. It is believed to be involved in modulating the circadian system (Pickard *et al.* 1987). This structure receives both an ipsilateral and a contralateral innervation, however, the contralateral side is usually more heavily innervated. (Argwala *et al.* 1989, Smale & Boverhof 1999)

Raphe nucleus

Serotonergic cells are associated with a wide variety of different functions, which is illustrated by the vast amount of structures that are innervated by the raphe nuclei (Morin 1999, Vertes *et al.* 1999); the SCN is also among the structures that receive projections from the raphe. The raphe is not considered to be necessary for entrainment, but it does have an effect on entrainment (Levine *et al.* 1986).

Direct, but sparse retinal projections going to the dorsal raphe nucleus have been demonstrated, the contralateral side receives a larger projection (Shen & Semba 1994, Reuss & Fuchs 2000).

Albino animals (Plate 3.1)

In general the contralateral projection of albino and pigmented animals appears to be similar (Zhang & Hoffman 1993). However in comparison with pigmented animals, the ipsilateral projection in albino animals is significantly smaller or even absent although the distribution of ipsilateral axons in the optic nerve seems to be alike (Jeffery 1989, Zhang & Hoffman 1993, Thompson *et al.* 1995).

Retinofugal projections to the ipsilateral side of brain are reduced in albino animals. Reduction in uncrossed retinofugal projections affects input to the lateral geniculate nucleus and superior colliculus (projections involved in binocular vision) but not SCN (Drager 1974).

Projections in mole-rats

The peripheral visual system (eye and optic nerve) of mole-rats is severely regressed. In contrast with other rodents, the eyes of mole-rats are minute, African mole-rats have external eyes measuring approximately 2mm (Eloff 1958). The blind mole-rat, *Spalax ehrenbergi*, an extreme case of reduction of the eye size occurs, the eye being less than 1mm in cross section and subcutaneous, embedded in the harderian gland. Despite the small size, the eyes of the mole-rat are anatomically normal, consisting of a lens, iris and retina. In addition, the anatomical structure of the retina has also proven to be consistent with that of other rodents. The nuclear cell - and plexiform layers are present (De Jong *et al.* 1990) and both a rod- and a cone-like visual pigment are expressed (Janssen *et al.* 2000). The structure of the photoreceptors is underdeveloped in appearance, although the rod pigment has been characterized as fully functional with a high homology with rod pigments of other rodents (Janssen *et al.* 2000).

The retinal ganglion cell layer is very sparse; the number of ganglion cells varies from 200 to 1000 depending on the species (Cooper *et al.* 1993a), and is distributed homogenously over the retina. These ganglion cell axons are reported to be unmyelinated up to the point of entry to the brain where after they become myelinated (Leder 1975, cited by Cooper *et al.* 1993a)

Mole-rats have retinal projections to most of the structures that receive innervation in standard rodent models, but the relative and absolute size of both the projections and the

structures they project to, differ. Structures involved with the primary visual system, are reduced by up to 90%, whereas the circadian structures are more or less the same size as in their aboveground counterparts (Bronchti *et al.* 1989, Cooper *et al.* 1993a).

Electrophysiological studies have implicated that mole-rats are not capable of image formation (Haim *et al.* 1983). Although auditory takeover of visual lateral geniculate nucleus and part of visual cortex have been suggested (Bronchti *et al.* 1989), these results are disclaimed (Cooper *et al.* 1993a). Nevertheless, these results have led to the conclusion that mole-rats are visually blind.

Most of the structures associated with the primary visual system are degenerated in mole-rats. Normally, the superior colliculus (SC) receives the largest part of the retinal projection, (more than 99% of the total projection). In mole-rats, this projection is severely regressed, with only about 20% of the retinal fibers projecting to the SC. The relative projections to the LGN, pretectum (PRT) and accessory optic system (AOS) are proportionally similar to other rodents, though the absolute size of these structures is considerably reduced (Cooper *et al.* 1993a).

In contrast, the bed nucleus of the stria terminalis (BNST) that usually receives a very sparse projection, is densely innervated in the mole-rat. The projection to this region shows both absolute and relative expansion (Cooper *et al.* 1993a).

In mole-rats, the SCN receives bilateral innervation from the retina. The number of retinal fibres projecting to the SCN is similar in both aboveground and underground mammals; approximately 30 to 130 retinal ganglion cells form the retinohypothalamic tract (RHT) (Cooper *et al.* 1993a). Proportionally, this projection is thus much larger in subterranean mammals, rendering almost 20% of the projection to the SCN, compared to less than 1% in other mammals.

There is thus an overall selective reduction of retinal innervation to structures of the visual system and an expansion of the circadian system.

Ontogeny of the mole-rat visual system

Regression of the visual system takes place post nately, development of the eye starts off normally, but degeneration of the visual system becomes apparent within the first two weeks after birth (Sanyal *et al.* 1990, Bronchti *et al.* 1991). Eye size of newborn and adult mole-rats

approximately similar, and the newborn mole-rat retina projects bilaterally to most of the usual targets (Bronchti *et al.* 1991). In young animals, projections to the SCN, ventral and dorsal LGN, pretectum and SC are seen, significant reduction of retinogeniculate projection is only observed in the adults. The retinal histogenesis however, proceeds normally resulting in a structurally reduced but well differentiated retina. (Sanyal *et al.* 1990).

African mole-rats

African mole-rats do not exhibit the same extreme form of structural and functional degeneration of the visual system as seen in *Spalax ehrenbergi*. *Spalax* possesses subcutaneous eyes with a size of about 1mm, whereas in its African counterparts, the eyes are visible externally being nearly twice as large (2mm) (Eloff 1958). It is therefore not unreasonable to expect that the internal anatomy of the visual system may show a less severe form of regression.

Objectives of this chapter

A study on the blind mole-rat *Spalax ehrenbergi*, a solitary subterranean animal, revealed that the retinal projections in these animals were roughly similar to those in other mammals, however, the relative and absolute size of the projections were different. This chapter aims to determine whether the social Giant Zambian mole-rat, *Cryptomys mechowi*, shows retinal projections to all the same visual structures as other rodents, and whether the different visual projections also exhibit selective expansion and regression as in *Spalax*.

Albino animals are known to have very small ipsilateral projections to visual structures. The second part of this chapter investigates the retinal projections of two albino mole-rats to determine whether the innervation of the visual and circadian structures differs considerably from that of other rodents, as well as other mole-rats.

MATERIAL AND METHODS

Intraocular injection

Three mole-rats (1 *C. mechowii*, 1 albino *C. h. pretoriae* and 1 albino *C. damarensis*) were anaesthetized with halothane vapours and a 0.3ml Ketamine Rompan injection. After dilating the pupil with atropine, animals received an intraocular injection into the vitreous body of the right eye. A quantity of 0.5-1.0 μ l cholera toxin (subunit B) horseradish peroxidase (CT-HRP) was injected and after a time lapse of 48 hours, animals were perfused.

CT-HRP

A cholera toxin horseradish peroxidase conjugate was used as the neuronal tracer for studying retinal projections. This conjugate is an enzyme and is detectable in neurons as a result of its enzymatic activity. It can be used as an anterograde and a retrograde tracer. Survival of 24-48 hours produced optimal transport of the tracer, thus effectively staining axons.

Perfusion

The animals were perfused in June 1998 in Pretoria, South Africa and immunohistochemistry was performed in Lyon, France starting February 1999. The animals were sacrificed by using an overdose of Halothane anaesthetic and a 0.3ml ketamine Rompan injection until the animal had expired, after which they were perfused through the heart with a solution of 0.9% sodium chloride at 37°C, followed by a Zamboni fixative solution of 4% Paraformaldehyde in a 0.1M phosphate buffer (pH 7.4) and 15% saturated picric acid (PFA). The heads remained in a PFA post fix for 1-2 days until treatment.

Histological Preparation

Prior to sectioning, the brains were removed from the skull and placed in 30% sucrose phosphate buffered solution overnight. A freezing microtome was used to cut 40 μ m thick coronal sections. The sections were divided into two series, one for long-term storage in an anti-freeze and one for immediate immunohistochemical staining.

Immunohistochemistry

The IHC procedure followed that normally used at the INSERM laboratory (Negroni, 1998). All steps performed using agitation:

#	Step	#	Time	Temp	Quantities and comments
1	PBS rinses	3x	10 min	4°C	With agitation
2	Alcohol-saline-H ₂ O ₂	1x	45 min	4°C	200ml dist H ₂ O 3.6g NaCl 200ml 100% alcohol 5ml H ₂ O ₂
3	PBS rinses	2x	10 min	4°C	With agitation
4	Normal serum incubation	1x	1 h	4°C	1.5 % NGS in PBSTA
5	Primary antibody		72 h	4°C	1 μ l anticholeratoxin (1:3.000) in 5ml 1.5% NGS- PBSTA
6	PBST rinses	2x	10 min	20°C	With agitation
7	Secondary biotinylated antibody	1x	2 h	20°C	anti-rabbit biot. conc.=1:200 / 18ml PBST
8	PBST rinses	2x	10 min	20°C	With agitation
9	AB complex (prepared 30 min before use)	1x	2 h	20°C	200 μ l A / 200 μ l B / 20ml PBST
10	PBST rinse	1x	10 min	4°C	With agitation
11	TRIS rinses	2x	10 min	4°C	With agitation
12	DAB-Nickel	1x	10-20 min	4°C	0.02% DAB with 0.5% Nickel sulphate in TRIS / + 0.001% H ₂ O ₂ (66 μ l H ₂ O ₂ (30%)/ 1.93ml distilled

					H ₂ O) Preincubation in DAB for 10 minutes without H ₂ O ₂ . Reaction with 200µl H ₂ O ₂ added until satisfactory level obtained
13	TRIS rinses	2x	10 min	4°C	After 2 rinses, sections were left in TRIS buffer overnight without agitation.

Table 1: Procedures for immunohistochemistry

The sections were mounted on gelatinized slides and after dehydration with a series of alcohol and Toluene baths, cover slips were attached with Depex.

Observations and analysis

A Leitz microscope (Aristoplan) equipped with a CCD camera was used to observe the SCN sections, using brightfield illumination and a magnification objective of 10x.

RESULTS

Giant mole-rat (*Cryptomys mechowii*)

This mole-rat was found to have retinal projections to both the primary visual system and the circadian system structures as described in other rodents, although the structures were not as densely innervated. Bilateral innervation was demonstrated, however the ipsilateral projection was particularly sparse and absent to some structures.

In coronal section the optic chiasma and tract can be seen at the base of the section. Bilateral staining of the optic tract was observed, but it was first visible on the ipsilateral side.

The ipsilateral side of the optic tract appeared more concentrated, while the contralateral side was visible as a longer thin line at the base of the SCN.

Staining of the SCN appeared simultaneously with that of the optic tract. The SCN can be seen as an approximately round, structure above the optic chiasma and ventral to the third ventricle. The SCN is a paired structure and bilateral staining was apparent although the contralateral side was less densely innervated. The label in the SCN was distributed over the entire structure but was the densest in the ventro-lateral region (Plate 2.2a). Staining of the SCN was visible in 4-5 sections, corresponding to the size of the SCN in other mole-rat species. It appeared that caudal of the SCN all label in the rest of the brain was found on the contralateral side.

Both the dorsal and ventral LGN received a dense contralateral innervation, while the dorsal LGN received a sparse ipsilateral projection as well. The dLGN was visible anterior to the vLGN, as expected. The IGL, located between the dLGN and vLGN, is likely to have received a small retinal projection, but it is difficult to distinguish the fibres which are close to or integrated with that of the vLGN (Plate 2.2b).

The olivary pretectal nucleus received a relatively dense innervation and was clearly visible. This oval nucleus was only visible on the contralateral side (Plate 2.2c).

Fibres of the superior colliculus started to appear above the pretectum, and became more densely innervated more caudally. In comparison with other rodents, this structure receives a very sparse innervation (Plate 2.2d). No projections were visible to the BNST or the accessory optic system, either on the contralateral or the ipsilateral side of the sections.

Albino highveld mole-rat (*Cryptomys hottentotus pretoriae*)

The retinal projections in the albino highveld mole-rat, *C. h. pretoriae* strongly resembles that of the giant mole-rat *C. mechowi*. Retinal afferents are found to the primary visual system, as well as the circadian system, and projections are not as dense as in other rodents. The ipsilateral projection is absent in all structures except the optic tract and the SCN.

The optic tract is visible at the base of the third ventricle, at first only on the ipsilateral side, and more caudal also on the contralateral side. In this animal, the optic tract anterior to the

chiasm is also more concentrated on the ipsilateral side, while on the contralateral side, it is stretched out as a thin line at the bottom of the section.

Bilateral staining of the SCN becomes visible a few sections after the optic tract could first be seen. The ipsilateral side of the SCN received a visibly denser innervation than the contralateral side, and is visible for longer. The SCN could be seen in 6-8 sections, which is relatively large (Plate 2.3a).

The dorsal and ventral LGN was densely stained on the contralateral side. No staining was visible on the ipsilateral side. The dLGN was visible before the vLGN, while the vLGN remained apparent after the dLGN could no longer be seen. Although the IGL can not distinctly be identified visually, it is possible that it does receive innervation, and that the IGL fibres are incorporated with that of the vLGN (Plate 2.3b).

The olivary pretectal nucleus was clearly visible on the contralateral side as an olive shaped structure just beneath the SC (Plate 2.3c). Label could also be seen in the superior colliculus. In comparison with other rodent species, the SC receives a relatively sparse, but still distinct retinal projection on the contralateral side (Plate 2.3d).

No other structures received visible innervation from the retina on either the ipsilateral or contralateral side.

Albino Damaraland mole-rat (*Cryptomys damarensis*)

In contrast with the other albino mole-rat species investigated, the albino Damaraland mole-rat received an extremely sparse retinal innervation. The optic chiasm and optic tract was strongly labeled and clearly visible as a thick line at the base of the third ventricle (Plate 2.4a). Label visible on either side of the base of the third ventricle could possibly be the SCN, however the label does not resemble the SCN as seen in other mole-rat species. The optic tract does not disappear to the sides of the section like normal, but the fibres spread throughout the basal hypothalamus before disappearing (Plate 2.4b).

DISCUSSION

Rodents have retinal projections to a number of structures involved with the visual system as well as the circadian system. Along with a reduction in eye size, mole-rats have modified retinal projections to almost all of these structures. In parallel with the finding that mole-rats are visually blind (Haim *et al.* 1983, Bronchti *et al.* 1989), structures involved with the primary visual system are severely regressed and the retinal projections to these structures are equally reduced and sparse.

In the giant mole-rat, the only structures from the classical visual system that receive retinal innervation are the dorsal and ventral LGN, the olivary pretectal nucleus and the superior colliculus. Whereas the projections to the LGN and the pretectum are still relatively large, the SC receives a very small projection in these animals, considering that more than 90% of the total projection is allocated to this structure in other rodents (Cooper *et al.* 1993a).

According to Bronchti *et al.* (1991), the only retinal projections found in the adult blind mole-rat *S. ehrenbergi*, were to the vLGN, some of the pretectal nuclei (the lateroposterior nucleus and the nucleus of the optic tract) and the superior colliculus. In a separate study on the same species, projections to all the visual and circadian structures could be identified, although not all the structures received bilateral innervation (Cooper *et al.* 1993a). Other fossorial mammals have retinal projections similar to that of aboveground rodents, however the innervations are reduced (Kudo *et al.* 1988, Covey *et al.* 1987, Herbin *et al.* 1994).

In the giant mole-rat, projections to the circadian system are present to the SCN and possibly the IGL. In comparison to the size of the projections that other structures of the brain receive, the SCN is well developed and receives a major projection from the retina. A similar projection to the SCN was found in the blind mole-rat, *S. ehrenbergi*, the IGL was not clearly visible either. However, in contrast to *C. mehowi*, this animal showed an additional projection to the BNST (Cooper *et al.* 1993a). In other subterranean rodents, the retinal projection pattern was found to be reduced but no significant change occurred in the proportion of neurons that innervates visual structures (Herbin *et al.* 1994).

Apart from the optic tract, the SCN and a very sparse projection to the dLGN, no ipsilateral projections were observed in the giant mole-rat. In other mammals, the ipsilateral

projections are usually smaller than the contralateral side, thus the absence of ipsilateral projections was not totally unexpected, considering the sparseness of the contralateral projections.

It is thus clear that *C. mechowii* displays retinal projections different from that of other rodents, and that the relative and absolute size of the projections are modified. *Cryptomys mechowii* shows a severe regression in the size of projections to structures concerning the visual system and nearly all ipsilateral projections are absent, however, the projections to the circadian system appears to be unaffected by this regression. Less structures in the social *C. mechowii* receive innervation than in the solitary mole-rat *S. ehrenbergi*, but this phenomenon correlates with results in the later chapters, providing further evidence that social species are less sensitive to light than their solitary counterparts.

In general, albino animals exhibit fewer retinal projections than normally pigmented animals, especially on the ipsilateral side (Drager 1974). Thus the albino highveld mole-rat receives surprisingly dense retinal projections to both the visual and circadian structures. Retinal fibres project to all the same structures as in the pigmented giant mole-rat, as well as an additional projection to the olivary pretectal nucleus. The retinal projections of this albino animal were equally dense to that of the giant mole-rat and thus resembled the projections of a pigmented animal, although of a different species.

In contrast with the albino highveld mole-rat, retinal projections of the albino Damaraland mole-rat were hardly visible at all, except for the optic tract which was strongly innervated, and dense labeling was visible. No structures could be positively identified as being innervated by the optic tract, although there was some label in the area of the SCN. No projections to the LGN, the superior colliculus or the pretectal nuclei could be seen. There is thus a significant difference between the two albino animals investigated, since projections in the albino highveld mole-rat corresponded to that of pigmented mole-rats, whereas innervation in the albino Damaraland mole-rat differed completely from all other animals investigated. Since only one *C. damarensis* was investigated, the experimental Damaraland mole-rat may not be a representative example of the species.

In conclusion, visual pathways of *C. mechowii* and the albino *C. h. pretoriae*, are not simply downscaled as a function of eye size. Structures of the circadian system such as the SCN,

receive proportionally much larger innervation, whereas visual structures such as the SC proportionally receive proportionally much smaller innervation.

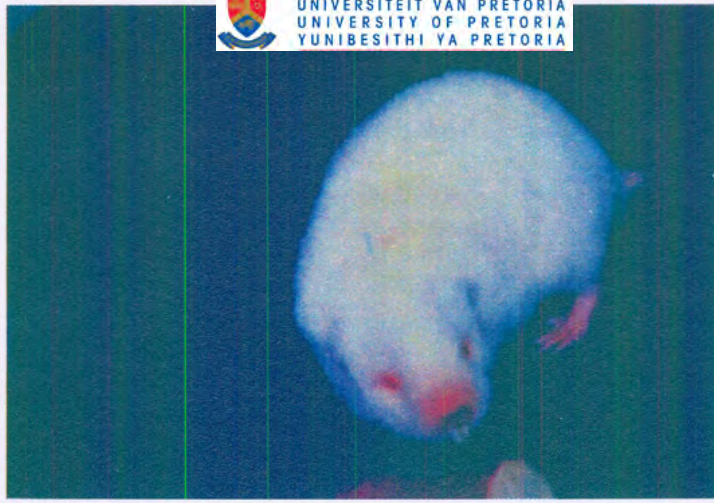


Plate 2.1: Photo of an albino *Cryptomys hottentotus pretoriae*.

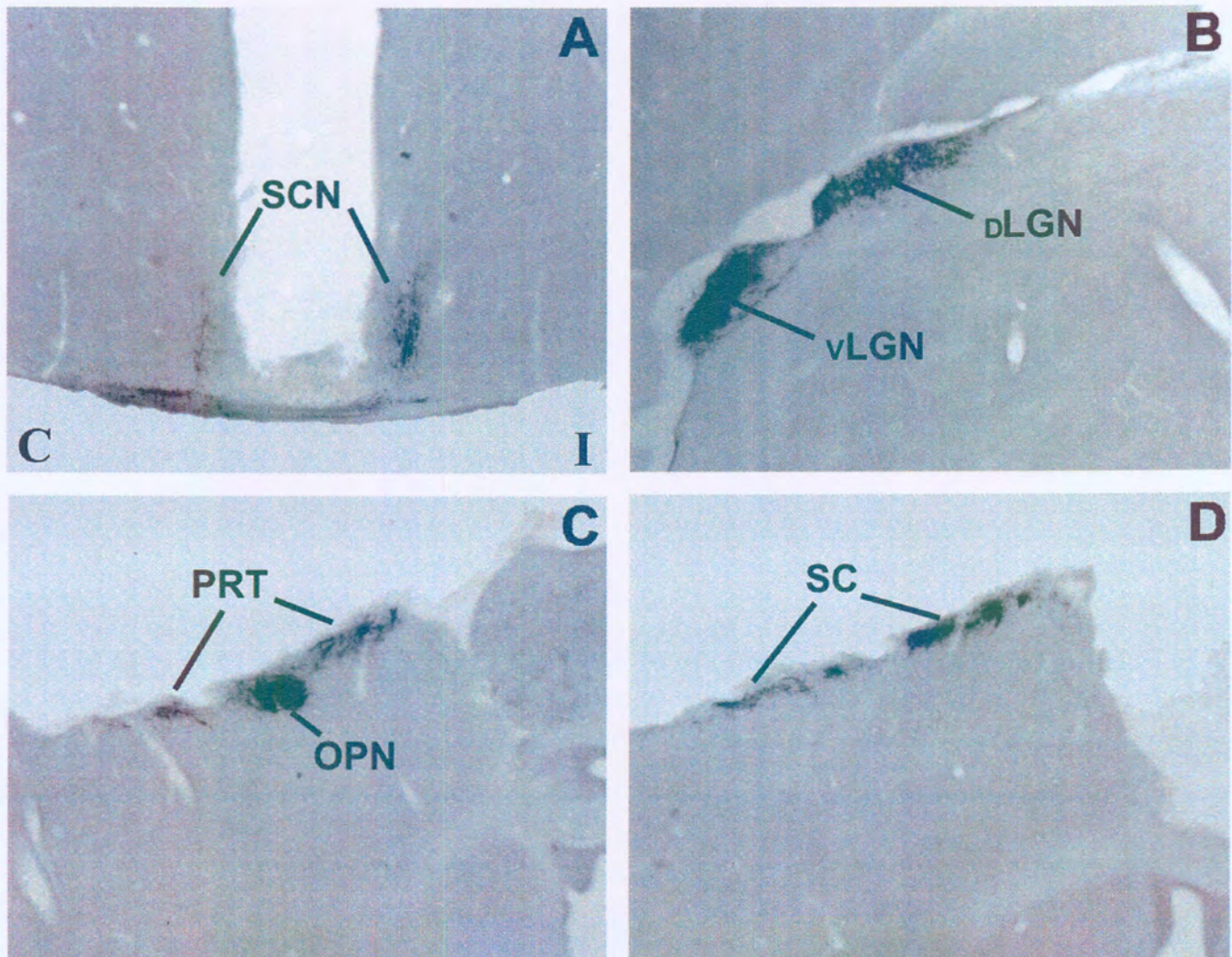


Plate 2.2: Retinal projections of the giant mole-rat *Cryptomys mechowii*, showing innervation of the SCN (A), the dLGN and vLGN on the contralateral side (B), the pretectum and olivary pretectal nucleus (OPN) on the contralateral side (C) and the superior colliculus on the contralateral side (D).

C - contralateral, I - ipsilateral

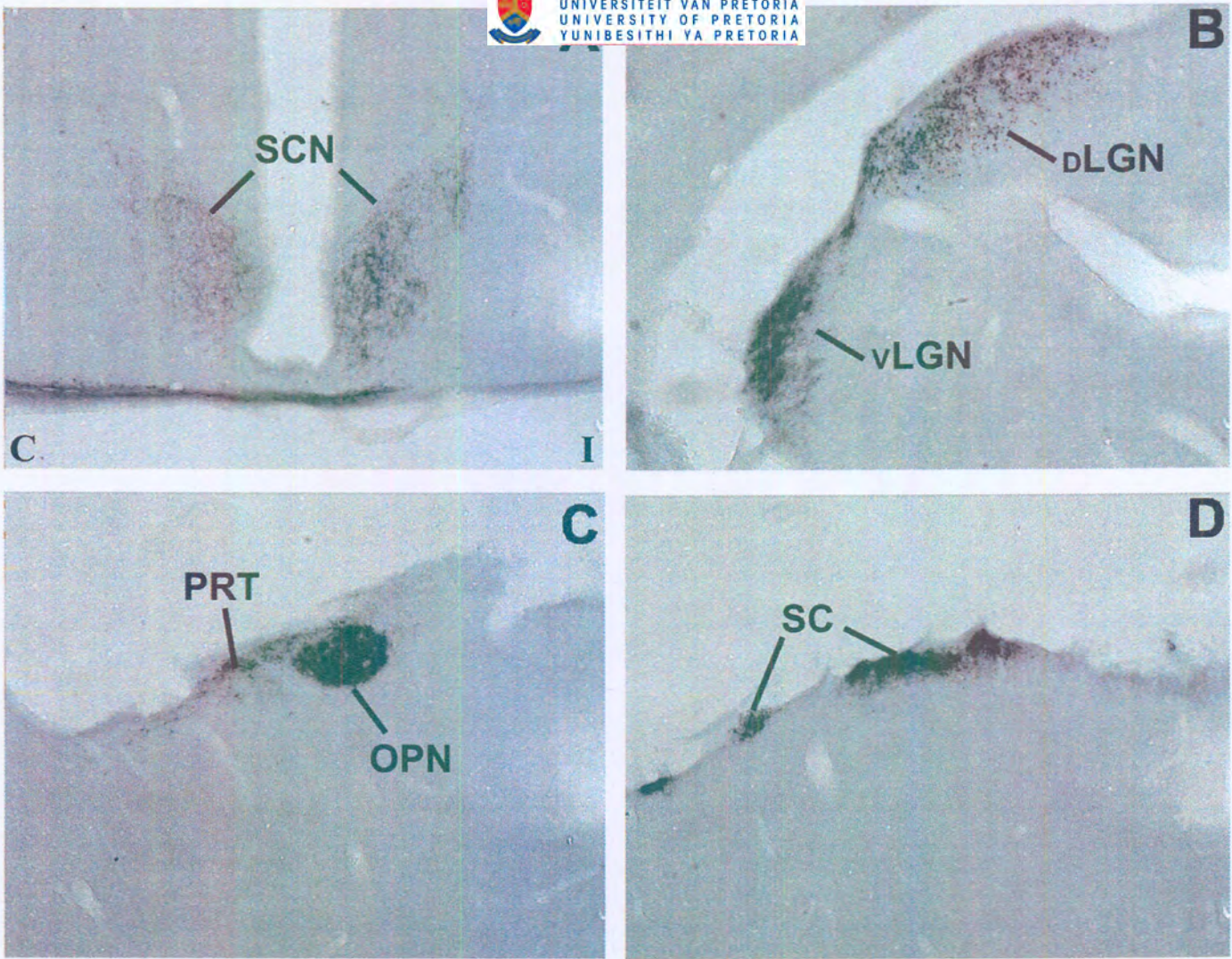


Plate 2.3: Retinal projections of the albino highveld mole-rat, *C. h. pretoriae*, showing innervation to the SCN (A), the dLGN and vLGN on the contralateral side (B), the pretectum and olivary preectal nucleus (OPN) on the contralateral side (C), and the superior colliculus on the contralateral side (D).

C - contralateral, I - ipsilateral

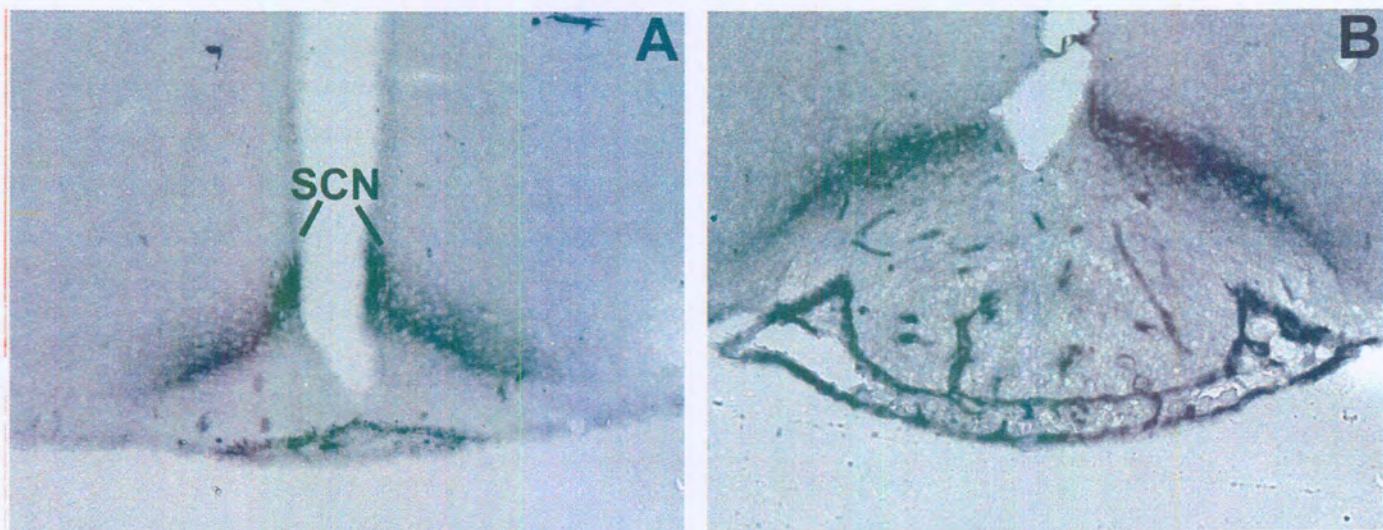


Plate 2.4: Retinal projections of the albino Damaraland mole-rat, *C. damarensis*, showing innervation to the area of the SCN (A) and the basal hypothalamus (B).

***CHAPTER 3: FOS EXPRESSION IN THE SCN OF
SOLITARY AND SOCIAL AFRICAN MOLE-RATS***

FOS EXPRESSION IN THE SCN OF SOLITARY AND SOCIAL AFRICAN MOLE-RATS

In mammals, the suprachiasmatic nucleus is the site of the endogenous circadian clock. Although the circadian clock is relatively well characterized, the cellular mechanism remains largely unknown. An increasing body of evidence suggests that gene expression is required for clock function, since single gene mutations in several species cause alterations in circadian rhythms (Ralph & Menaker 1988, Dunlap 1990, Vitaterna *et al.* 1994). Therefore, to understand the basis of circadian rhythms, it is necessary to identify the genes and proteins that are associated with the clock.

Neuronal genes are characterized in two general groups, the early immediate genes (IEG's) and the late response genes. Several early immediate genes have been identified in the SCN. These genes are expressed rapidly and transiently in response to light stimulation and the transcribed mRNA's have a very short half-life (Sheng & Greenberg 1990). Transcription of immediate early genes are induced within five minutes after the initiation of the light pulse, peaks after 30 minutes, and the gene levels are back at basal level 2 hours post stimulation (Kornhauser *et al.* 1990). Early immediate gene protein products activate the transcription of late response genes. Late response genes are transcribed more slowly over several hours and generally depend upon new protein synthesis (Sheng & Greenberg 1990).

Hence, immediate early genes function as 3rd messengers, encoding for transcriptional regulatory factors controlling the expression of a cascade of late response genes that convert short-term extra-cellular changes into long-lasting cellular changes (Morgan & Curran 1989,1991).

Transcription of IEG's within the SCN is spatially specific, in response to light it is only expressed in the SCN and no other retino-recipient structures, and it is temporally specific, since it is phase dependent (Hastings *et al.* 1995). These characteristics are of importance as it provides functional markers of neuronal activity in the SCN in response to light.

The early immediate gene, *c-fos*

The proto-oncogene *c-fos* is one of a series of early immediate genes whose induction within the SCN is believed to be related to the daily phase shifts required for entrainment to the external environment. Several lines of evidence support the idea of Fos involvement in light-induced phase shifts. Firstly, the distribution of light activated Fos immunoreactive cells in the SCN maps the area of retinal innervation. In the SCN, Fos can only be induced by light stimulation, it is not expressed in response to non-photic stimuli (Mead *et al.* 1992). In response to light, Fos is induced only in retinorecipient structures of the circadian system in the brain (Caldelas *et al.* 1998). In addition, the expression of Fos is gated by the circadian clock in such a manner that it is only expressed during times when light also causes behavioural phase shifts (Fig. 3.1). The amplitude of the behavioural phase shifts and levels of Fos induction in the SCN correspond positively to intensity of the light pulse.

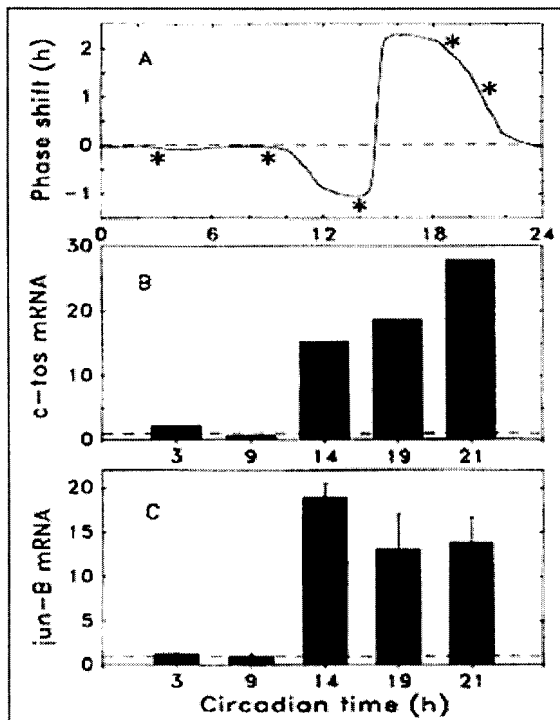


Figure 3.1a

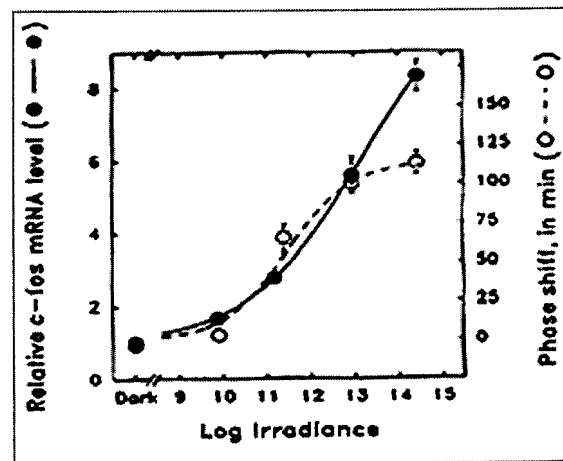


Figure 3.1b

Figure 3.1a,b: Illustration of the correlation between behavioural phase shifts and immediate early gene expression in the SCN. (From Kornhauser *et al.* 1992,1996)

Furthermore, the minimum light intensity needed for induction of Fos is virtually indistinguishable from that needed to elicit behavioural phase shifts (Fig. 3.1, Kornhauser *et al.* 1990). The glutamate agonist NMDA mimics the effect of light on Fos and behavioural rhythms at specific times of the circadian cycle (Zheng *et al.* 1999) and NMDA receptor antagonists block both fos and activity phase shifts (Colwell *et al.* 1991). Finally, antisense oligonucleotides against *fos* & *jun* blocks light induced activity phase shifts (Wollnik *et al.* 1995). Given the correlation between light induced phase shifts and the pattern of Fos induction in the SCN, it is very likely that Fos might be in the pathway involved in light induced phase shifting.

Mechanism of expression and action of *c-fos*

Gene induction in the SCN neurons is accomplished through signal cascades that are activated by the transient phosphorylation of proteins that activates transcription. Light can stimulate the phosphorylation of the transcriptional factor CREB in the SCN neurons, but only during the subjective night, indicating that CREB phosphorylation is gated by the circadian clock. This suggests that gating by the circadian clock occurs upstream from CREB phosphorylation. Furthermore, the period when phosphorylation of this protein is possible, matches the period when Fos can be induced in the SCN, linking it with gene transcription. Phosphorylation of CREB appears to be essential for the transcription of *c-fos* mRNA, and can also initiate behavioural phase shifts (Ginty *et al.* 1993, Ding *et al.* 1997). Light induces P-CREB to a maximal level in 10 minutes, this level is maintained for 30 min, and basal levels are once again obtained 2h after stimulation (Ding *et al.* 1997). Without stimulation the level of P-CREB is very low. Phosphorylated CREB binds to promotor site Ca/CRE, which is the principal site of transcriptional activation of *c-fos* by light (Sheng *et al.* 1990). P-CREB can activate the CRE's of a number of genes, including that of *c-fos* and *jun-b*.

Since the transcriptional factor of *c-fos* and *jun-b* genes is gated by the circadian clock, the expression of these genes is necessarily also gated by the circadian clock. The transcribed mRNA's of the Fos and Jun genes are transported to the cytoplasm of the neuron where they encode for the proteins Fos and Jun. The protein products are transported back to the nucleus, where the half live does not exceed 2h (Sheng & Greenberg 1990, Fig. 3.2 & 3.3).

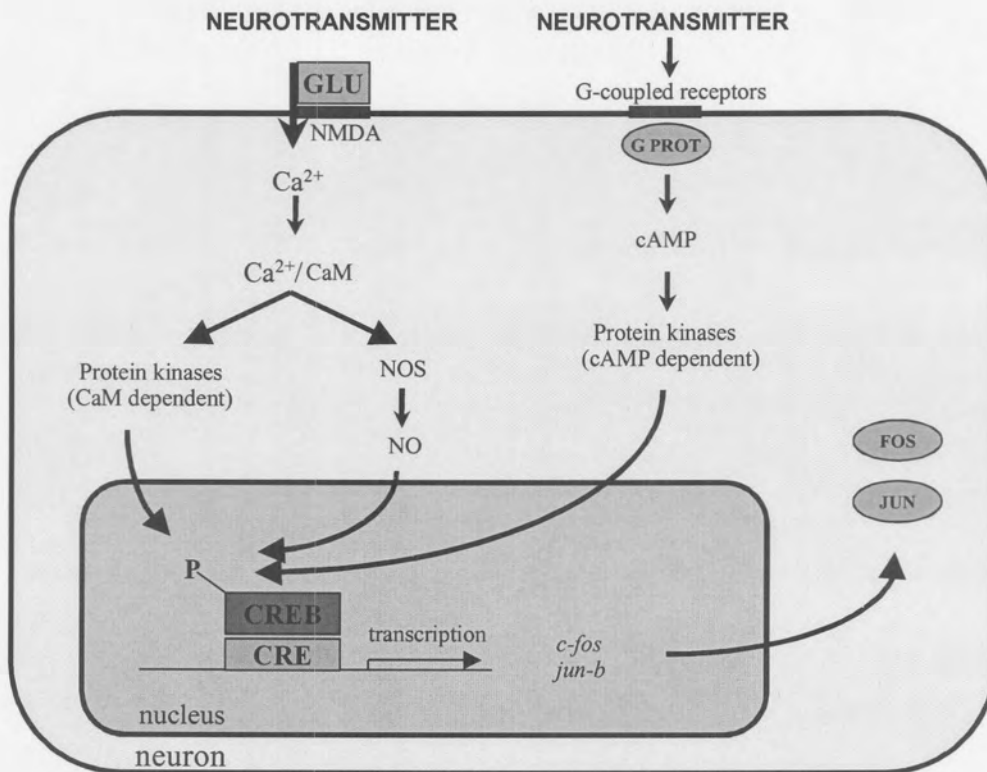


Figure 3.2: Schematic representation of Fos expression in a SCN neuron.

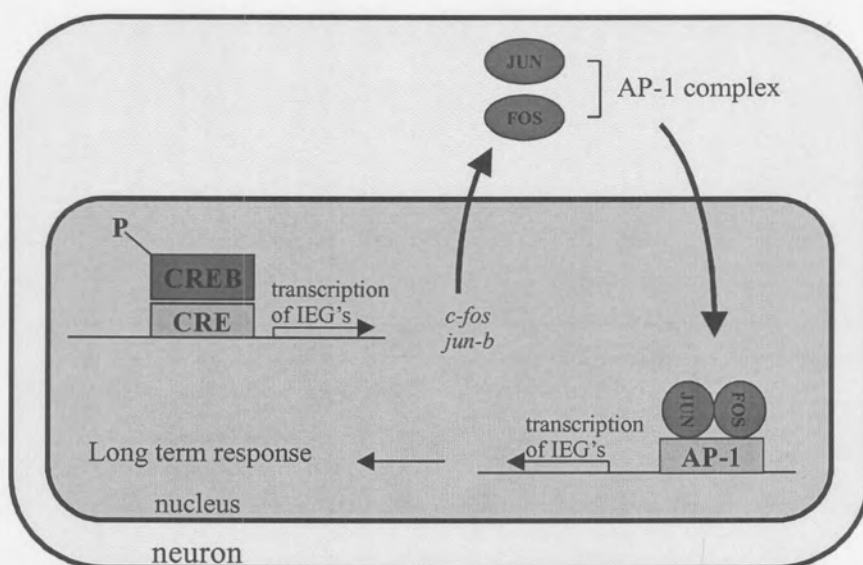


Figure 3.3: Schematic representation of the action of Fos protein.

In the nucleus, the Fos and Jun proteins form a heterodimeric complex known as activator protein-1 (AP-1) that interacts with the activator protein-binding site (AP-1), coding for the transcriptional factor AP-1. This transcriptional factor can suppress the promoter genes of *c-fos* and *jun-b*, thus Fos and Jun are capable of negatively auto-regulating their own transcription.

Fos expression over the circadian cycle

In a LD cycle, *c-fos* has a pattern of expression peaking around subjective dawn, with lower levels occurring during the rest of the light period, and basal levels of Fos are expressed during the dark period. In constant darkness, endogenous Fos expression is largely restricted to the dorso-medial part of the SCN, in contrast to light induced Fos expression that takes place mainly in the ventro-lateral part (Sumova *et al.* 1998). The relative abundance of Fos expression in the SCN is much lower than that in response to light stimulation (Schwartz *et al.* 2000) (Fig. 3.4).

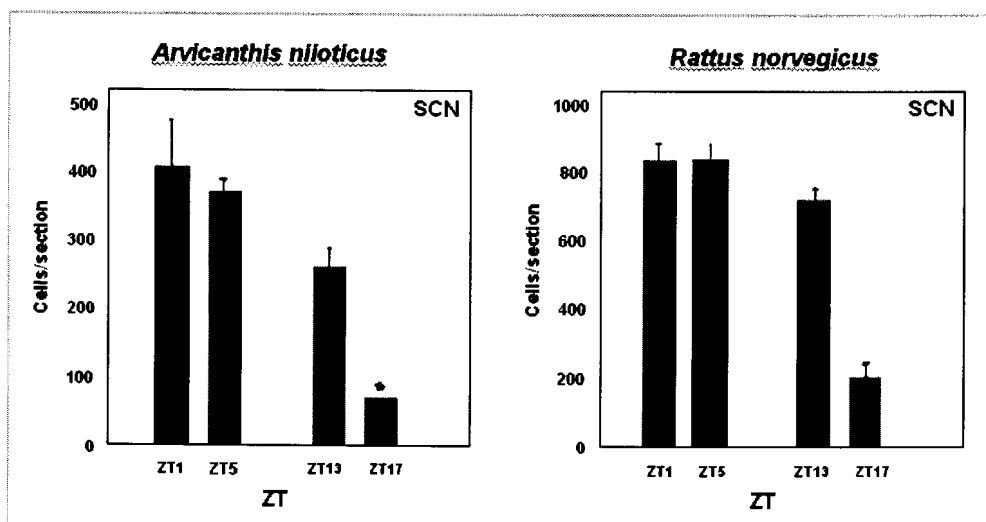


Figure 3.4: Mean (\pm SEM) number of Fos+ cells per section in the SCN of *Arvicantis niloticus* (left) and *Rattus norvegicus* (right) at four different circadian times. Lights were on from ZT 0 – ZT 12. (from Nunez *et al.* 1999)

Spontaneous Fos expression in a LD cycle differs from the pattern of light induced Fos, which is strictly phase dependent, it is expressed in response to light stimulation during subjective night, not during subjective day (Kornhauser *et al.* 1996, Cooper *et al.* 1998). Both the spontaneous and light induced expression of *jun-b* mRNA display similar induction patterns to that of Fos (Kornhauser *et al.* 1992), and following light stimulation, Fos & Jun are co-localized in SCN neurons in the ventro-lateral region (Takeuchi *et al.* 1993) These findings support the idea that these two genes are closely related in function (See fig. 3.1, Kornhauser *et al.* 1992).

In rodents, Fos expression in the SCN is considered to be a cellular correlate of light induced behavioural phase shifts (Takahashi *et al.* 1984). Non-photoc stimuli (e.g. behavioural manipulations) also cause behavioural phase shifts but do not induce Fos expression in the SCN (Mead *et al.* 1992). However, certain stimuli can cause Fos expression in the IGL (Janik & Mrosovsky 1992). The expression of Fos is gated by the circadian clock only in the SCN, and not in other retinorecipient areas. Fos expression in the IGL is not phase dependent in either nocturnal or diurnal rodents (Krajnak *et al.* 1997).

Difference in Fos expression in nocturnal and diurnal animals

The majority of work on Fos expression has been done on nocturnal animals, nevertheless, the little experimentation on diurnal rodents suggests that the daily pattern of Fos expression in nocturnal and diurnal rodents is in general very similar.

The endogenous rhythm of Fos expression in the SCN of the diurnal *Arvicanthus niloticus* was found to peak in the early subjective day and remain more or less at basal levels during the rest of the subjective day and night (Katona *et al.* 1998). This pattern of expression resembles that found in nocturnal rodents (Guido *et al.* 1999).

Moreover, the diurnal *Octodon degus* exhibited a decrease in Fos expression during subjective day in response to light stimulation (Krajnak *et al.* 1997). In addition, electrical and metabolic activity in the SCN is similar in nocturnal and diurnal animals (Nunez *et al.* 1999).

Circadian time

The effect of light on the circadian system over the course of a 24-hour day is displayed

by a phase response curve. Nocturnal rodents have a characteristic light-induced phase response curve, during subjective day there is an insensitive period, where light has no effect on the circadian system, phase delays occur in early subjective night and phase advances in late subjective night. The amplitudes of the phase shifts vary according to different species, but the general form of the PRC is representative for all above ground nocturnal rodents. Subterranean species appear to display the same characteristic phase response curve for Fos as other rodents. The solitary *Spalax ehrenbergi* exhibits Fos expression in the same clock dependent way as aboveground rodents, although the density of cellular expression is lower (Tobler *et al.* 1998, See Fig. 3.1).

Light intensity

The sensitivity of the circadian system to light depends on the previous history of the specific animal, thus causing inter species variations. The threshold for visual response also varies between species, ranging from approximately 0.1 lux in the rat to 10 lux in the hamster, and saturation of the visual system occurs at around 500 lux (Nelson & Takahashi 1991). These intensities compare to those occurring around dawn and dusk.

Since both the intensity and duration of the light pulse determine the cellular response in the SCN, one would expect that when pulse duration is kept constant and intensity is elevated, the Fos expression will become more robust accordingly.

Animals exposed to increasing light intensities exhibit light activated cells that show an increased discharge rate. There is a threshold value after which the cell will not increase discharge rate (Meijer *et al.* 1986).

Duration of pulse

Since the circadian system needs longer integration times than the visual system, the circadian clock is unresponsive to very short light pulses (<1s). The responsiveness of the circadian system increases with pulse duration until it reaches a saturation level where after the level of Fos expression in the SCN declines again. In hamsters, peak mRNA Fos expression in

the SCN is reached with a light pulse duration of around 30 minutes, longer pulse durations do not render any higher levels of Fos expression (Kornhauser *et al.* 1990, See Fig. 3.1).

Similar to the effects of light intensity, if the light intensity is to be maintained at a constant level and the pulse duration lengthened, an increase in Fos expression is also expected. Thus there is an interaction between pulse duration and intensity, the circadian response depends on the total number of photons received.

Objectives of this chapter

The fossorial environment offers far less variation in environmental factors such as light, temperature & humidity changes. The objectives of this chapter were to investigate the responsiveness of the SCN to light, as well as to differences in light intensity and duration, and lastly to assess whether ambient temperature affects the responsiveness of the SCN.

The aims of this chapter were to firstly determine whether *Georychus capensis* and *Cryptomys hottentotus pretoriae* are sensitive to light using Fos as an assay, and if so, whether these species display differential sensitivity towards light during the subjective day and night. Secondly, the response of the circadian system to increasing light irradiance was investigated in a solitary species, *Georychus capensis* and the social mole-rat species, *Cryptomys damarensis*, *Cryptomys hottentotus hottentotus* and *Heterocephalus glaber*. Thirdly, the effect of light pulse length on Fos expression in the SCN was tested on *Cryptomys hottentotus pretoriae*. Finally, as a pilot study, the effect of temperature on the Fos expression in the SCN was examined in three social species, *Cryptomys darlingi*, *Cryptomys hottentotus pretoriae* and *Heterocephalus glaber*.

MATERIAL AND METHODS

Experiments

Experiment 1: Fos expression over the circadian cycle

A solitary species, *G. capensis* (n=18) and a social species *C. h. pretoriae* (n=20) were used to determine whether the circadian system is sensitive to light illumination and if so, whether the response is gated by the circadian clock.

Experimental animals were maintained on a 12 hour light:12 hour dark (12L:12D) cycle for three to four weeks at 27°C. On the day of the experiment, the animals were kept in total darkness (DD), such that the time of normal light onset ZT0 (Zeitgeber Time 0) was used as the beginning of the circadian time, CT0. The period of CT0 to CT12 corresponded to subjective day (normally the light period), and the period CT12 to CT24 to subjective night (normally the dark period). The animals were then exposed to a monochromatic light pulse at different times of the circadian cycle (CT4, CT10, CT16, CT22). The fifteen minute light pulse was presented at a wavelength of 500nm with an intensity of 1.0×10^{15} photons.cm⁻².s⁻¹. Subsequently the stimulated animals were returned to darkness and perfused 1 hour after beginning of stimulation. The dark control animals were treated similarly, except they did not receive any light stimulation (Fig. 3.5).

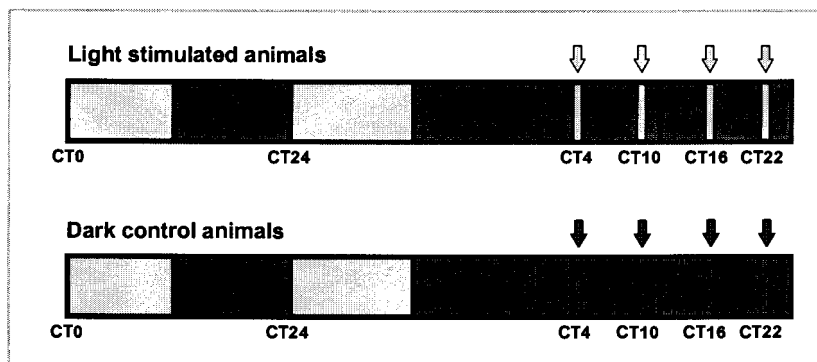


Fig. 3.5: Schematic representation of light pulses presented to stimulated animals (yellow arrows), as well as the corresponding ‘no pulses’ to the dark control animals (grey arrows) during the first experiment.

Experiment 2: Variation in Fos expression with different light intensities at CT16

One solitary species, *G. capensis* (n=15) and three social species *Cryptomys damarensis* (n=13), *Cryptomys hottentotus hottentotus* (n=25) and *Heterocephalus glaber* (n=12) were examined for circadian responses when the animals were presented with light stimulation of increasing irradiance. Before the experiment, the mole-rats were maintained on a circadian cycle of 12L:12D for two weeks, where after they were kept in total darkness at 27°C on the day of the experiment as described above. Animals were divided into groups that received light pulses at CT16 corresponding to intensities ranging from 1×10^{14} photons.cm⁻².s⁻¹ to $3,1 \times 10^8$ photons.cm⁻².s⁻¹, increasing in 1 log unit steps. The stimulation lasted for fifteen minutes, and the animals that served as dark controls received the same treatment without the light pulse (Fig.3.6).

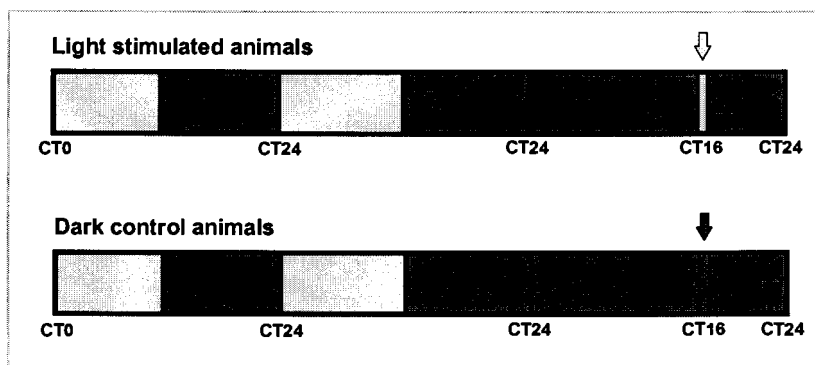


Figure 3.6: Schematic representation of light pulses presented to stimulated animals (yellow arrow), as well as the corresponding 'no pulses' to the dark control animals (grey arrow) during the second experiment.

Experiment 3: Variation in Fos expression with different light pulse durations at CT16.

The species *Cryptomys hottentotus pretoriae* (n=25) was investigated to determine whether the duration of illumination affects the Fos induction in the SCN. The same initial procedure was followed as pretreatment before the experiment. Animals were subjected to light pulses (3.1×10^{14} photons.cm⁻².s⁻¹) at CT16 ranging from 1.5 minutes to 90 minutes. Again dark

control animals were not subjected to light stimulation but otherwise received the same treatment than the stimulated animals (Fig. 3.7).

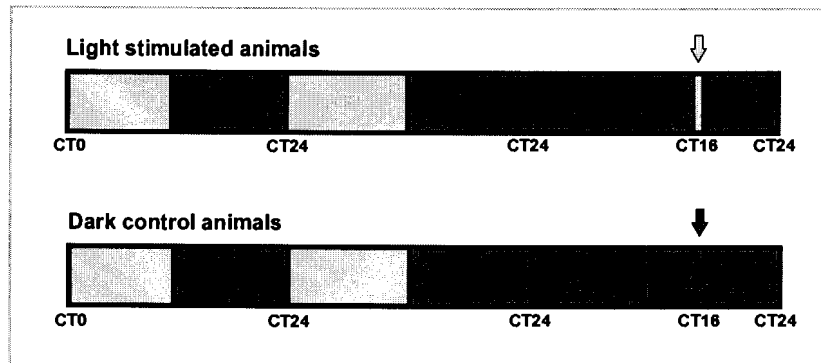


Figure 3.7: Schematic representation of light pulses presented to stimulated animals (yellow arrow), as well as the corresponding ‘no pulses’ to the dark control animals (grey arrow) during the third experiment.

Experiment 4: Variation in Fos induction with different ambient temperatures at CT16.

As a pilot study, three social species were studied to determine whether temperature has an influence on the Fos induction in the SCN in response on light illumination. As pretreatment to the experiment, the mole-rats *Cryptomys darlingi* (n=6), *Cryptomys hottentotus pretoriae* (n=9) and *Heterocephalus glaber* (n=6) were kept in conditions of 12L:12D for two weeks where after they were kept in constant darkness at 27°C on the day of the experiment as specified above. Experimental animals were divided into a 27°C and 15°C group, and a dark control group. One hour before the light pulse, the 15°C group was transferred to a chamber with ambient temperature of 15°C. The dark controls and the 27°C group were maintained at 27°C. Experimental groups were given a fifteen-minute light pulse of 3.1×10^{14} photons.cm⁻².s⁻¹ at CT16, and dark control animals received the same protocol without the light pulse (Fig.3.8).

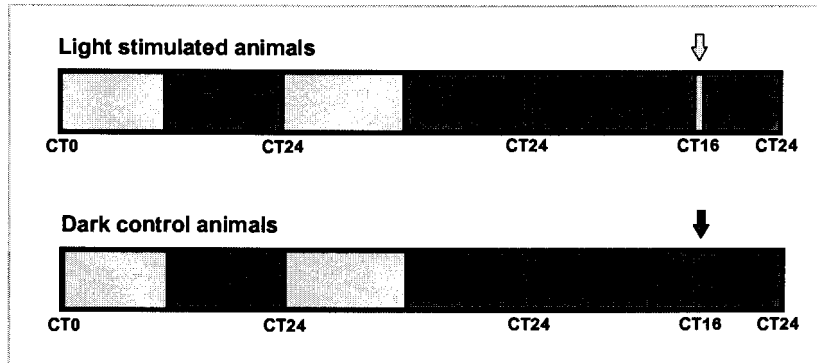


Figure 3.8: Schematic representation of light pulses presented to stimulated animals (yellow arrow), as well as the corresponding ‘no pulses’ to the dark control animals (grey arrow) during the fourth experiment.

Perfusion and Fixation

The animals were perfused in Pretoria, South Africa and were processed immunohistochemically in Lyon, France. The animals were sacrificed using Halothane anaesthetic and a 0.3ml subcutaneous Ketamine Rompan injection until the mole-rat had expired, after which they were perfused intracardially with a solution of 0.9% sodium chloride at 37°C, followed by a Zamboni fixative solution containing 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4) and 15% saturated picric acid (PFA). The heads were then removed from the bodies and placed in a PFA post-fix for 1-2 days at 4°C until further treatment.

Histological Preparation

Prior to sectioning, the brains were removed from the skull and placed in 30% sucrose phosphate buffered solution overnight. A freezing microtome was used to cut 40 µm thick coronal sections. The sections were divided into two series, one for long-term storage in an anti-freeze and one for immediate immunohistochemical staining.

Immunohistochemistry

The IHC procedure followed that normally used in the INSERM laboratory (Negroni, 1998). All steps performed using agitation:

#	Step	#	Time	Temp	Quantities and comments
1	PBS rinses	2x	10 min	20°C	With agitation
2	Alcohol-saline-H ₂ O ₂	1x	45 min	20°C	200ml dist H ₂ O 3.6g NaCl 200ml 100% alcohol 5ml H ₂ O ₂
3	PBS rinses	2x	10 min	20°C	With agitation
4	Normal serum incubation	1x	1 h	4°C	1.5 % NGS in PBSTA
5	Primary antibody		72 h	4°C	200µl C-Fos (1:10.000) in 1.5% NGS- PBSTA
6	PBST rinses	2x	10 min	20°C	With agitation
7	Secondary biotinylated antibody	1x	2 h	20°C	anti-rabbit biot. conc.=1:200 / 18ml PBST
8	PBST rinses	2x	10 min	20°C	With agitation
9	AB complex (prepared 30 min before use)	1x	2 h	20°C	200µl A / 200µl B / 20ml PBST
10	PBST rinse	1x	10 min	4°C	With agitation
11	TRIS rinses	2x	10 min	4°C	With agitation
12	DAB-Nickel	1x	10-20 min	4°C	0.02% DAB with 0.5% Nickel sulphate in TRIS / + 0.001% H ₂ O ₂ (66µl H ₂ O ₂ (30%)/ 1.93ml distilled H ₂ O) Pre-incubation in DAB for 10 minutes without H ₂ O ₂ . Reaction with 200µl H ₂ O ₂ added until satisfactory level obtained
13	TRIS rinses	2x	10 min	4°C	After 2 rinses, sections were left in TRIS buffer overnight without agitation.

Table 3.1: Procedures for immunohistochemistry

The brain sections were mounted on gelatinized slides and after dehydration with a series of alcohol and Toluene baths, cover slips were attached with Depex.

Observations and analyses

A Leitz microscope (Aristoplan) equipped with a CCD camera was used to analyze the SCN sections, using brightfield illumination and a magnification objective of 10x. The quantitative analysis was done by calculating the optical density and surface area of the immunoreacted cells in the SCN, by making use of an image analysis program (Visiolab 1000, Biocom, Les Ulis, France). The image analysis program was used rather than counting the cells visually, for three main reasons:

- The image analysis program is much faster than counting cells manually, and enables one to analyze all the sections, instead of only a few selected ones.
- The threshold of the label is defined at the beginning of the analysis, and remains constant for the rest of the analysis, whereas, if cells were to be counted by eye, the subjective definition of the threshold would vary according to observer and various factors (lighting conditions, degree of label).
- The density analysis takes into account the relative amount of label of individual cells, whereas the counting method gives equal weight to unequally labelled cells.

(For overview see Rieux *et al.* 2001)

Statistical analysis

The mean and standard error was calculated for each individual stimulated and dark control group. The non-parametric Kruskal-Wallis ANOVA was used to compare all the groups to each other within each series to test for any differences in the level of Fos expression. When the Fos expression levels were significantly different according to the Kruskal-Wallis ANOVA, the Mann-Whitney U-test was performed to determine which individual groups were significantly different from each other. Statistical significance was maintained at $p < 0.05$. A regression line was fitted to the applicable graphs to observe any trends.

RESULTS

Experiment 1: Fos expression over circadian cycle

a) Cape mole-rat (*Georychus capensis*)

The time points CT 0 to CT 12 corresponded to subjective day and CT12 to CT24 to subjective night. Dark control animals did not have any noticeable Fos induction during any of the experimental conditions. Light stimulation during early subjective day at CT4 resulted in a small amount of Fos induction. At late subjective day (CT10), no difference between the dark control animals and the stimulated animals could be observed. The dark control sample size for the two groups during subjective day (CT4 and CT10) was one, and thus did not permit statistical analysis. During the subjective night, the Fos induction was visibly higher for both the experimental conditions than during subjective day. The highest level of Fos expression occurred in early subjective night (CT16). According to the Kruskal-Wallis ANOVA, there was not a significant difference between any of the groups ($p=0.0807$). A Mann-Whitney U-test that was performed on the CT16 stimulated group and the dark controls which confirmed this result ($p=0.0833$). The non-significant results obtained in this species may be due to the small sample sizes.

By clumping the two experimental groups for subjective day (CT4 and CT10) and the two subjective night groups (CT16 and CT22) together, a significant difference was found between the two stimulated groups (Mann-Whitney U-test, $p=0.0065$). In addition, both the experimental conditions were significantly different from the dark controls. The subjective night values were markedly higher than that for subjective day (Mann-Whitney U-test, subjective day/dark control: $p=0.046$, subjective night/dark control: $p=0.011$). (Fig. 3.9 & 3.10, Plate 3.1, also see Appendix A, Table 3.2 & 3.3)

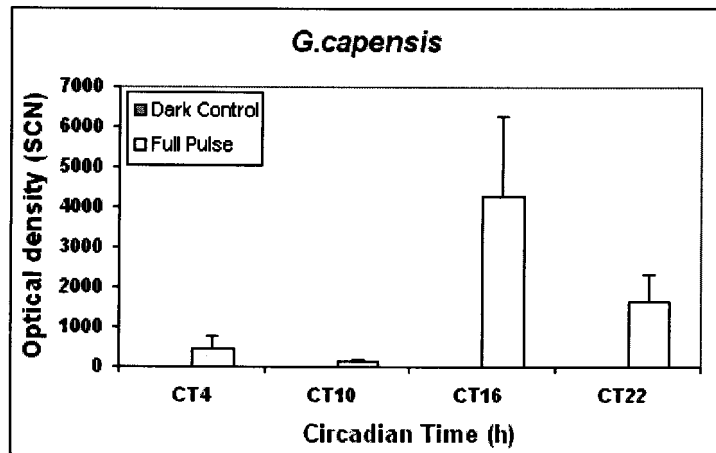


Figure 3.9: Fos expression in the SCN of *Georychus capensis* at different circadian times.

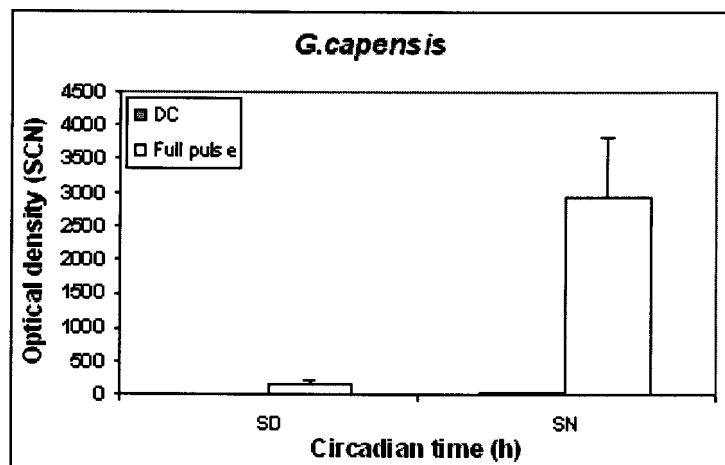


Figure 3.10: Fos expression in the SCN of *Georychus capensis* during subjective day and subjective night.

b) Highveld mole-rat (*Cryptomys hottentotus pretoriae*)

Over the whole 24-hour cycle, the dark control animals had relatively high values. The dark control animals had Fos expression levels close to that of the stimulated animals, there was no significant difference between the two datasets. The Fos expression in the experimental

animals did not differ statistically from the dark control animals during either the subjective day or subjective night (Kruskal-Wallis ANOVA, $p=0.333$). In contrast with *G.capensis*, the additive values of subjective day (CT4 and CT10) and subjective night (CT16 and CT22), revealed no visible or statistical difference between dark controls and stimulated animals (Mann-Whitney U-test, subjective day/dark control: $p=0.522$, subjective night/dark control: $p=0.136$). Neither was there a difference between the two stimulated groups (Mann-Whitney U-test, $p=0.631$) (Fig. 3.11 & 3.12, Plate 3.2, also see Appendix A, Table 3.4 & 3.5).

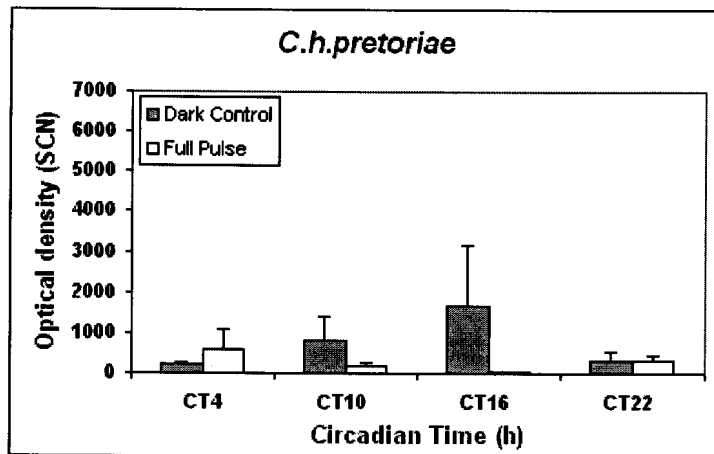


Figure 3.11: Fos expression in the SCN of *Cryptomys hottentotus pretoriae* at different circadian times.

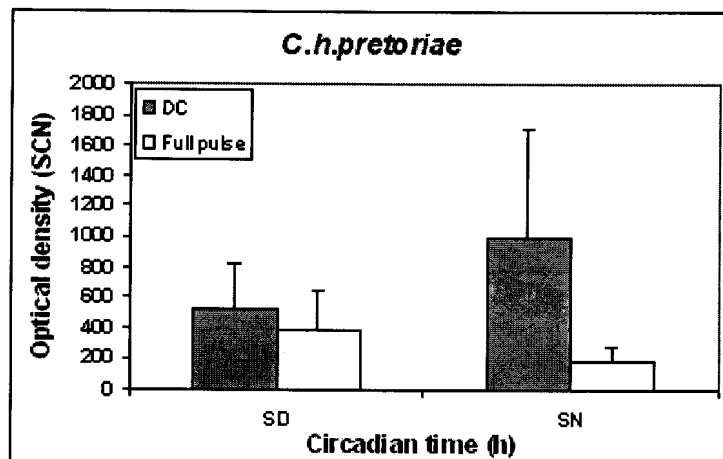


Figure 3.12: Fos expression in the SCN of *C.h.pretoriae* during subjective day and subjective night.

Experiment 2: Variation in Fos induction at different light intensities at CT16.

a) Cape mole-rat (*Georychus capensis*)

Dark control animals had extremely low levels of Fos expression. The amount of Fos expression in the SCN increased with the intensity of illumination. However, the highest irradiance did not render the highest level of Fos induction. Overall there was a tendency of increased Fos expression with higher light intensities. (Regression line $y=81.515x-9.822$, $R^2=0.353$) The experimental groups had noticeably higher amounts of Fos induction in the SCN. This was statistically substantiated, as all the experimental groups had values significantly different from the dark control animals (Kruskal-Wallis ANOVA, $p=0.0469$). It is possible that the Fos expression may have reached saturation at ND, in the present conditions of stimulation (Fig. 3.13 & Plate 3.3, also see Appendix A, Table 3.6).

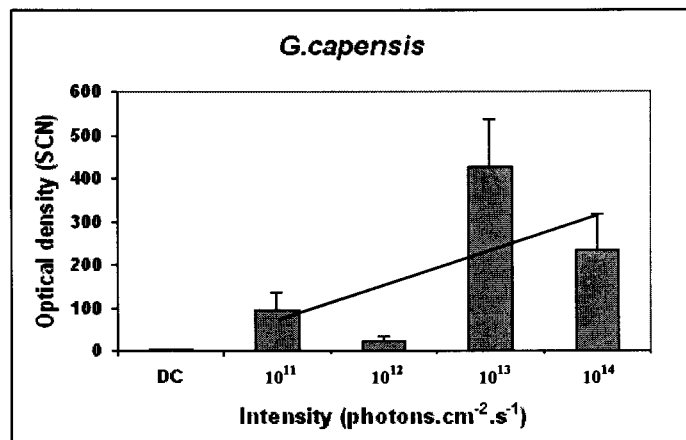


Figure 3.13: Fos expression in the SCN of *Georychus capensis* with increasing light intensities.

b) Common mole-rat (*Cryptomys hottentotus hottentotus*)

The Fos induction in the dark control animals was relatively high in comparison with other species. The Fos expression in response to increasing light intensities was variable. There

was an increase in Fos expression for the irradiances 3.1×10^8 to 3.1×10^9 photons.cm⁻².s⁻¹. Fos induction at intensity 3.1×10^{10} photons.cm⁻².s⁻¹ was very low, followed by an increase in the level of Fos for irradiances from 3.1×10^{11} to 3.1×10^{12} photons.cm⁻².s⁻¹, and a slight decrease for the irradiances of 3.1×10^{13} to 3.1×10^{14} photons.cm⁻².s⁻¹. A slight overall increase in Fos expression was observed with increasing illumination intensity (Regression line $y=346.84x+2493.6$, $R^2=0.151$). The level of Fos was not high in any of the groups, although there was more Fos in the SCN of light stimulated animals than in the dark controls. This difference was not significant in any of the groups (Kruskal-Wallis ANOVA, $p>0.05$) (Fig. 3.14 & Plate 3.4, also see Appendix A, Table 3.7).

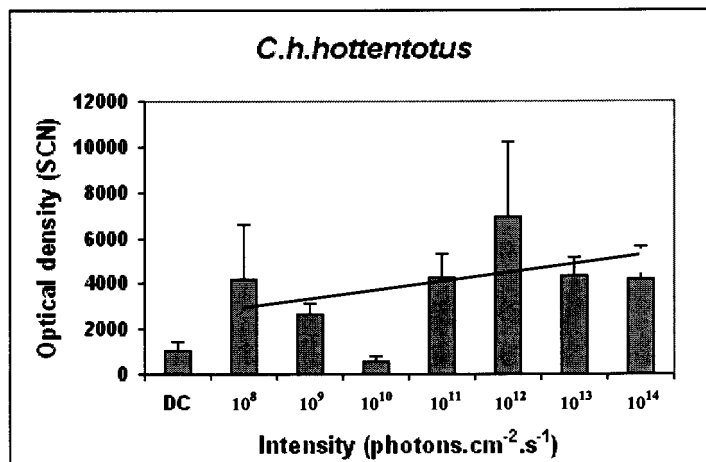


Figure 3.14: Fos expression in the SCN of *Cryptomys hottentotus hottentotus* with increasing light intensities.

c) Damaraland mole-rat (*Cryptomys damarensis*)

Compared to other above ground rodents (mouse, hamster) the level of light induced Fos in the SCN was visually not very dense in either the dark control or the light stimulated animals. The response of Fos induction in the SCN to increasing light intensities was variable, but showed an elevation with intensities from 3.1×10^9 to 3.1×10^{11} photons.cm⁻².s⁻¹, and thereafter decreased at irradiances of 3.1×10^{12} and 3.1×10^{13} photons.cm⁻².s⁻¹, followed by a sharp

increase at an intensity of 3.1×10^{14} photons.cm⁻².s⁻¹. There was an overall increase in Fos induction in response to increased light irradiance (Regression line $y=772.86x-446.03$, $R^2=0.376$). There was no statistical difference between the amount of Fos expression in dark control animals and any of the stimulated groups (Mann-Witney U-test, $p=0.0552$). Visually, there was a relatively large difference between the dark control group and the group with the highest irradiance levels, but it was not statistically different (T-test, $p=0.056$) (Fig. 3.15 & Plate 3.5, also see Appendix A, Table 3.8).

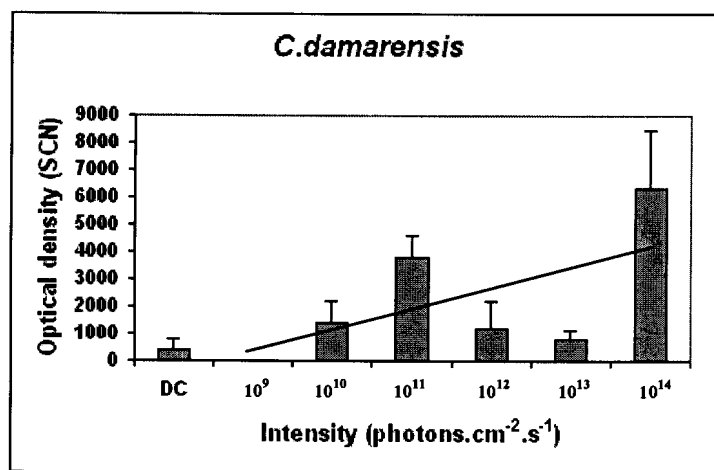


Figure 3.15: Fos expression in *Cryptomys damarensis* with increasing light intensity.

d) Naked mole-rat (*Heterocephalus glaber*)

A large variance in Fos expression of the dark control animals was found. The mean amount of Fos in the dark control animals appeared to be higher than the mean observed in any of the stimulated animals, due to one dark control value. The exclusion of this data point would not shed more light on the interpretation of the data, and was therefore left in the dataset. There was no visual or statistical difference between the dark control group and any of the experimental groups (Kruskal-Wallis ANOVA, $p=0.6861$).

The Fos induction in the light stimulated animals did not appear to exhibit any relation with increasing light intensities. The level of Fos induction remained similar from irradiances of 3.1×10^{14} through to 3.1×10^{10} photons.cm⁻².s⁻¹ (Regression line $y=8.49x+31.49$, $R^2=0.0257$). The amount of Fos induction was overall very low and did not show any pattern (Fig.3.16 & Plate 3.6, also see Appendix A, Table 3.9).

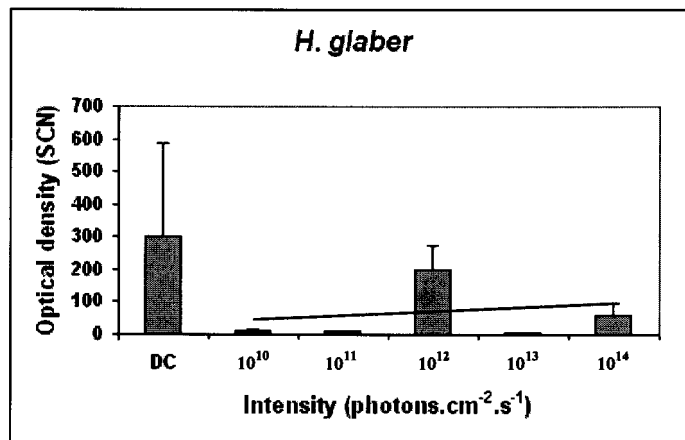


Figure 3.16: Fos expression in *Heterocephalus glaber* with increasing light intensities.

Experiment 3: Variation in Fos induction with different pulse durations at CT16 Highveld mole-rat (*Cryptomys hottentotus pretoriae*)

Fos induction observed in the dark control animals was relatively low. When stimulated, there was a general increase in Fos expression with an increase in pulse duration (Regression line $y=902.44x-534.75$, $R^2=0.705$). The different duration times (1.5, 4.75, 15, 47.5, 90 min) were not significantly different from one another (Kruskal-Wallis ANOVA, $p>0.05$). However, when the separate groups were compared to the dark control animals, the longest duration of 90 minutes was significantly different from the dark controls (Mann-Whitney U-test, $p=0.027$) (Fig. 3.17 & Plate 3.7, also see Appendix A, Table 3.10).

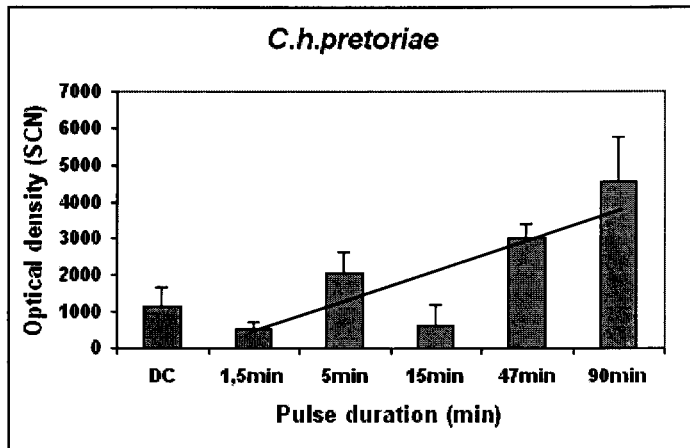


Figure 3.17: Fos expression in the SCN of *Cryptomys hottentotus pretoriae* with different lengths of pulse duration.

Experiment 4: Variation in Fos induction with different temperatures at CT16

a) Mashona mole-rat (*Cryptomys darlingi*)

There was a positive correlation between temperature and Fos induction in this species. The dark control animals had no Fos expression in the SCN, and there was a visible difference in Fos expression for animals in the 15°C, as well as the 27°C group. Comparison between all three groups was statistically different (Mann-Whitney U test, $p=0.0214$). Results of a t-test confirmed a significant difference between the dark control group and the 27°C group (t-test, $p=0.0009$), but no statistical difference between the dark controls and the 15°C group (t-test, $p=0.0739$), and likewise no difference between the two light stimulated groups (t-test, $p=0.0596$) (Fig. 3.18 & Plate 3.8, also see Appendix 3.11).

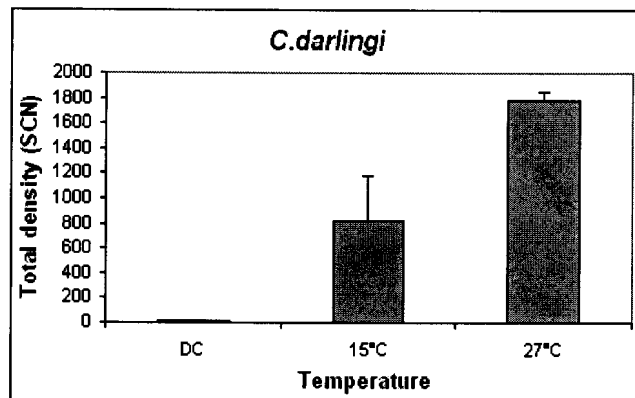


Figure 3.18: Fos expression in the SCN of *Cryptomys darlingi* with different temperatures.

b) Highveld mole-rat (*Cryptomys hottentotus pretoriae*)

The dark control animals, maintained at 27°C, had higher levels of Fos induction in the SCN than the 15°C group, but lower than the 27°C group. However, neither of the two stimulated groups was significantly different from the dark control animals (Mann-Whitney U-test, $p > 0.05$). Fos expression in the animals stimulated at 27°C was significantly higher than animals that were stimulated at 15°C (Mann-Whitney U-test, $p = 0.049$) (Fig. 3.19 & Plate 3.9, also see Appendix A, Table 3.12).

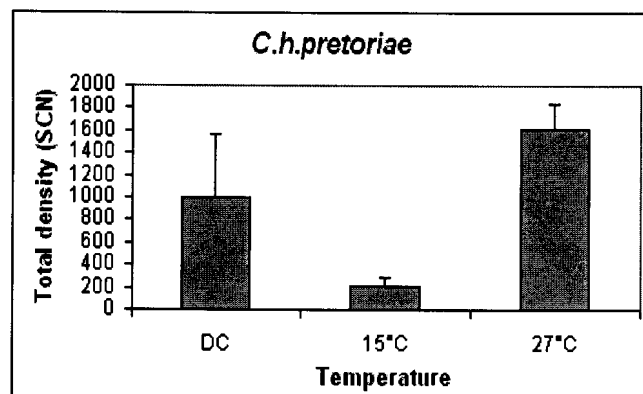


Figure 3.19: Fos expression in *Cryptomys hottentotus pretoriae* with different temperatures.

c) Naked mole-rat (*Heterocephalus glaber*)

The dark control animals of this experiment overlap with those of the intensity study. Thus the dark control values were relatively high, again higher than the experimental readings. The stimulated groups had very low levels of Fos expression in the SCN, the two groups had nearly equal amounts of induced Fos. No statistical difference was found between any of the groups (Kruskal-Wallis ANOVA, $p=0.5283$) (Fig. 2.20 & Plate 3.10, also see Appendix A, Table 3.13).

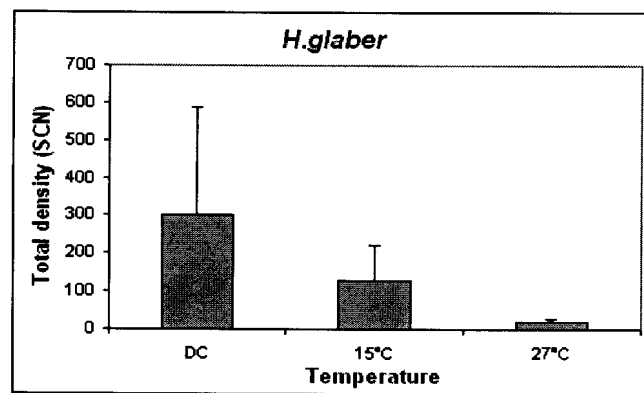


Figure 3.20: Fos expression in the SCN of *H. glaber* with different temperatures.

DISCUSSION

a) C-Fos expression over the circadian cycle

In mammals, photoreceptive pigments have only been identified in the eye. The question as to whether mole-rats can perceive changes in light or not, has arisen since these rodents have a severely regressed visual system. The eyes are minute and despite the anatomically complete retina (Janssen *et al.* 2000), the small size results in a grossly reduced amount of photo pigments.

In addition, mole-rats are not frequently exposed to light, raising the question of circadian rhythmicity and light entrainment in these animals.

Fos expression in both nocturnal and diurnal rodents is dependent on the phase of the circadian clock. In nocturnal and diurnal rodents, photic induction of Fos is low during subjective day, and higher during the subjective night (See Fig.3.1). Fos expression in the SCN in response to light occurs mostly in the ventro-lateral part in neurons that receive innervation from the retina.

The results of this study demonstrate that *Georychus capensis* displays sensitivity towards light changes. *Georychus capensis* has been described as nocturnal (Lovegrove *et al.* 1995), and the results of Fos expression in response to light obtained from this experiment corresponds with that of other rodents, very little Fos expression occurs during the subjective day, the Fos induction in early subjective day is more than that later in the day, and highly elevated levels of Fos expression occurs during subjective night. The number of Fos immunoreactive cells in the SCN was relatively low compared to other rodents. Fos is, like in other aboveground rodents, only expressed during certain times of the circadian cycle. The circadian clock thus gates the circadian response of Fos expression in these animals. Moreover, these findings are in accord with the early immediate gene expression of another solitary mole-rat, *Spalax ehrenbergi*, which displays a similar Fos induction pattern (Tobler *et al.* 1998). Fos expression in the SCN of these solitary mole-rat species also corresponds to that of classical rodent models such as rats (Rusak *et al.* 1992) and hamsters (Kornhauser *et al.* 1990). Thus the Fos expression in *G. capensis* is comparable to that of other rodents, although expressed at a lower intensity.

In contrast with the solitary species, *Cryptomys hottentotus pretoriae* does not appear to be very sensitive to light, the data obtained from this experiment showed no significant difference between stimulated and dark control groups. Accordingly, there was no indication of differential sensitivity to light at different times of the circadian cycle. Compared to other mole-rat species these results resemble those found in *C. h. hottentotus*, *C. damarensis* and *H. glaber*. None of these species displayed a significant difference between any of the stimulated and dark control groups. However, in contrast with *C. h. pretoriae*, when comparing the summated values of subjective day to that of subjective night, the Fos expression in the SCN of the stimulated animals proved to be significantly different from the dark controls in *C. h. hottentotus* and *C. damarensis*. This may be related to the small sample sizes used in the experiments. On the other hand, the

naked mole-rat, *H. glaber* did not exhibit any sensitivity towards light at any time of the day, or reveal evidence of gating by the circadian clock, resembling *C. h. pretoriae* (Negroni 1998).

The lack of response to light in *C. h. pretoriae* can be attributed to a number of factors. Firstly it can be inferred that the photic system is incapable of efficiently transmitting light information to the circadian clock. This could be as a result of malfunctional or absent components inside either the eye or the retinohypothalamic tract leading to the SCN. However, considering that these animals did show a response to light in some of the experiments (discussed later), this argument does not hold. A more plausible alternative could be that the light pulse presented was either lacking in duration or intensity. Considering that the animals in the experiments undertaken by Negroni (1998) received hour-long light pulses, it might be reasonable to assume that the 15-minute pulse presented in this experiment was insufficient to allow proper integration of light information to the SCN.

Therefore, according to these experiments the following can be concluded:

- Light information evidently reaches the SCN of *Georychus capensis* and is sufficient for the induction of Fos protein, and in addition the Fos expression is gated according to the phase of the clock, although statistical analysis of the individual groups does not reflect a significant difference from the dark control group, this is clearly due to the small sample sizes.
- It does not appear as if the SCN of *C. h. pretoriae* effectively integrates light information in response of retinal stimulation at the given duration and intensity of light. Subsequently, no evidence could be found that the circadian clock has any gating effect on the expression of Fos in the SCN.

b) Variation in Fos induction with different light intensities at CT16

The phase response curve provides an indication of when the circadian system is maximally sensitive to light. In nocturnal rodents, light pulses during the early subjective night elicit the highest levels of Fos in the SCN. Likewise, this is also the time of maximum Fos expression in mole-rats, even though the level of expression is not always significantly different from the dark controls. Consequently light pulses were presented at CT16 when investigating the effect of increasing light intensity on the Fos induction in the SCN.

It has been suggested that the additive number of photons reaching the SCN determines the immunoreactive response, thus both the duration and intensity has to be taken into account (Dkissi-Benyahya *et al.* 2000). Therefore, when maintaining a constant duration of light stimuli one would expect the Fos response to increase along with an increase in light intensity.

The solitary species *G. capensis* showed a significant increase in Fos expression in response to greater light intensities. The dark control values of the *G. capensis* were so low that all the experimental conditions proved to be significantly different. Again, the Fos expression in the SCN of the solitary *G. capensis* displays a similar trend to that of other rodents (Kornhauser *et al.* 1990, Rusak *et al.* 1992).

In the social mole-rat, *Cryptomys damarensis*, an overall increase of Fos immunoreactive cells in the SCN was found as the light intensity increased. However, a significant response to light could only be seen at the highest irradiance level presented. In a previous study conducted by Negroni (1998), a lower light intensity elicited significant Fos expression in the SCN of this species. However, the animals in that experiment received a light pulse of one hour, indicating that duration of the stimulation is also an important factor in the response of the SCN to light. Nonetheless, the sample sizes for both experiments were very small, and could also contribute to the difference observed.

Both *C. h. hottentotus* and *H. glaber* demonstrated an increase in Fos induction with increased light intensity, however neither of these species displayed any values significantly different from the dark control animals.

Considering the early subjective day peak in the spontaneous Fos expression rhythm in the SCN, it is possible that light does not have an effect on the Fos expression in the SCN of *H. glaber*. The observed Fos in the SCN could be a result of the spontaneous rhythm or basal levels of Fos, since the expression was very low.

The following conclusions could be deduced from the experiments:

- *Georychus capensis* has a lower threshold for significant Fos induction in the SCN compared to the other mole-rat species in this experiment. This higher sensitivity relates with the results of experiment 1, verifying that this species is more sensitive to light than the other mole-rat species.
- *Cryptomys damarensis* appears to be somewhat more sensitive to light than the other social species, showing a threshold for Fos expression at 1×10^{15} at the given duration of stimulation, at least 3 log units higher than that of *G. capensis*.

- Light stimulation did not have any significant effect in the SCN in the two social species *C. h. hottentotus* and *H. glaber*. It can thus be assumed that the circadian clock within these species is less sensitive to light than the solitary *G. capensis* and the social *C. damarensis*.

The minimum intensity necessary for significant Fos expression in the SCN in nocturnal rodents is very low, around 1×10^8 to 1×10^9 photons.cm⁻².s⁻¹. Diurnal animals require an intensity of 2 or three log units higher. The results of this set of experiments vary according to species, but it demonstrates that in general, higher light intensities induce a greater level of Fos expression in the SCN of mole-rats. All of the species except *H. glaber* exhibited an overall increase in Fos induction with increasing light intensities.

c) Fos expression with different light pulse durations

The light pulse was presented at CT16 as the highest Fos levels are expressed in the SCN at this time. Since both intensity and duration are important in the eventual level of Fos expression, the effect of increasing pulse length on *C. h. pretoriae* was investigated. Again if the additive effect of intensity and duration determines the level of Fos expression in the SCN, the Fos induction should increase with prolonged duration of stimulation if the intensity is maintained at a stable level. These results illustrate that only very long pulse durations have a significantly larger effect on the Fos expression in the SCN of *C. h. pretoriae*.

In this experiment, the respective pulse durations were not significantly different from one another, and with exception of the longest duration (90 minutes), neither did it differ from the dark control group. Comparing data from *C. h. pretoriae* to that of *C. damarensis* (Negroni, 1998), the circadian clock of the latter species appears to be more sensitive to light. Like *C.h.pretoriae*, the dark stimulated groups did not differ significantly from each other. The dark control group is significantly different from all experimental conditions.

It is therefore clear that only very long durations of light stimulation result in significant Fos expression in the SCN of *C. h. pretoriae*. Most likely this occurrence is responsible for the lack of sensitivity observed in the previous experiments. This supports the notion that the circadian clock of solitary species is more capable of integrating light information, and has a lower threshold for response to light stimuli.

d) Fos expression at different ambient temperatures

Although light serves as the primary entraining factor in most animals, other stimuli such as temperature may also be used as an entraining agent. Several species are known to be able to entrain their locomotor activity to temperature cycles (Goldman *et al.* 1997, Liu *et al.* 1998, Pohl 1998). The solitary mole-rat *Spalax ehrenbergi*, exhibits entrainment to temperature cycles (Goldman *et al.* 1997). Though the experimental conditions were somewhat exaggerated from what is experienced in field conditions, this experiment did reveal that temperature entrainment is possible in this species.

According to these results, it appears that temperature has an effect on the light induced Fos expression in the SCN only in those species that show sensitivity to light as well. In *Cryptomys darlingi*, there was a visual difference in Fos expression between the dark control group and both the stimulated groups, but this was not confirmed statistically.

Cryptomys h. pretoriae displayed very interesting results. Both the dark control (27°C) and the stimulated group at 27°C had a higher amount of Fos induction in the SCN than the stimulated animals at 15°C. This suggests that temperature has a relatively dramatic effect on the Fos expression in the SCN. It appears that a higher ambient temperature induces greater amounts of Fos expression in the SCN. Nevertheless, a control group at 15°C and an increased sample size are ideally needed for a complete picture, without which it is difficult to say anything conclusive about the results.

Heterocephalus glaber did not exhibit visible Fos expression in the SCN in dark control animals or either of the experimental groups. Unlike the other mole-rat species, the naked mole-rat is a heterothermic animal, its body temperature possessing similar values to ambient below 26°C. Given that it has been shown that other heterothermic animals display differences in circadian period length at different ambient temperatures (Freeman & Zucker 2000), it would be expected that this would hold true for the naked mole-rat. Since this species did not show prominent Fos expression in previous experiments in response to light, where animals were also maintained at 27°C, and other species proved to express less Fos at lower ambient temperatures, these results are not totally unexpected.

Certain populations of SCN cells are known to be sensitive to temperature, they exhibit an increase in firing rate in response to elevated temperatures (Burgoon & Boulant 2001). From

certain results of this study (*C. h. pretoriae*) it seems possible that higher temperatures result in a higher Fos expression in the SCN.

Since homeothermic animals maintain a stable body temperature, it is likely that it is not necessary for the SCN to be temperature compensated as it is 'temperature buffered' from a near constant body temperature (Burgoon & Bowant 1998). Studies have also shown that there is a difference in sensitivity of the SCN towards temperature depending on the different life histories of animals. Temperature has a much larger effect on the SCN firing rate in rats with a stable body temperature than on ground squirrels that hibernate (Ruby & Heller 1996). Since mole-rats are neither hibernators nor are exposed to rapid and large temperature changes, the SCN is less likely to be overtly sensitive to temperature, whether it be for temperature compensation or temperature entrainment. Daily temperature fluctuations in the tunnels are also relatively small in comparison with above ground (Bennett *et al.* 1988), a further reason for temperature insensitivity in the SCN.

On the one hand it seems reasonable to assume that temperature could play a more significant role in the entrainment of subterranean species than in other animals as the reductive features of the eye clearly indicates that light is not extremely important factor in the mole-rats and they are not frequently exposed to it. On the other hand, it can be argued that mole-rats are also not exposed to large daily temperature fluctuations (Bennett *et al.* 1988), significant changes in temperature are seasonal. For most of these species seasonal changes are important as some inhabit arid areas and only certain seasons are suitable to extend tunnel systems etc., while others are seasonal breeders and need to find breeding partners.

There is thus no reason to believe that these animals should be more sensitive to temperature than light. Studies on the temperature sensitivity of the circadian system of *Spalax ehrenbergi* indicate that although this species is able to entrain to circadian temperature cycles, a larger proportion of animals are entraining to light stimulation (Goldman *et al.* 1997). It also suggests that light is still the primary zeitgeber for entrainment in mole-rats despite the large reduction in the visual system.

These studies at different ambient temperatures were pilot studies to determine whether further investigation would be meaningful. Since the sample sizes were very small, nothing conclusive can be said at this point. Two of the species showed promising results, however the sample sizes would have to be increased for statistical purposes.

Taken together, these findings support a hypothesis proposing that social mole-rat species tend to be less sensitive to light than solitary species. The Fos induction over the circadian cycle of the solitary species investigated was comparable to that of other solitary mole-rats species as well as aboveground rodents. In contrast it appears as if the circadian clock of social species does not integrate light efficiently (Fig. 3.21).

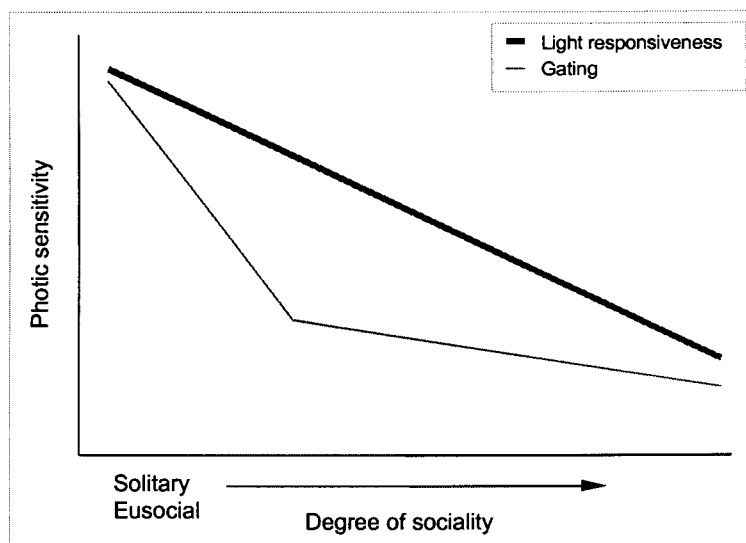


Figure 3.21: Schematic representation of the decline in photic sensitivity in the different mole-rat species

This trend was also observed in the remainder of the experiments. All the mole-rat species except *H. glaber* presented a general increase in responsiveness of immunoreactive cells in the SCN in reaction to increased light, but the slope of the increase decreases with increasing social organization. A longer pulse duration renders a higher Fos response in social mole-rat species, possibly indicating that these species requires a longer integration time for Fos responses.

Lower ambient temperatures result in a reduction of the Fos expression in the SCN of some mole-rats, however, this response also declines with increasing sociality.

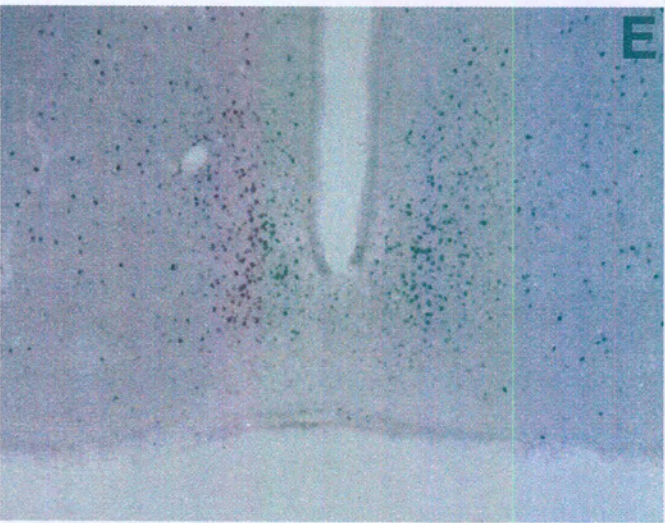
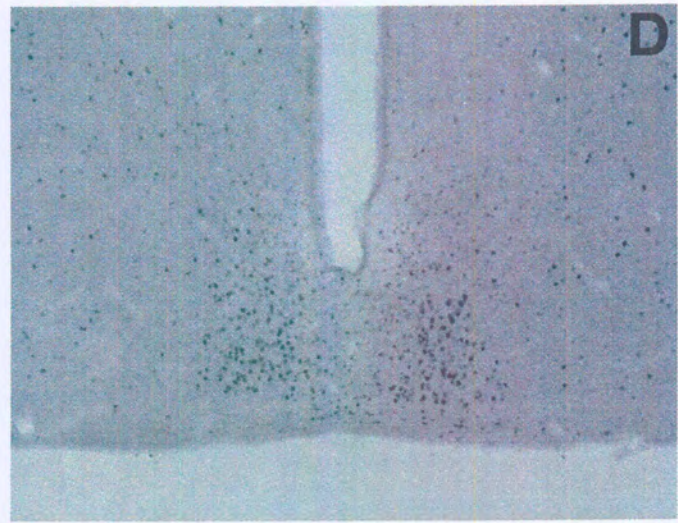
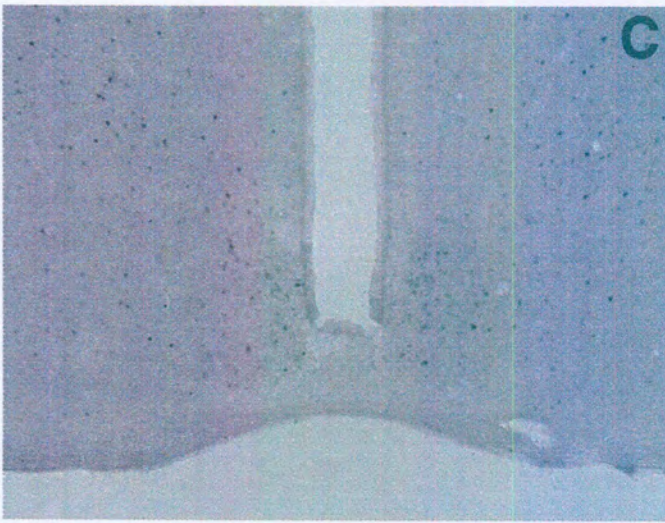
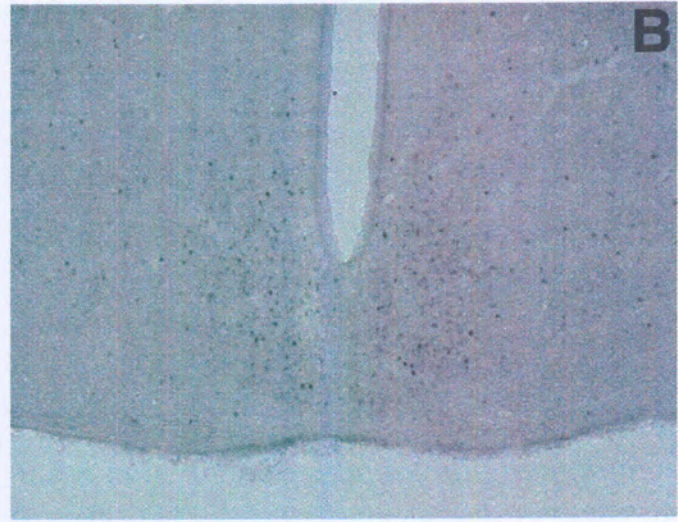
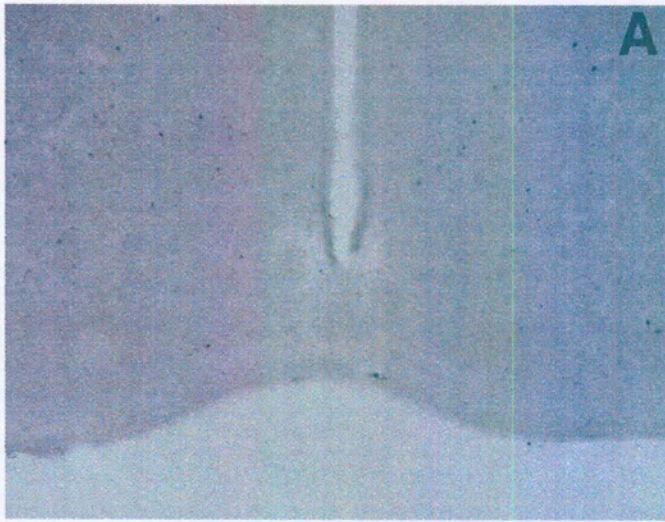


Plate 3.1: Fos induction in the SCN of *Georychus capensis* during different circadian times.

A - Dark control, B - CT4, C - CT10, D - CT16, E - CT22.

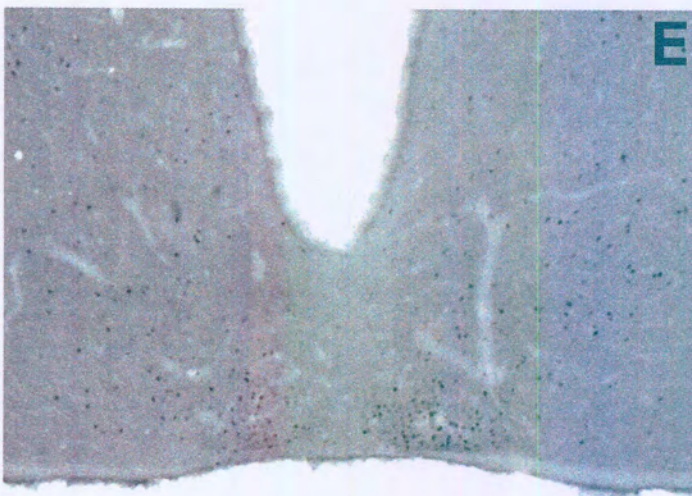
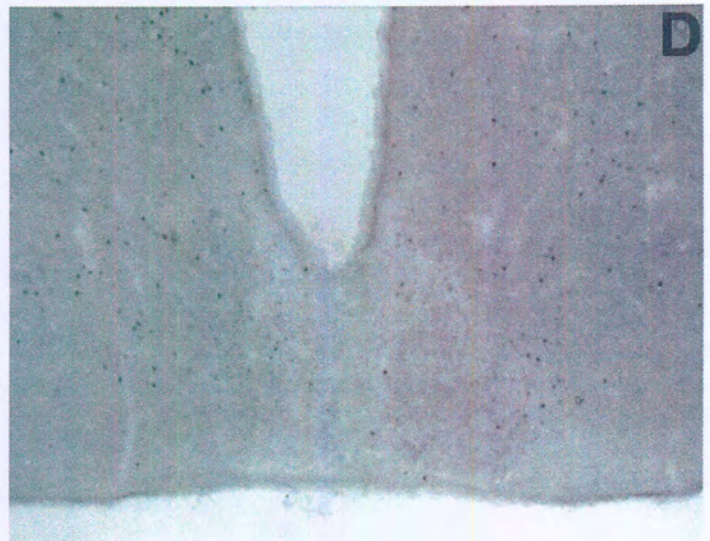
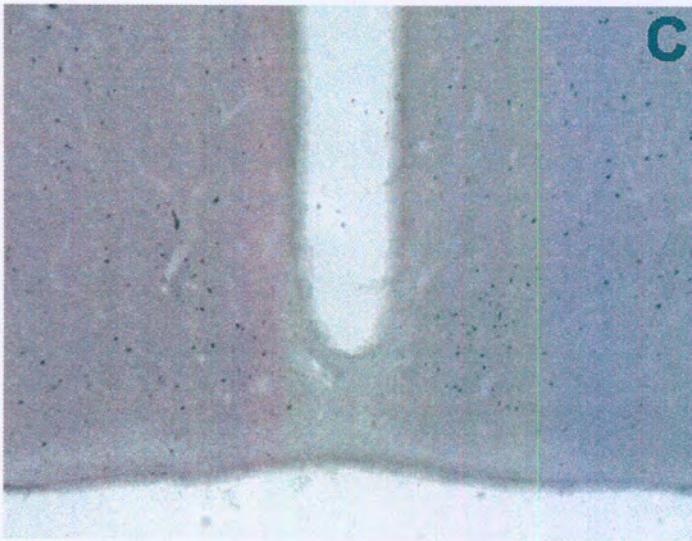
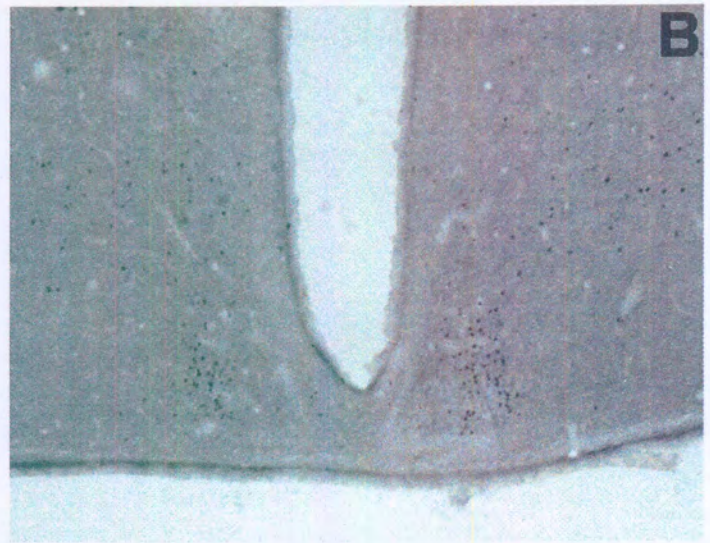
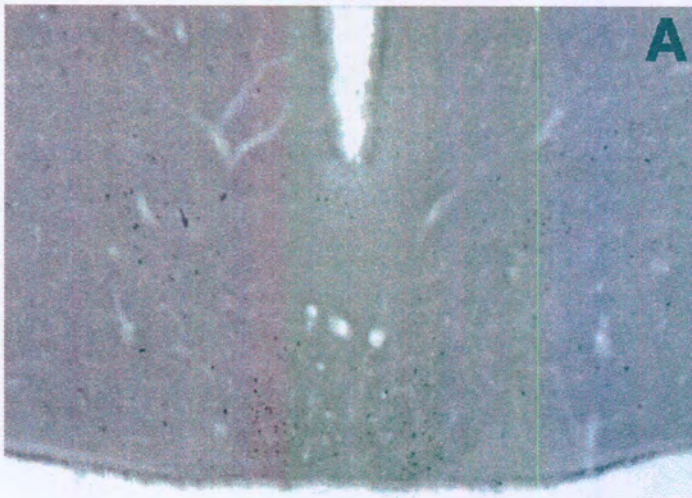


Plate 3.2: Fos induction in the SCN of *Cryptomys hottentotus pretoriae* during different circadian times.

A - Dark control, B - CT4, C - CT10, D - Ct16, E - CT22.

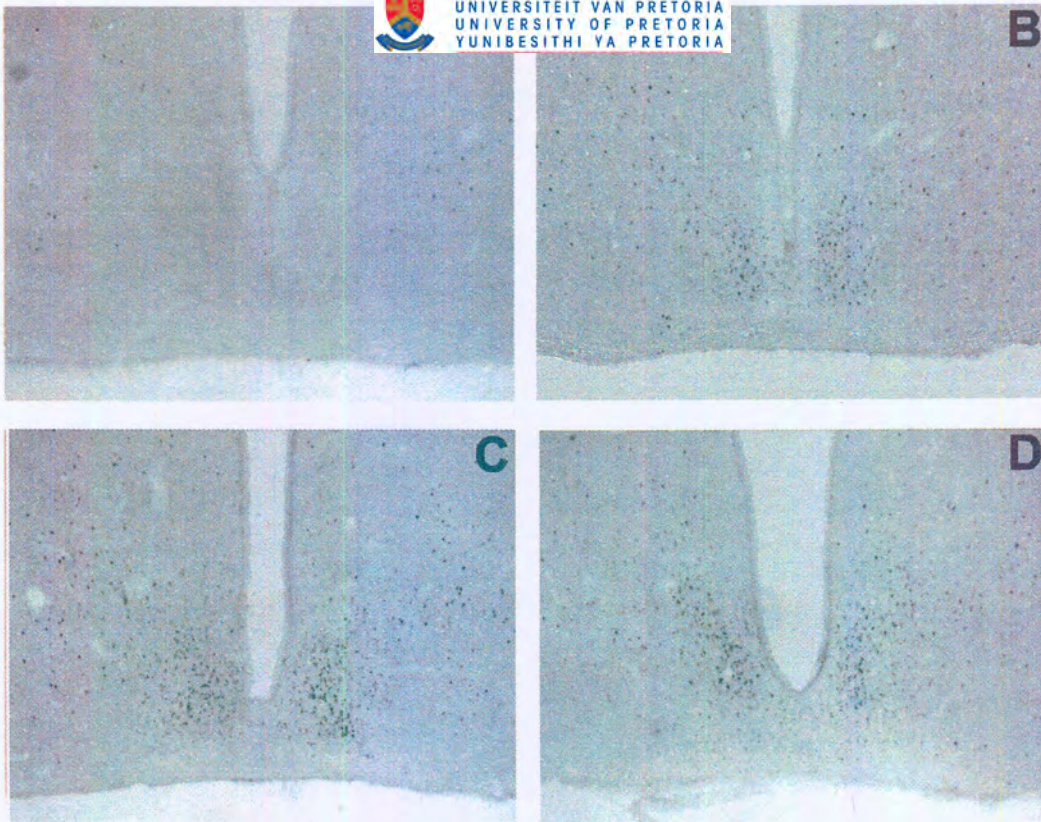


Plate 3.3: Fos induction in the SCN of *Georychus capensis* with different light intensities.

A - Dark control, B - 3.1×10^{11} photons/cm²/sec, C - 3.1×10^{13} photons/cm²/sec, D - 3.1×10^{14} photons/cm²/sec.

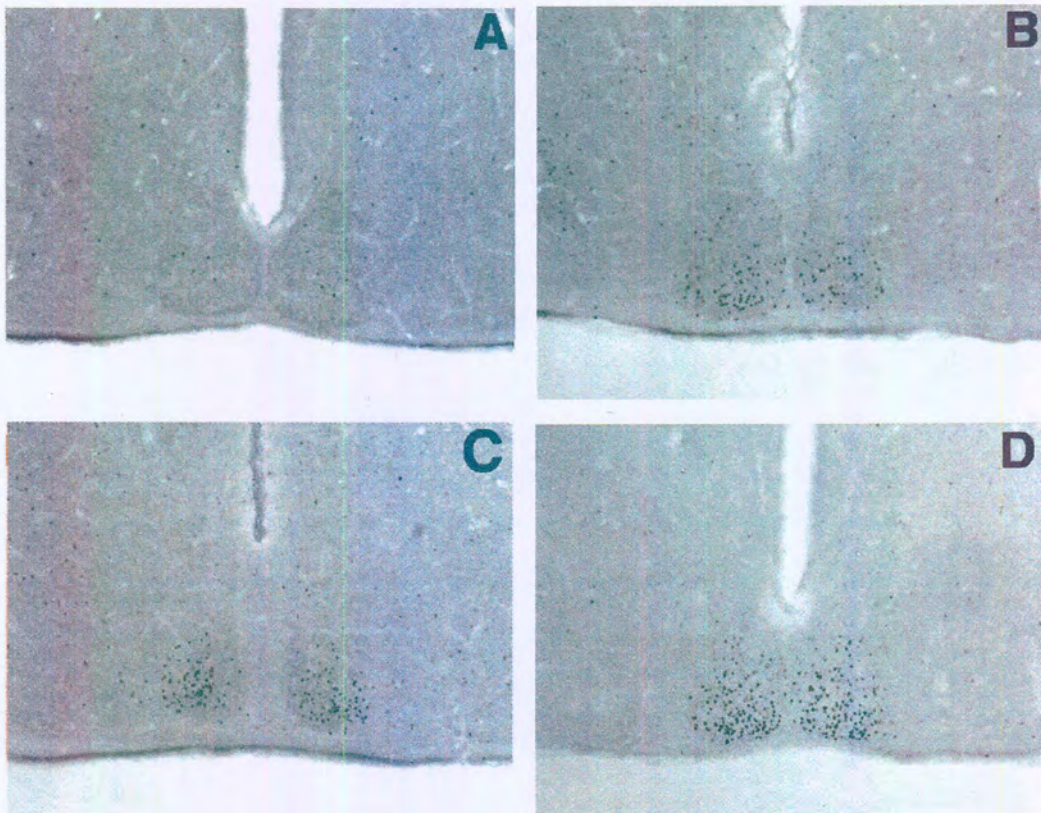


Plate 3.4: Fos induction in the SCN of *Cryptomys hottentotus hottentotus* with different light intensities.

A - Dark control, B - 3.1×10^9 photons/cm²/sec, C - 3.1×10^{10} photons/cm²/sec, D - 3.1×10^{13} photons/cm²/sec.

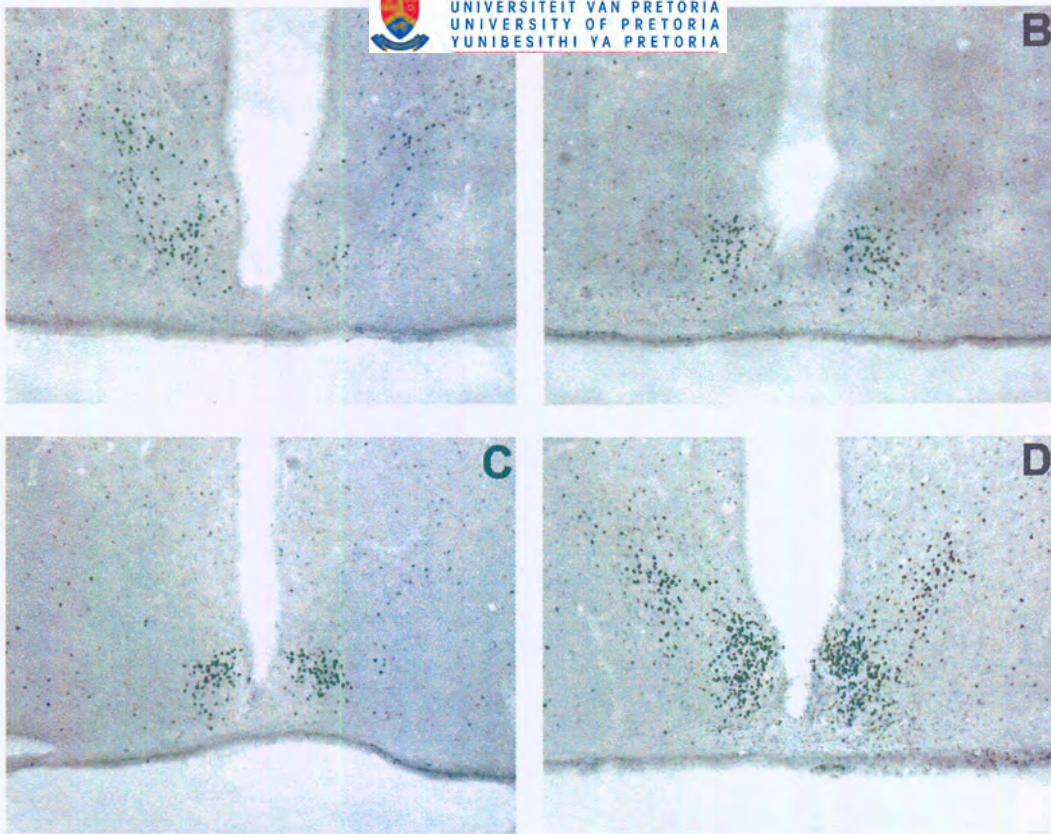


Plate 3.5: Fos induction in the SCN of *Cryptomys damarensis* with different light intensities.

A - Dark control, B - 3.1×10^{10} photons/cm²/sec, C - 3.1×10^{12} photons/cm²/sec, D - 3.1×10^{14} photons/cm²/sec.

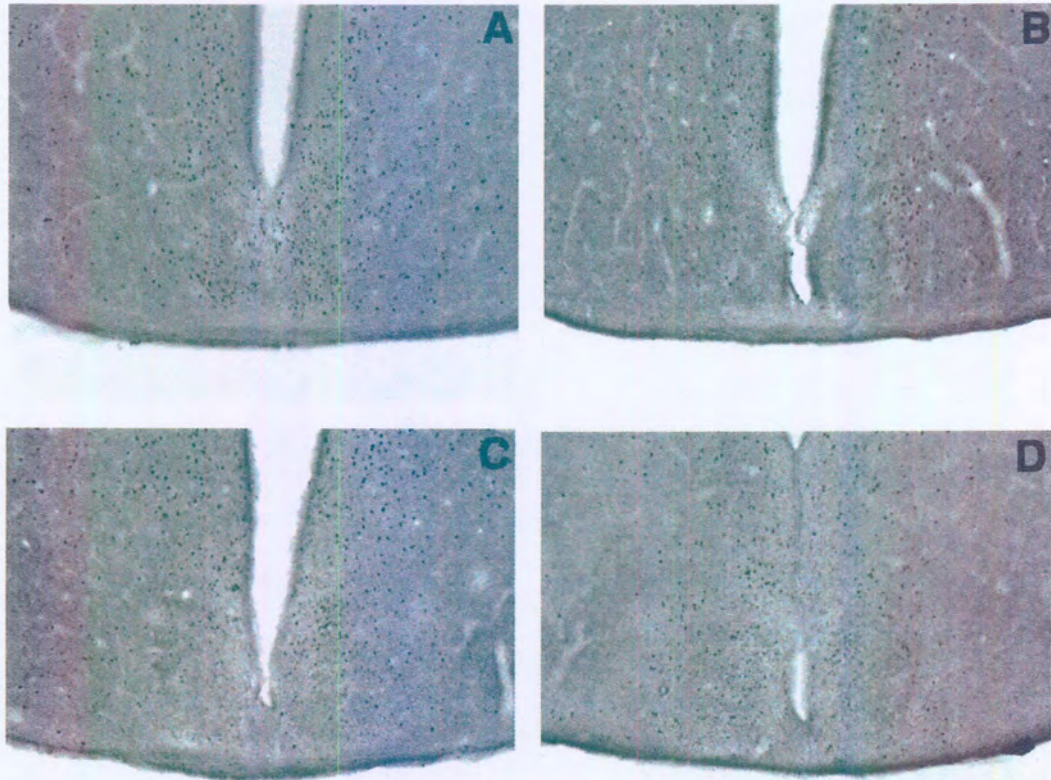


Plate 3.6: Fos induction in the SCN of *Heterocephalus glaber* with different light intensities.

A - Dark control, B - 3.1×10^{10} photons/cm²/sec, C - 3.1×10^{12} photons/cm²/sec, D - 3.1×10^{14} photons/cm²/sec.

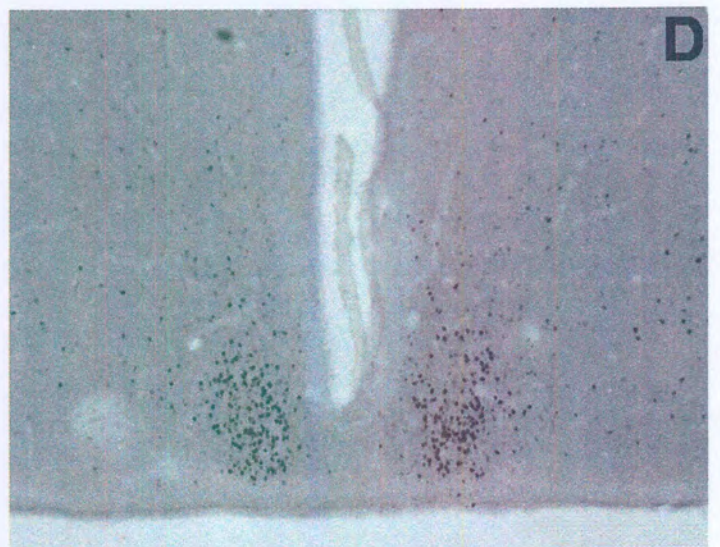
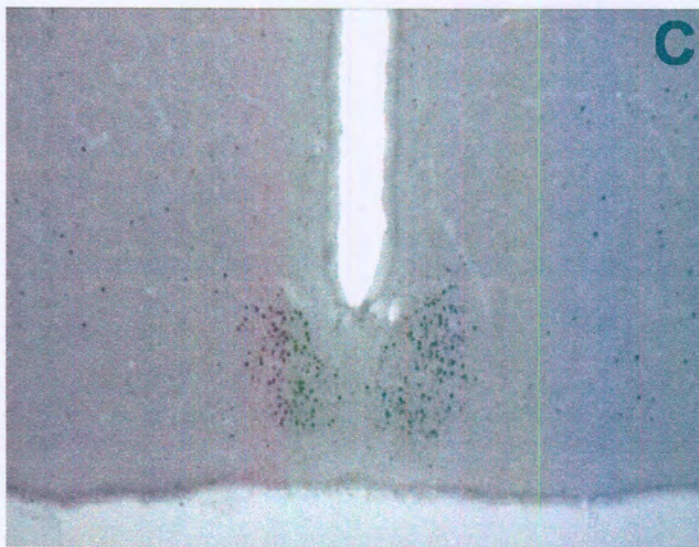
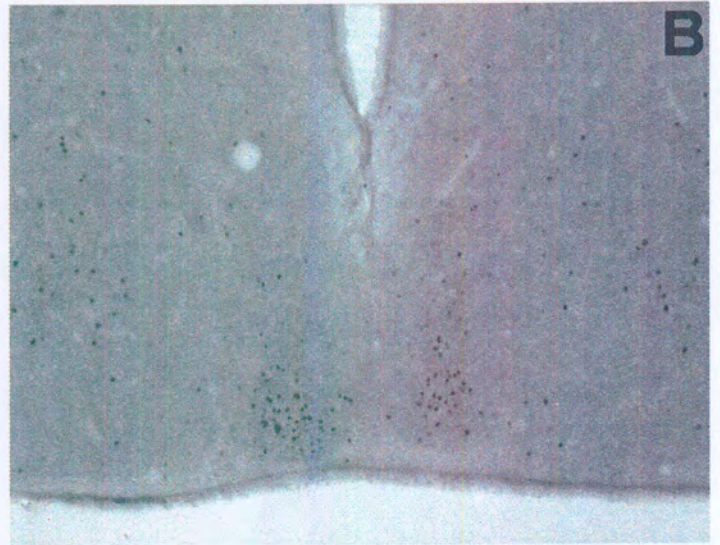
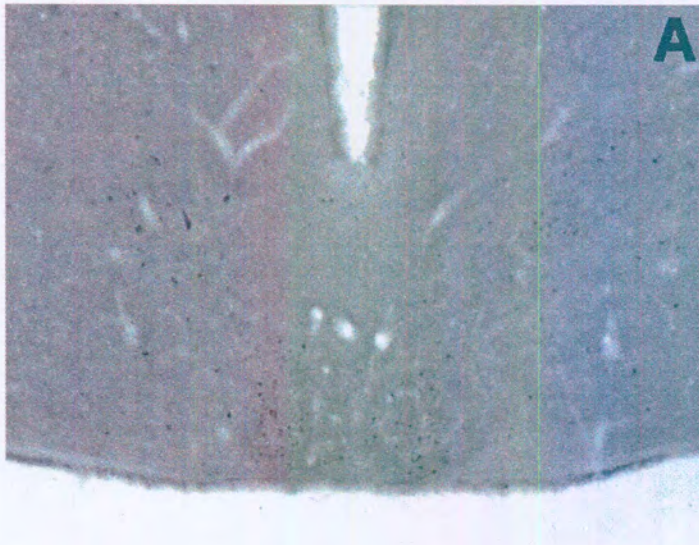


Plate 3.7: Fos induction in the SCN of *Cryptomys hottentotus pretoriae* with constant light intensity and different pulse duration.

A - Dark control, B - 1.5 minutes at 3.1×10^{14} photons/cm²/sec, C - 47 minutes at 3.1×10^{14} photons/cm²/sec, D - 90 minutes at 3.1×10^{14} photons/cm²/sec.

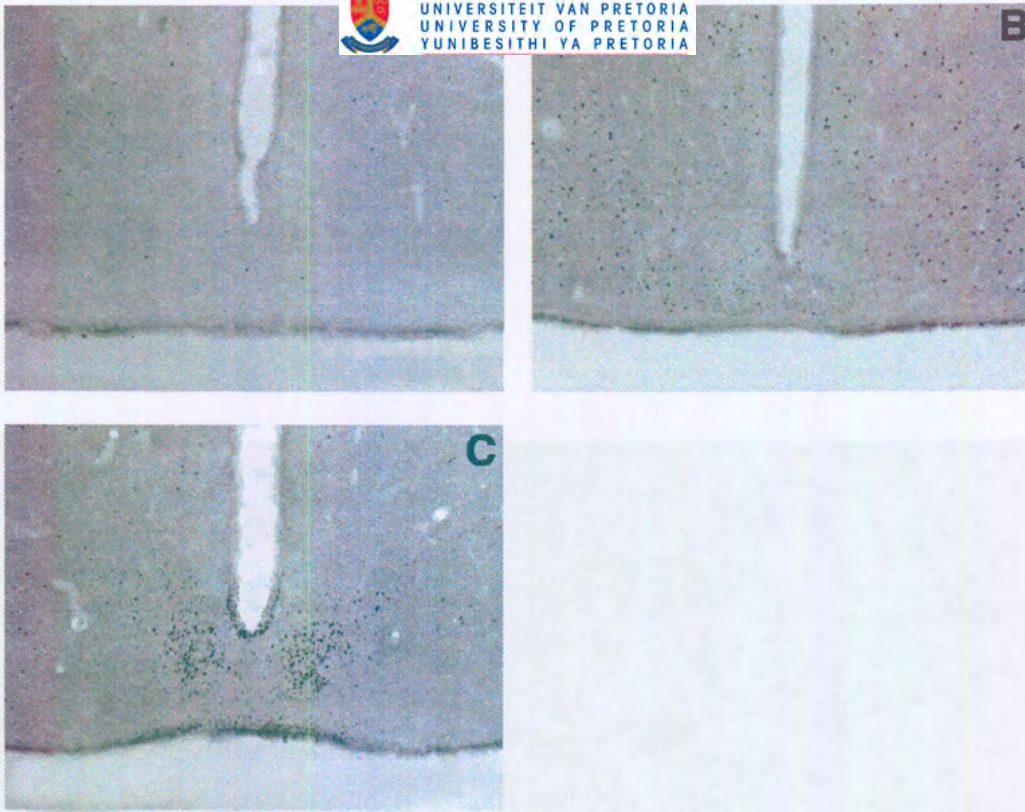


Plate 3.8: Fos induction in the SCN of *Cryptomys darlingi* at constant light intensity but different temperatures.

A - Dark control at 27°C, B - 15°C at 3.1×10^{14} photons/cm²/sec,
C - 27°C at 3.1×10^{14} photons/cm²/sec.

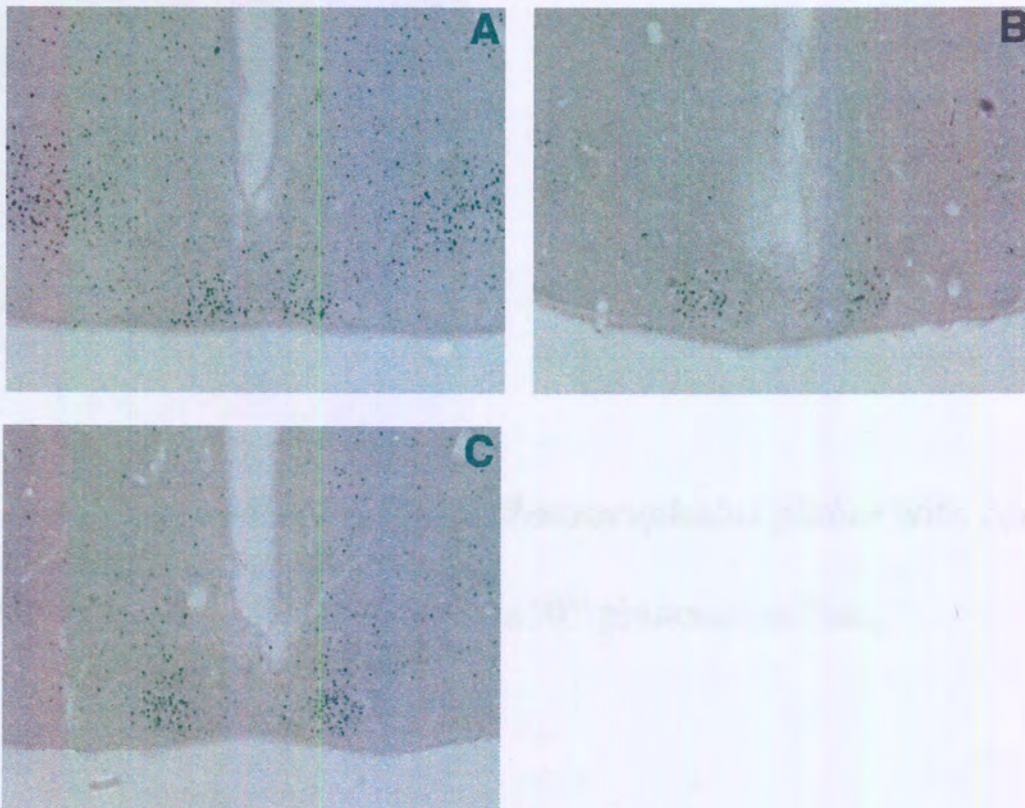


Plate 3.9: Fos induction in the SCN of *Cryptomys hottentotus pretoriae* with constant light intensity but different temperatures.

A - Dark control at 27°C, B - 15°C at 3.1×10^{14} photons/cm²/sec,
C - 27°C at 3.1×10^{14} photons/cm²/sec.

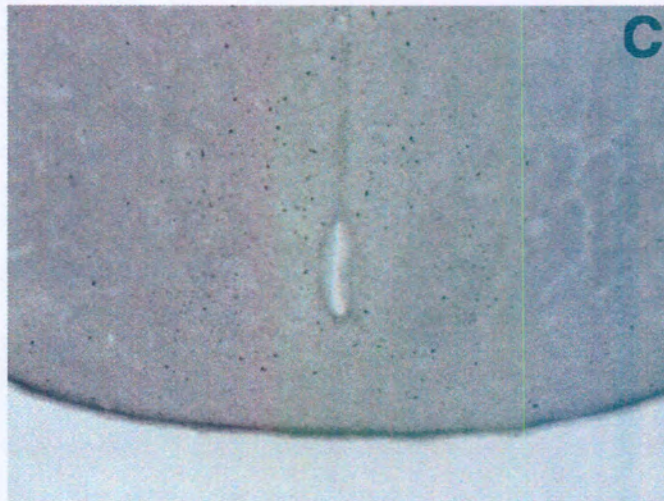
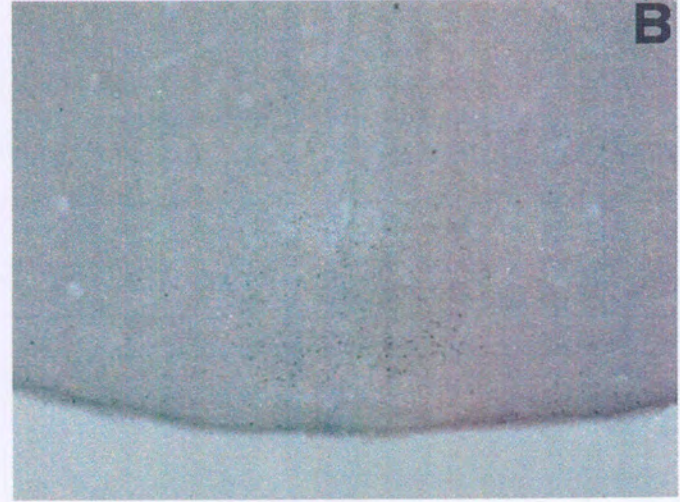
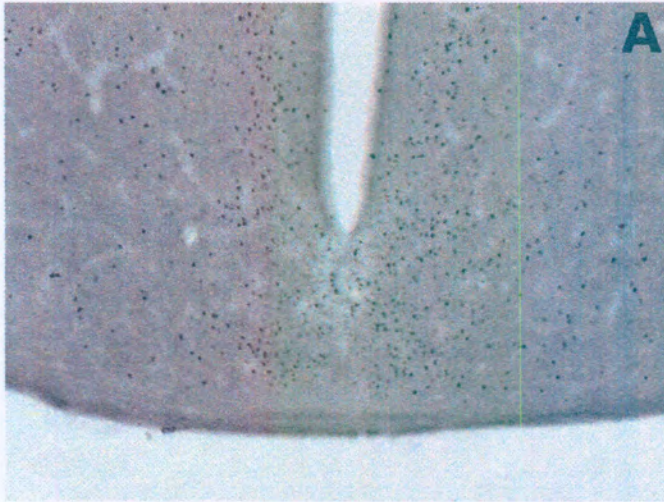


Plate 3.10: Fos induction in the SCN of *Heterocephalus glaber* with constant light intensity and different temperatures.

A - Dark control at 27°C, B - 15°C at 3.1×10^{14} photons/cm²/sec,

C - 27°C at 3.1×10^{14} photons/cm²/sec.

**CHAPTER 4: LOCOMOTOR ACTIVITY RHYTHMS
IN AFRICAN MOLE-RATS**

LOCOMOTOR ACTIVITY RHYTHMS IN AFRICAN MOLE-RATS

Circadian rhythms are present in all mammals and circadian rhythmicity is encountered in a wide variety of biochemical, physiological and behavioural processes. It has been suggested that circadian rhythms have developed as an adaptive feature to improve survival by allowing the organism to anticipate changes in environmental conditions. In order to avoid competition and predation, the activities of animals are distributed unevenly over time and space. Behavioural activity patterns of most species can be classified as nocturnal, diurnal or crepuscular depending on the time of day when they are active. Nocturnal animals concentrate the largest proportion of their activity during the night time, diurnal animals during the day, whereas crepuscular animals exhibit two bouts of activity around dawn and dusk. Animals exhibit nocturnal or diurnal activity rhythms as a result of two processes, entrainment and masking. An expressed rhythm is often the product of both masking and entraining effects.

Masking and entrainment

Masking is a direct response to light on behaviour since it does not involve the circadian pacemaker. Animals in which the SCN has been lesioned, can still regulate their activity according to light and dark patterns in the environmental cycle, however, they no longer have the ability to express endogenous rhythms in constant conditions (Ibuka *et al.* 1977, Mosko & Moore 1979).

In essence masking is a change in activity in response to a light or dark pulse. Masking can be classified as either negative or positive, according to the direction of the effect. Positive masking results in an increase in activity in response to light in a diurnal species, and dark in a nocturnal species. Negative masking describes a decrease in activity as a result of dark in a diurnal species and a similar effect of light in a nocturnal species. Masking can obscure clock-controlled responses, hence it is important to distinguish between masking and entrainment in activity studies. Unlike entrainment, masking does not cause a permanent alteration of activity. The use of constant lighting conditions and skeleton photoperiods are therefore appropriate methods to eliminate the effects of masking (Mrosovsky 1999).

In contrast to masking, entrainment has an indirect response on behaviour since it is the phase of the clock that is first altered prior to the affect on behaviour (Mrosovsky *et al.* 1999). Entrainment causes permanent alterations in activity involving long-term cellular and molecular changes. Entrainment takes place by means of shifts in the phase and changes in period of circadian rhythms in response to external cues. Phase shifting can take place via a variety of photic and non-photoc stimuli. Of the three major projections that reach the SCN, one is of photic origin whereas the remaining two are both photic and non-photoc.

Photic entrainment

The daily alternating cycle of light and dark is the primary environmental cue whereby organisms synchronize their circadian rhythms. Photic information reaches the circadian pacemaker from the eye via the retinohypothalamic tract (RHT), the main photic pathway.

By experimentally altering the phase of the photoperiod, the effects of the circadian pacemaker can be determined. The magnitude and direction of a phase shift in photoperiod determines whether and how quickly an organism will re-entrain to a shifted photo cycle. It appears that animals are more likely to re-entrain to a large phase delay than a phase advance and the larger the phase shift, the longer the animals take to re-entrain (Ruby *et al.* 1998).

Photic entrainment is influenced by the afferent fibers from the intergeniculate nucleus (IGL) and raphe nuclei. Although these innervations are not essential for photic entrainment, the IGL affects the rate of entrainment and the raphe nuclei modulate the phase angle of entrainment.

Activity patterns of different species

Mammals entrain their activity rhythms to environmental light/dark cycles by means of phase shifting of the circadian clock. Hence the twilight zones, the transition periods between day and night, are important modulators for entrainment. Since the circadian pacemaker gates the response to light, phase shifts generally only take place during subjective night. These behavioural activity rhythms are closely related to immediate gene expression in the circadian clock.

When light stimulation is presented together with a non-photic stimulus, the two stimuli usually influence one another's phase shifting effect. Behaviour (non-photic) suppresses neuronal activity at every phase of the circadian cycle. Neuronal activity exhibits a circadian rhythm with high firing rate during the day and a lower firing rate during the night. The magnitude of this suppression shows circadian variation with greater suppression occurring during the subjective day. Light pulses oppose the suppression of neuronal activity. The resulting discharge level is a summation of the two responses (Schaap & Meijer 2001).

Under constant conditions the activity rhythms of mammals free-run according to their own endogenous clock. Constant light has a lengthening effect on the free-running rhythm, and prolonged periods under LL may cause splitting of the activity rhythm, although this is only noticed in a small portion of the population.

Activity patterns in mole rats

Since mole-rats are exposed to minimal changes of the environment and have a severe degeneration of the visual system, early investigations reported that these animals were insensitive to light. However, more recently a number of mole rat species have been investigated, and all of them have revealed evidence of expressing circadian rhythmicity (Bennett 1992, Lovegrove *et al.* 1993, Lovegrove & Papenfus 1995, Goldman *et al.* 1997, Tobler *et al.* 1998). Most of these studies have investigated locomotor activity rhythms and found indications of an ability to entrain to light cycles, although the results vary somewhat.

Goldman *et al.* (1997) found that the Mediterranean mole rat *Spalax ehrenbergi* generally exhibited diurnal activity patterns. Some mole-rats that at first displayed nocturnal activity, later changed their activity to diurnal (Tobler *et al.* 1998). Goldman *et al.* (1997) reported that 20% of the mole-rats in their study were arrhythmic, whereas the remainder of the animals displayed circadian fluctuations of which between 40-50% had robust rhythms. Activity patterns were mostly monophasic. Some of the animals with robust rhythms were also able to entrain to temperature cycles. Thus these animals are able to entrain their activity to light dark cycles and exhibit free-running rhythms under constant conditions.

Lovegrove *et al.* (1995) showed, the solitary mole-rat, *Georychus capensis* from Natal is able to entrain to light cycles and displays nocturnal locomotor activity. This species clearly

perceives light under constant lighting conditions and exhibits free running rhythms. In constant light some mole-rats also revealed evidence of splitting of the activity pattern, a phenomena that sometimes occurs under prolonged constant conditions.

Investigating activity patterns in singly housed common mole-rats, *Cryptomys hottentotus*, Hickman (1980) found this species to exhibit a larger proportion of activity during the night, although there was not a large difference in the total amount of activity displayed during the day and night. A colony of *C. h. hottentotus* did not appear to exhibit a well defined nocturnal or diurnal locomotor activity pattern. Recordings of individuals in the colony confirmed this observation (Bennett 1992).

Entire colonies of *C. damarensis* were found to exhibit activity rhythms (Lovegrove 1993). In contrast with the other social species of mole rats, this species exhibited diurnal patterns in locomotor and feeding behaviour. It is, however, not clear whether all the individual animals expressed circadian rhythmicity, or whether only some animals within the colony showed rhythmic behaviour, which may act as a social cue for the rest of the group to become active.

Riccio & Goldman (2000) showed that approximately 65% of naked mole rats, *Heterocephalus glaber*, exhibited wheel running activity with mostly nocturnal locomotor patterns. Of the running animals, two thirds entrained to the LD cycle, whereas the rest were arrhythmic or exhibited free-running activity. Most of the mole-rats shifted their activity according to changes in the LD cycle, and when presented with a 1 hour light pulse in constant darkness, 65% entrained to the light schedule, but none entrained to a 23 hour light cycle. All animals free ran when maintained in constant conditions.

The ability to entrain to environmental signals supports the notion that these animals must possess functional photoreceptors in the retina as well as a complete and functional retinohypothalamic tract. The presence of endogenous rhythms under constant conditions confirms the ability of the circadian clock to generate rhythms. Although there is considerable variation amongst the individuals of the different species, all display some degree of rhythmicity.

Objectives of this chapter

Studies have been performed on a number of fossorial mammals such as mole-rats, ground squirrels, and mole lemmings. The majority of these animals displayed circadian

rhythmicity, as well as endogenous rhythms under constant conditions. Given the lower responsiveness to light in the suprachiasmatic nucleus of mole-rats compared to other rodents, the experiments in this chapter were designed to investigate:

- Whether solitary and social species of African mole-rats display circadian rhythmicity by entraining their circadian activity to light cycles;
- whether this rhythmicity is maintained under constant conditions;
- and to determine whether activity is altered when light/dark regimes are changed.

Both seasonally and aseasonally breeding social species with different life histories were used in the experiments to enable comparison between the social species as well as the solitary species.

MATERIAL AND METHODS

Animal maintenance

Experimental animals were either freshly caught (*Georychus capensis* and *Cryptomys hottentotus pretoriae*) or existing wild caught and subsequent laboratory maintained animals (*Cryptomys damarensis*). The animals were housed singularly in containers (60x30x30cm), which were provisioned with wood shavings. The cages were fitted with Perspex divisions to create topside-open tunnels and 15x15cm closed nest boxes (Fig. 4.1).

Cages were cleaned once every 2 weeks during light cycles, and once a month during DD cycles. Animals were fed on chopped sweet potatoes, carrots, gem squash and apples once a day at random times. An additional annex was constructed in front of the climate room to prevent light from entering the experimental chamber during feeding times. Animals were maintained in temperature and light controlled chambers at 26°C. The general health of the animals was monitored during feeding and cleaning times.

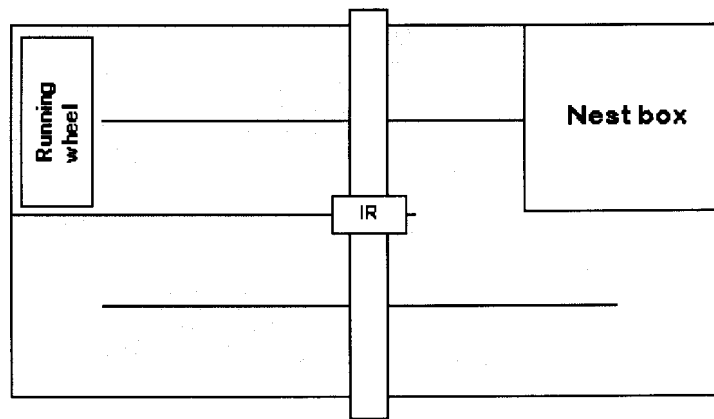


Figure 4.1: Schematic representation of the cage setup with either e-mitter, running wheel or passive infra red captor (IR).

Three containers were placed on energized receivers covering the whole surface of the cage, to receive signals from implanted e-mitters within the mole-rats, 4 containers were fitted with running wheels with magnetic switches and 4 containers with infra red detectors fitted in the middle of the container on a fixed platform such that the range covered the whole width of the cage and the animals had to pass underneath to reach the food or the nesting chamber. Cages were spaced out under a light source of approximately 500 lux (Fig. 4.2).

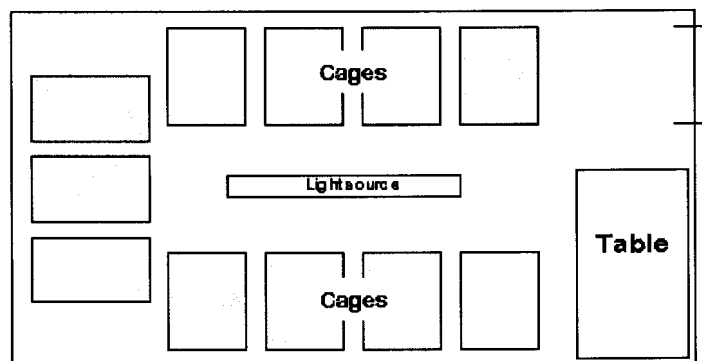


Figure 4.2: Schematic representation of the cage layout in the climate room.

Behavioural recordings were done in Pretoria, South Africa. Activity was measured once a minute, and the actograms were double plotted to facilitate visualization of rhythms.

Experimental protocol

Experiment 1: Entrainment and endogenous rhythms of *Georychus capensis*.

Prior to experimentation, animals were maintained on a 12L:12D light cycle for approximately 8 weeks (Lights on at 08h00, lights off at 20h00). The e-mitters were implanted 12 weeks prior to recordings. The solitary species, *Georychus capensis* was maintained on a 12L:12D cycle and a constant temperature of 26°C for approximately 10 days, until a clear on and offset were observed in the activity rhythm. This was done to investigate whether this species was able to entrain to a light cycle. Once the animals were entrained to the cycle, the light regime was changed to constant darkness (DD). This was maintained for approximately 14 days to investigate the endogenous rhythm within the animal. The third part of the experiment involved re-entraining the animals to the previous 12L:12D light cycle for 7 days. During this stage, four extra mole-rats were coupled to the system, with running wheels. The light cycle was then shifted 6 hours forward (Lights on at 14h00, lights off at 02h00). Animals were kept on this cycle for approximately a month to observe phase shifts to the new light cycle. The next step was to change the light cycle 9 hours backwards and simultaneously shorten the night part to 8 hours and consequently lengthen the day period to 16 hours to investigate whether the activity would follow this change in lighting conditions (Lights on at 05h00, lights off at 21h00). This cycle was maintained for about a month. The final part of the experiment was to inverse the previous light schedule, resulting in a 16 hour night and 8 hour day (Lights on at 21h00, lights off at 05h00) (Fig. 4.3).

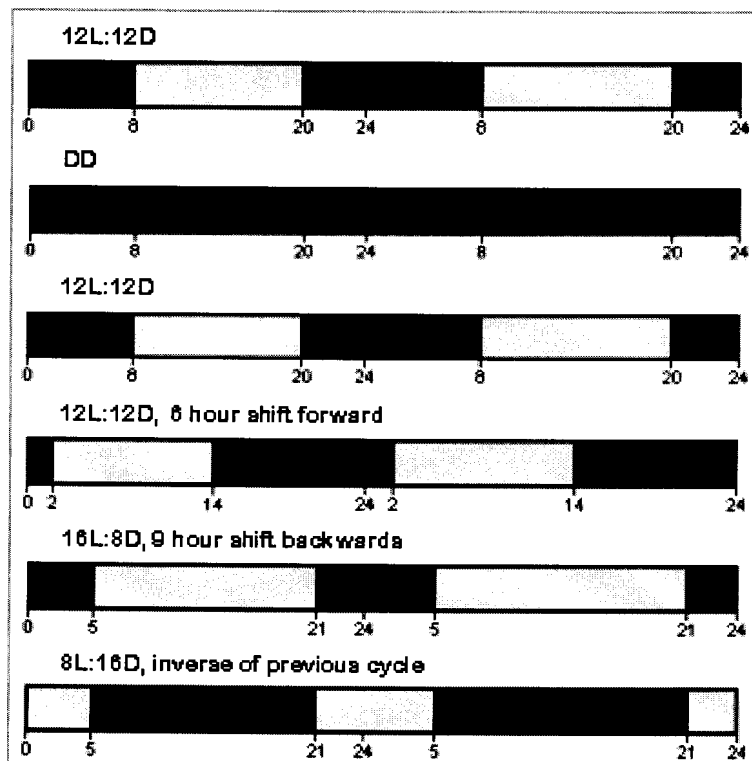


Figure 4.3: Representation of light schedules employed in the first experiment.

In this study only females were used, other studies have indicated that there are no differences in activity patterns between sexes (Ben-Shlomo *et al.* 1995).

Experiment 2: Entrainment and endogenous rhythms of *Cryptomys hottentotus pretoriae*.

Animals were maintained on a light cycle of 12L:12D for a period of 2 months prior to commencing the recordings. One mole-rat was implanted with an e-mitter, two mole-rats were housed with running wheels and four other cages were fitted with infra-red captors.

Mole-rats were recorded on the 12L:12D light cycle for a month until clear rhythms could be seen (Lights on at 06h00, lights off at 18h00). When the mole-rats were entrained to the 12L:12D cycle, the light cycle was changed to total darkness (DD). The animals were kept on this cycle for one month to ensure that endogenous rhythms had time to manifest themselves.

After DD, animals were re-entrained to the previous 12L:12D cycle for a month, thereafter followed by an inverse of the LD cycle (Lights on at 18h00, lights off at 06h00). This cycle was also maintained for approximately one month. The last part of the experiment involved animals being maintained in constant light (LL) for one month (Fig. 4.4).

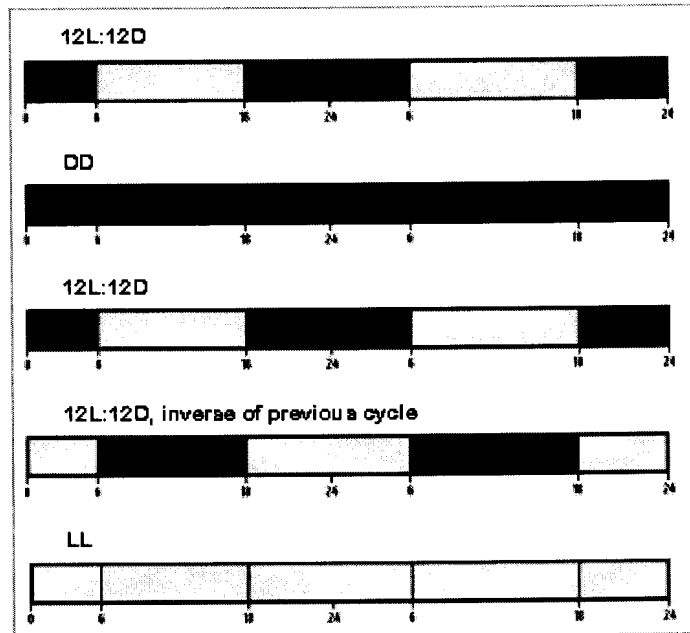


Figure 4.4: Representation of light schedules in the second experiment.

Experiment 3: Entrainment and endogenous rhythms of *Cryptomys damarensis*.

The experimental animals were housed under a light cycle of 12L:12D for two months before starting the recordings. Two mole-rats received e-mitter implants, two had running wheels and four infra red captors.

After entraining the animals to a 12L:12D cycle for a month, (Lights on at 06h00, lights off at 18h00), animals were subjected to constant darkness conditions (DD) for approximately a month. This was done in order to investigate whether the animals exhibit endogenous rhythms and to determine the free running period. Afterwards, the mole-rats were re-entrained to the

previous 12L:12D cycle for a month, and the light cycle was subsequently inverted (Lights on at 18h00, lights off at 06h00) to examine whether the animals shift their activity according to the external light stimulation. Since the animals were then already entrained to a 12L:12D light cycle, the fourth experiment could start without entraining first. The light cycle was then switched to constant light (LL) conditions to observe whether there are any changes in the rhythm under such a light regime (Fig. 4.5).

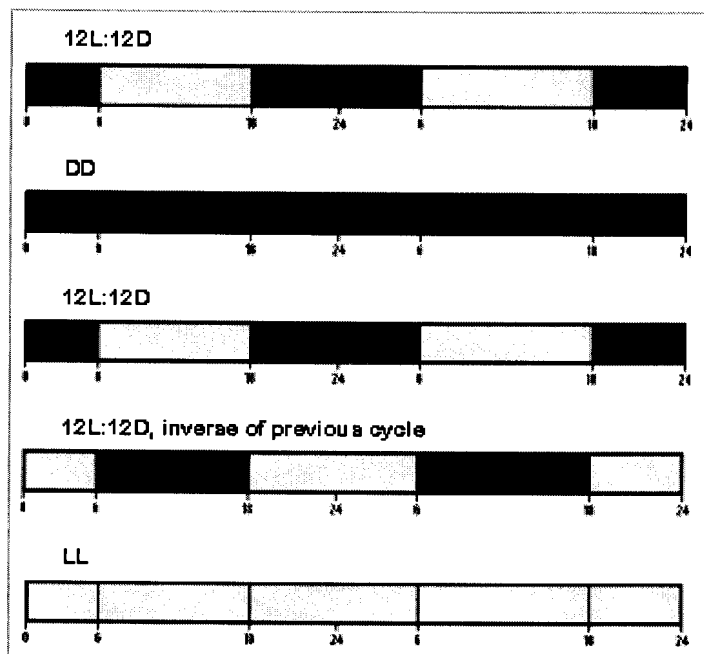


Figure 4.5: Representation of the light schedules in the third experiment.

Analysis

Recordings from the activity captors were fed directly into a computer, and data was processed to a suitable format for use in other programs. ActiView and Clocklab software were employed to analyze behavioural patterns and generate actograms. Activity was double plotted on actograms with the number of days on the y-axis and the circadian time on the X-axis. The period of the endogenous rhythm was obtained where possible, using a tau cursor line by means of the 'eye ball method'. The percentage activity during a certain phase of the light cycle was calculated

for 12L:12D, 16L:8D and 8L:16D cycles where applicable. Microsoft Excel macro programs were used for this purpose.

RESULTS

Activity captors:

Three different types of activity captors (E-mitters, IR captors and running wheels) were used to compare the differences in activity recording. Activity was clearly visible on any of the three types of captors, the running wheels provided the clearest on- and offsets of activity, while it was generally more difficult to determine the on- and offsets with e-mitter recordings.

In previous experiments, variability was noticed in the number of animals that ran on wheels (Goldman *et al.* 1997, Tobler *et al.* 1998). In *G. capensis*, 4 of the 5 animals tested for wheel running, exhibited wheel running. The variation was thus not large, although the sample size was somewhat small.

Experiment 1: Entrainment and endogenous rhythms of *Georychus capensis*.

a) Temporal activity preference

The majority of the experimental animals displayed nocturnal activity patterns throughout the experiment. After being subjected to constant conditions, one animal became arrhythmic while a second changed its activity from nocturnal to diurnal. These changes occurred fairly early in the experiment and remained constant for the remainder of the experimental regime. The activity patterns could therefore be divided into three categories, stable nocturnal activity (7 animals), stable diurnal activity (2 animals) and arrhythmic behaviour, resulting in approximately

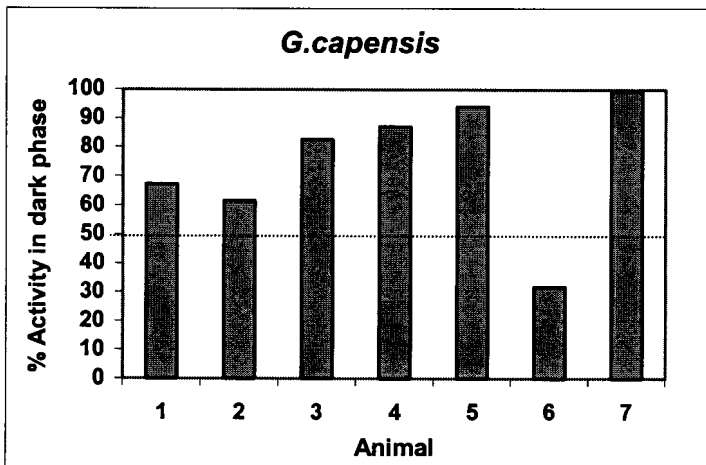


Figure 4.6: Percentage of activity in *G. capensis* during the dark phase of a 12L:12D light cycle.
 e-mitters: animals 1-3
 IR captors: animals 4-7

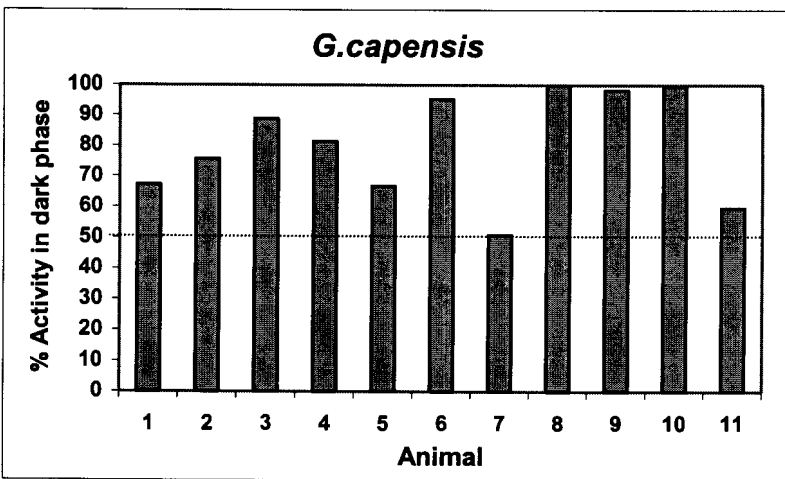


Figure 4.7: Percentage of activity in *G. capensis* during the dark phase of a 16L:8D light cycle.
 e-mitters: animals 1-3
 IR captors: animals 4-7
 Running wheels: animals 8-11

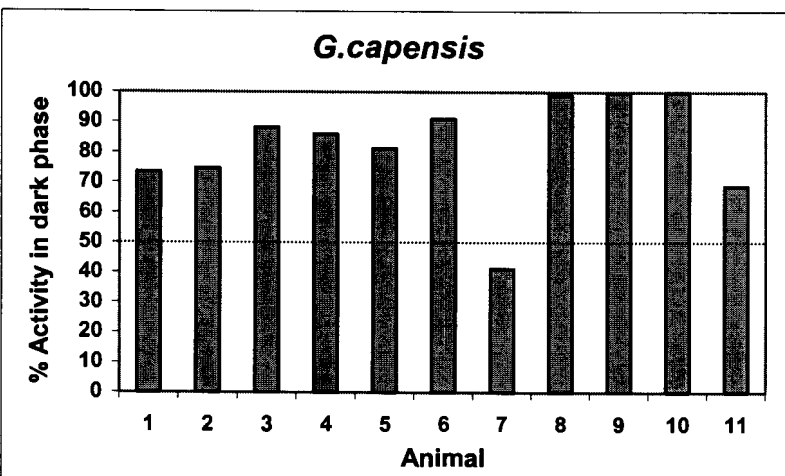


Figure 4.8: Percentage of activity in *G. capensis* during the dark phase of a 8L:16D light cycle.
 e-mitters: animals 1-3
 IR captors: animals 4-7
 Running wheels: animals 8-11

equal amounts of activity during the light and dark part of the light cycle (2 animals). Thus it is clear that the majority of the animals express nocturnal activity (Fig. 4.8, 4.9, 4.10). The amount of activity expressed during the light and dark phases did not change with alteration of day and night length.

b) Entrainment & synchronization

Relatively large variation was observed between the animals. Approximately 80% of the animals entrained to light cycles, whereas of these about 50% displayed clear entrainment. The remainder showed arrhythmic behaviour. Most of the animals exhibited either a clear on or offset of activity, but rarely both. Activity was generally monophasic with a single bout of activity during a circadian cycle. Individual variation was observed within the phase angle of entrainment, some animals were active from the change in the light cycle until the next change, while others concentrated most of their activity towards the end of the phase (Figs. 4.9, 4.10).

Georychus capensis

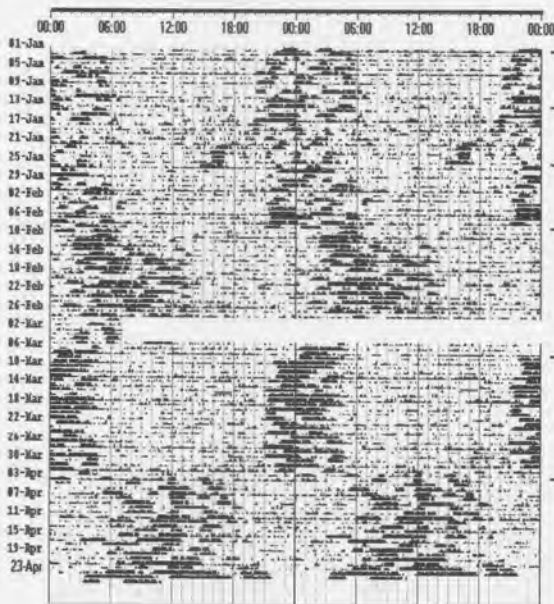


Figure 4.9: Actogram of a mole-rat showing good entrainment. (Mole-rat G3)

12L:12D
DD
12L:12D
12L:12D,
6 hour
shift
16L:8D,
9 hour
shift
8L:16D,
inverse

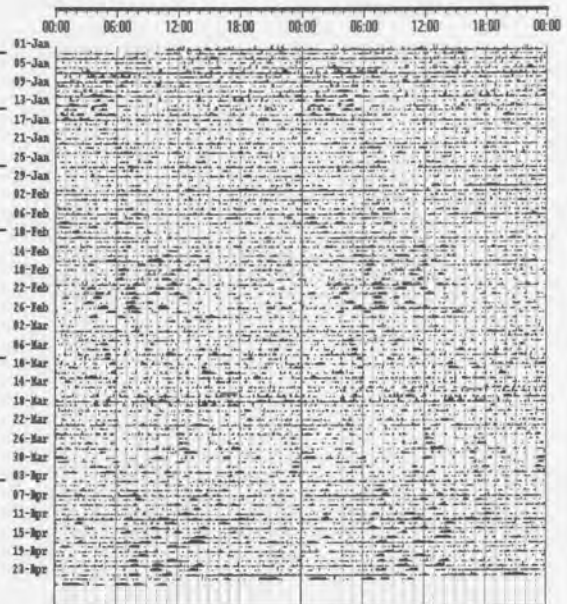


Figure 4.10: Actogram of a mole-rat that did not display good entrainment. (Mole-rat G1)

To determine whether mole-rats are able to remain entrained to changed light cycles, light schedules were shifted forward and backwards. The results indicate that activity was altered according to the shifts in the light cycles. By altering the duration of the day and night, it was apparent that the amount of activity was contracted and expanded according to the length of day and night. Most animals altered their activity immediately after a change in the light schedule, whereas some mole-rats took a couple of days to become fully entrained. Mole-rats showing robust activity rhythms generally entrained faster than animals exhibiting less clear rhythms. Some of the mole-rats remained arrhythmic despite the lighting regimes they were exposed to (Figs. 4.11, 4.12).

Georychus capensis

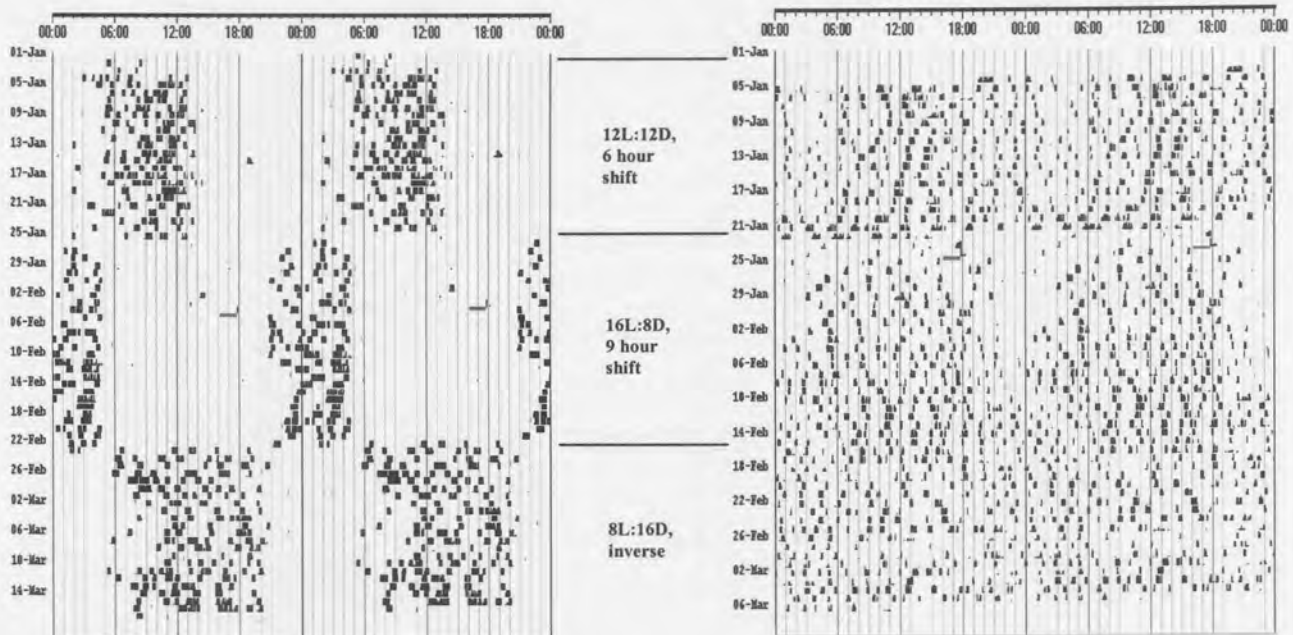


Figure 4.11: Example of phase shifting according to shifts in the light cycles and activity is adjusted to different durations in the light regimes. (Mole-rat G8)

Figure 4.12: No entrainment to the different light regimes. (Mole-rat G11)

c) Constant conditions

Although the period of DD was somewhat short, mole-rats that showed clear entrainment to the light cycle, also expressed endogenous rhythms. The endogenous rhythms were generally slightly shorter or around 24 hours long ($\tau=23.95\pm 0.154$, $n=4$). One of the mole-rats that at first expressed nocturnal activity, shifted its activity to occur during the subjective day. The diurnal mole-rat continued to display the majority of its activity during the subjective day. Of the mole-rats that entrained less readily to the first light cycle, one exhibited an endogenous rhythm, while the other two did not show any visible endogenous rhythms. One arrhythmic mole-rat continued to exhibit activity over the whole 24 hour period (Figs. 4.13, 4.14).

Georychus capensis

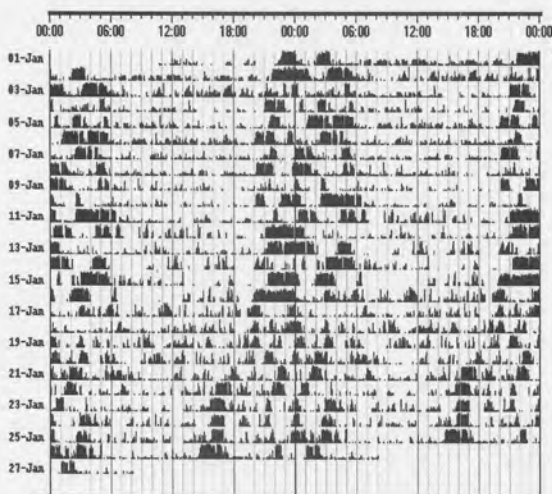


Figure 4.13: Example of an actogram of a free-running mole-rat. (Mole-rat G3)

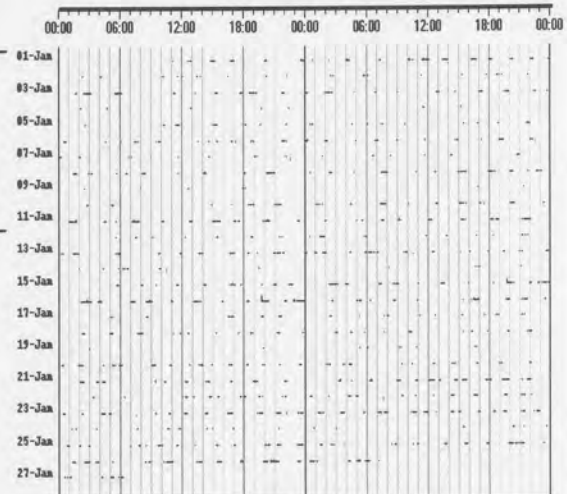


Figure 4.14: An example of an actogram of a mole-rat that did not display a free-running rhythm. (Mole-rat G5)

Experiment 2: Entrainment and endogenous rhythms of *Cryptomys hottentotus pretoriae*

a) Temporal activity preference

The animals with distinguishable rhythms mainly displayed nocturnal activity (Fig.4.15). None of the animals changed their activity preference during the course of the experiments. One animal displayed two bouts of activity, one towards the end of subjective day and another towards the end of subjective night during the LD cycles. The diurnal activity bout seemed to disappear under constant DD conditions, leaving only the subjective night bout of activity during this period, but as soon as the animals were placed under a LD cycle again, the daily bout reappeared. Another animal appeared to be arrhythmic, but did display an endogenous rhythm after some time under constant conditions.

In general, these animals could be divided into three categories, nocturnal activity (3 animals), arrhythmic animals (3 animals) and one animal with distinct bouts of activity in both the light and dark part of the LD cycle. In comparison with *G. capensis*, a much larger proportion of animals were arrhythmic, and the activity was not as defined or restricted to the dark phases of the light cycle.

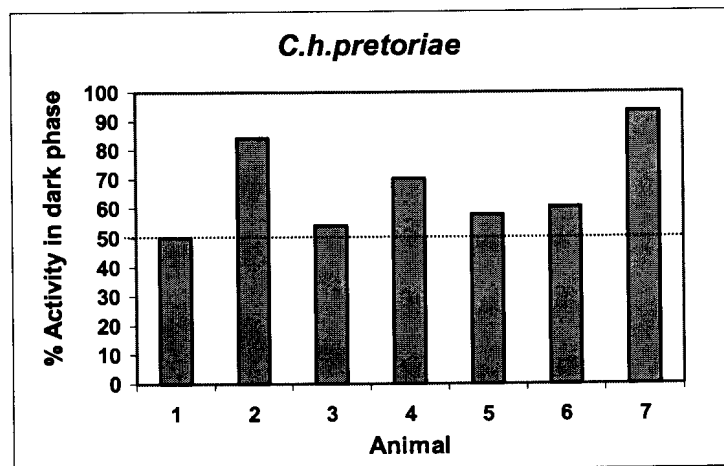


Figure 4.15: A graph illustrating that *C. h. pretoriae* displays the largest percentage of its activity during the dark phase of the light cycle, confirming that the species is nocturnal.

b) Entrainment

About fifty percent of the animals entrained to the 12L:12D light cycle, and this was to varying degrees. The entraining animals mostly displayed monophasic activity patterns. However, one animal appeared to exhibit an ultradian rhythm with distinct activity bouts at the end of the light and dark phases of the light cycle, although the nocturnal activity bout was longer. One animal appeared to display some splitting of its activity after about a month and a half in 12L:12D, both bouts of activity manifested during the dark phase (Fig. 4.16, 4.17). Generally a clearer offset of the activity was evident, with the activity concentrated at the end of the subjective night.

Cryptomys hottentotus pretoriae

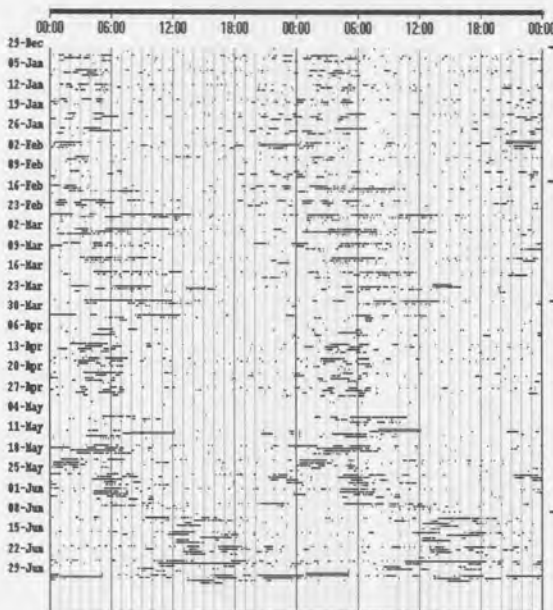


Figure 4.16. An example of a mole-rat with good entrainment and some indications of splitting in the activity. (Mole-rat P1)

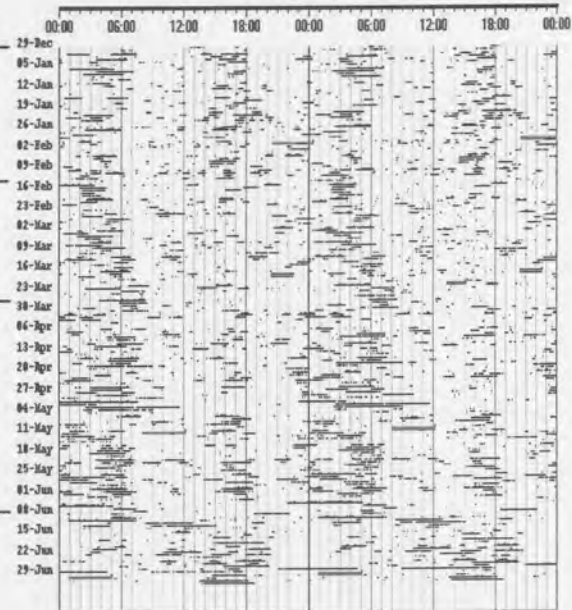


Figure 4.17: An example of a mole-rat that shows ultradian bouts of activity. (Mole-rat P6)

The light cycle was inverted to determine whether the animals could modify their activity accordingly. Animals that entrained their activity to the light cycles, altered their activity along with the shift in light cycles. The mole-rat exhibiting two bouts of activity also altered its activity so that the larger nocturnal bout was again during the dark phase of the light cycle. A mole-rat

that was arrhythmic under the LD cycle, suddenly started to exhibit a nocturnal rhythm after the LD cycle was inverted. The alteration in activity did not happen immediately, but occurred during the course of a few days, this being in marked contrast to *G. capensis* (Fig. 4. 18, 4.19).

Cryptomys hottentotus pretoriae

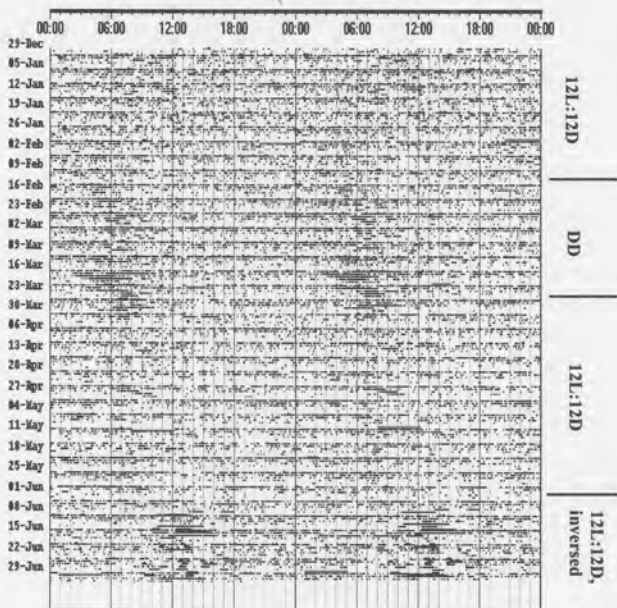


Figure 4.18: An actogram of a mole-rat with arrhythmic behavioural patterns under LD conditions but displays a free-running rhythm. (Mole-rat P7)

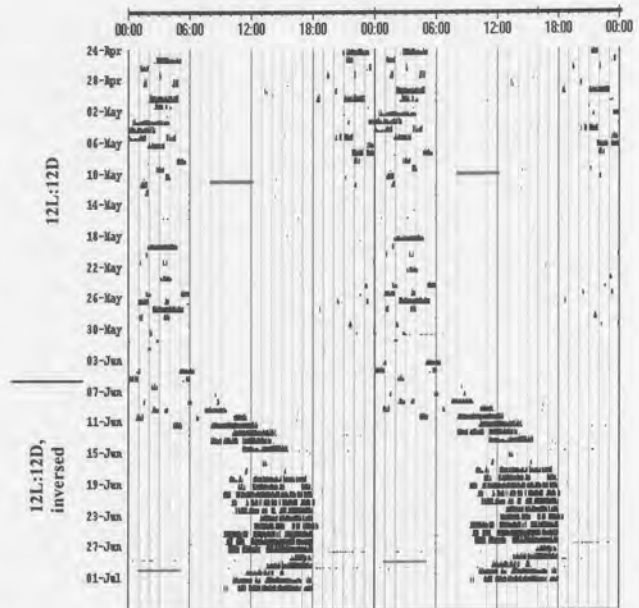


Figure 4.19: An actogram of a mole-rat that shifted its activity according to shifts in the light cycle. (Mole-rat P4)

c) Constant conditions

All mole-rats that showed entrainment expressed endogenous rhythms, mostly slightly longer than 24 hours ($\tau=24.09\pm 0.105$, $n=5$). One animal appeared to be arrhythmic under the LD cycle, but displayed an endogenous rhythm after a few days in DD. The remaining mole-rats that did not entrain to LD cycles, did not exhibit endogenous rhythms. The animal with the diurnal and nocturnal bouts of activity did not display a distinctive peak in diurnal activity during DD, although there was still some activity during the subjective day.

Generally entrainment seemed to disintegrate quite rapidly under constant light conditions. Very few animals remained entrained under this light schedule (Figs. 4.20, 4.21).

Cryptomys hottentotus pretoriae

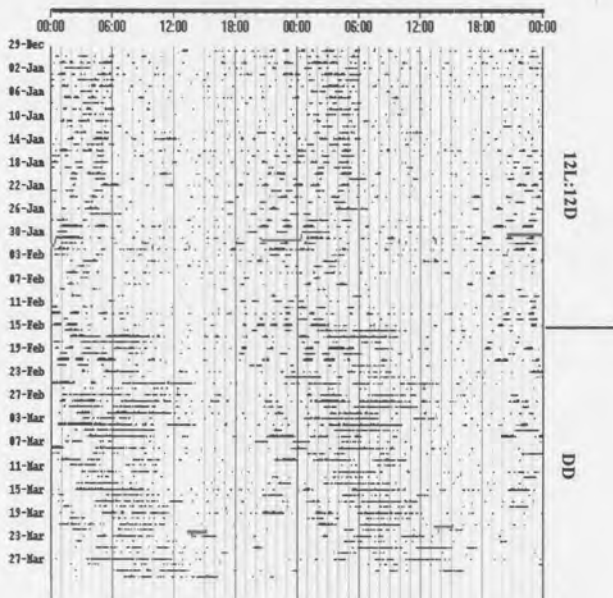


Figure 4.20: An example of a mole rat with a clear endogenous rhythm in DD. (Mole-rat P1)

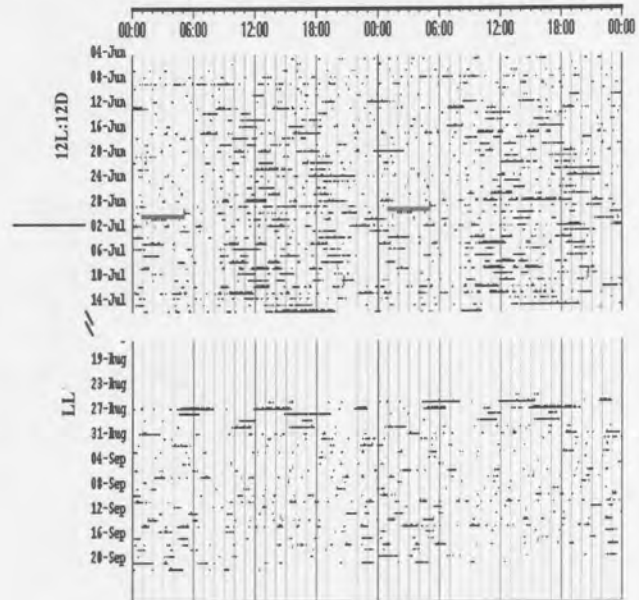


Figure 4.21: An example of a rhythm becoming arrhythmic under LL conditions. [Clear space is missing data](Mole-rat P2)

Experiment 3: Entrainment and endogenous rhythms of *Cryptomys damarensis*

a) Temporal activity preference

The majority of the animals had a preference for diurnal activity and the pattern displayed during the first light cycle was not altered in later cycles (Fig.4.22). Activity patterns could be classed in three categories, namely diurnal (4 animals), nocturnal (1 animal) and arrhythmic (3 animals). The nocturnal animal appeared to change its activity to display a diurnal rhythm after some time. In comparison to the solitary species, activity was not as restricted to the light phase

Although a greater proportion of activity occurred during the light phase, there was a fair amount undertaken during the dark phase.

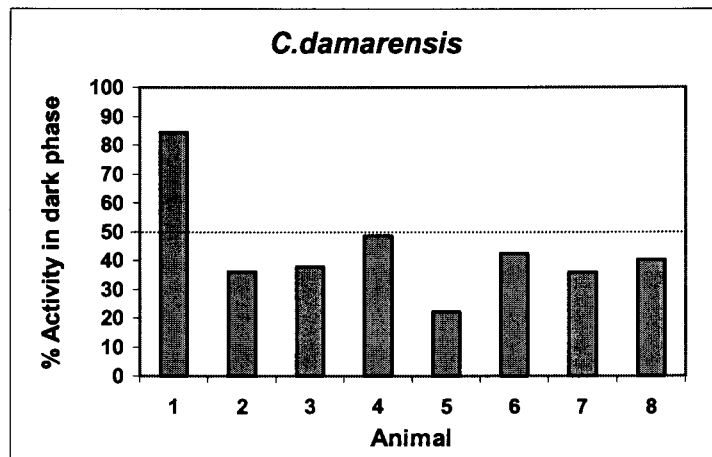


Figure 4.22: A graph illustrating that *C. damarensis* displays the largest percentage of its activity during the light phase of the light cycle, confirming that the species is diurnal.

b) Entrainment and activity rhythms

Approximately fifty percent of the animals entrained well to the first light schedule, the remaining animals being weakly entrained or arrhythmic. Large variability was noticeable in this species. One animal that showed a good free-running rhythm under DD, appeared to lose rhythmicity after being transferred back to a LD cycle, the rhythm reappearing after approximately a month seemingly still free running for about 10 days before re-entraining to the LD cycle (Figs. 4.23, 4.24).

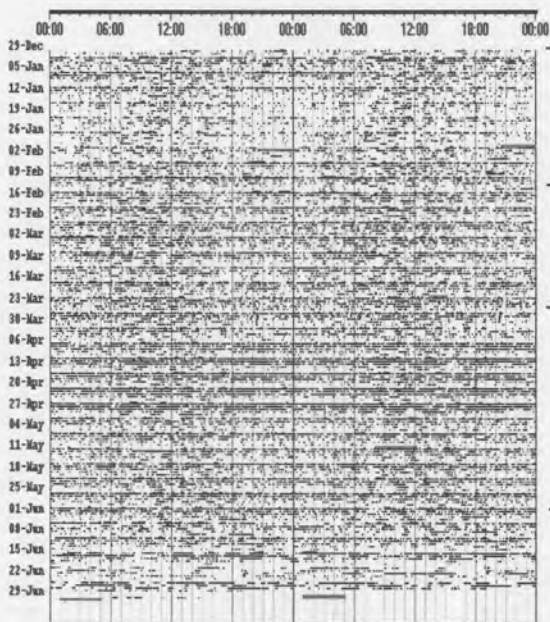
Cryptomys damarensis

Figure 4.23: An example of a mole-rat that shows some degree of entrainment. (Mole-rat D7)

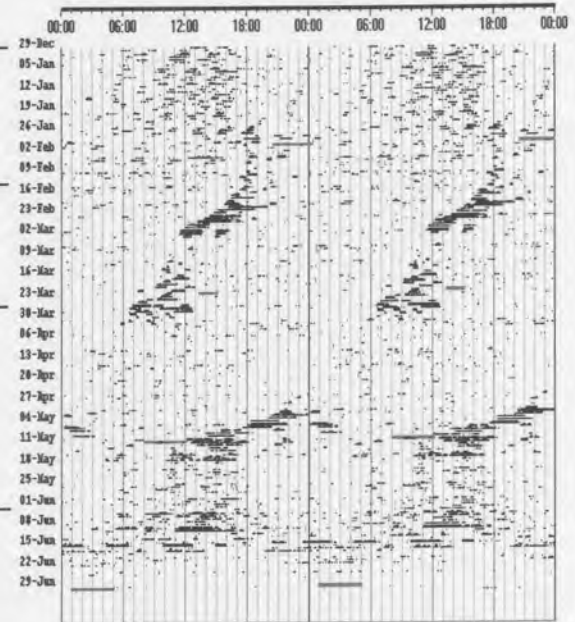


Figure 4.24: An example of a mole-rat that appears to have lost its ability to entrain for a while. (Mole-rat D2)

Most animals re-entrained to shifts in the light cycle, animals not expressing rhythms remained arrhythmic. One animal was entrained to the LD cycle before DD, but after being returned to the LD cycle, it showed less and less activity until it appeared to become arrhythmic. Another animal that showed a fairly clear rhythm to the first LD cycles, no rhythm during DD, finally became arrhythmic after the inversion of the light cycle (Figs. 4.25, 4.26).

Cryptomys damarensis

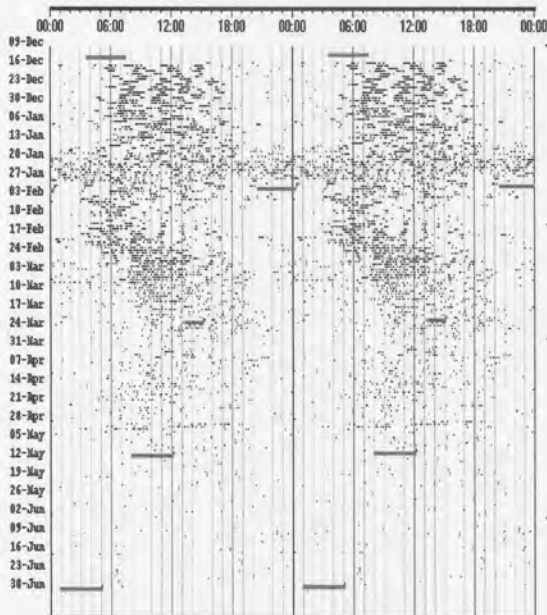


Figure 4.25: An example of a mole-rat that lost rhythmicity after being entrained. (Mole-rat D5)

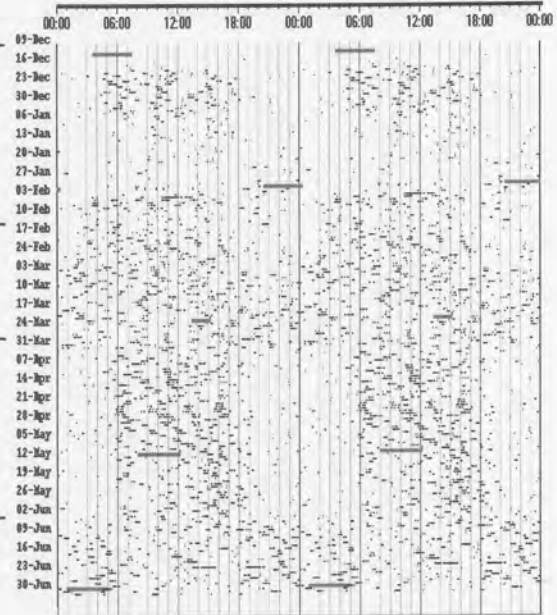


Figure 4.28: An example of a mole-rat that did entrain to LD cycles but did not display an endogenous rhythm. (Mole-rat D6)

c) Continuous conditions

Mole-rats that showed entrainment to the LD cycles, exhibited endogenous rhythms. Three animals displayed clear endogenous rhythms during the subjective day, but two had less clear rhythmic patterns. Of the animals with clear rhythms, one was shorter than 24h, whereas the other was 24h and one slightly longer than 24h. Two animals with weak rhythms both had rhythms of about 24 hours ($\tau = 23.95 \pm 0.075$, $n=6$). The rest of the animals were arrhythmic under DD. Of the arrhythmic animals, one appeared to show a weak rhythm for about 17 days whereafter it lost rhythmicity. The animal that showed nocturnal activity during the first LD cycle, shifted its activity to diurnal just before going into DD, and subsequently appeared to show free running from a phase angle in subjective day.

In conditions of constant light, only one animal retained a weak rhythm, all other animals completely lost rhythmicity almost immediately. As in *C. h. pretoriae*, the animals did not exhibit

endogenous rhythmicity under constant light conditions. The rhythms disintegrate quickly and completely (Figs. 4.27, 4.28).

Cryptomys damarensis

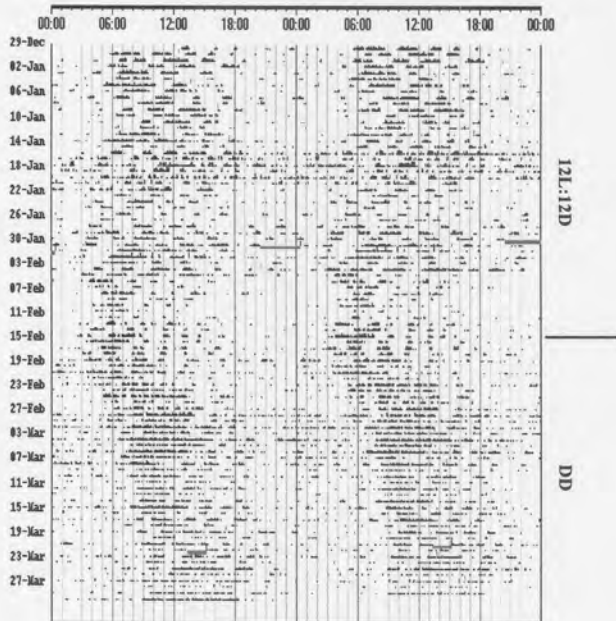


Figure 4.27: A mole-rat with a good endogenous rhythm in DD (Mole-rat D5)

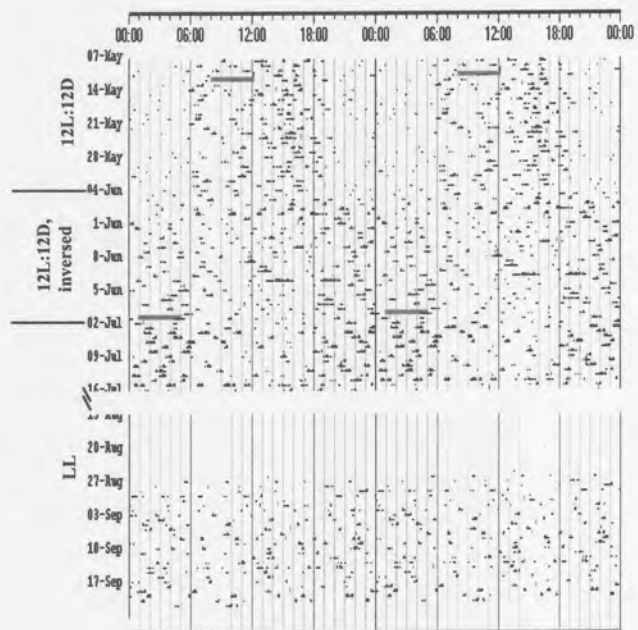


Figure 4.28: An example of a disintegrating rhythm under constant light conditions. [Clear space is missing data](Mole-rat D6)

DISCUSSION

a) Temporal activity distribution

Mole-rats are able to perceive the difference between light and dark, since most individual animals display either nocturnal or diurnal activity patterns. The solitary *G. capensis* clearly shows a preference for nocturnal activity, the majority of the animals exhibited a distinct

nocturnal activity pattern during the course of this experiment. Likewise animals that displayed diurnal activity were clearly diurnal. This study confirms the findings of Lovegrove & Papenfus (1995), who found all of their experimental *G. capensis* to be nocturnal. Although the same species, Lovegrove's experimental animals were from a different population, occurring in Natal. It appears that the more extensively studied *S. ehrenbergi*, also a solitary species, displays somewhat less clear-cut patterns in daily activity. Goldman *et al.* (1995) indicated that all their animals displayed diurnal activity, whereas Tobler *et al.* (1998) stated that the majority of their experimental subjects were nocturnal. Nevertheless, long-term observations revealed that most of these nocturnal animals altered their activity and became diurnal.

Social species are also able to perceive changes in light and dark. Both species in this experiment displayed activity rhythms. In contrast with the solitary species, social species appear to display a much smaller difference between the amount of activity during light and dark phases of the light cycle. The activity is thus not as restricted to a specific phase of the cycle. In the present study all the entraining *C. h. pretoriae* expressed nocturnal activity, contrasting earlier studies that found a lack of distinct activity rhythms (Bennett 1992, Hickman 1980).

The majority of the entraining *C. damarensis* exhibited diurnal activity, this pattern was also noted in a study by Lovegrove (1993) using colonies, instead of single animals. As in *C. h. pretoriae*, the activity in this species was more uniformly distributed across the 24 hour period. From these results it can be inferred that social species are less capable of displaying distinct nocturnal or diurnal activity rhythms when compared to solitary species, and this could be interpreted as a weaker link between the pacemaker and its output rhythms. However, a study on *H. glaber*, the naked mole-rat, has revealed that most of the animals exhibited robust nocturnal activity rhythms (Riccio and Goldman 2000), but this may be as a result of pre-scanning the animals and only using good wheel running animals.

The reason why some mole-rat species exhibit nocturnal activity patterns and some diurnal patterns remains unknown. There is no predation pressure on these subterranean species strong enough to cause selective nocturnal and diurnal activity patterns. It may simply reflect the fact that light is an unusual situation for a subterranean mole-rat. Thus assumingly some unknown evolutionary pressure in the past is the foundation of the selective temporal distribution of activity in mole-rats.

b) Entrainment of activity rhythms

The results obtained from this study suggest that most individual *G. capensis* display circadian rhythmicity of locomotor activity. Not all of the animals exhibited clear cut rhythms, about 80% of the animals entrained to the light cycles. This percentage is comparable to that of *S. ehrenbergi*, a solitary mole rat species from North Africa and the Middle East (Goldman *et al.* 1997), which also exhibits a relatively high percentage of entrainment amongst test animals. Social species are also able to perceive changes in light conditions and synchronize their activity to a light cycle. However, in comparison with the solitary *G. capensis* a larger proportion of the experimental animals of the two social mole rat species were unable to entrain to LD cycles. In social species, approximately half of the animals exhibited entrainment, indicating that light reaches the SCN. The low percentage of entrainment implies a weak link from the clock to its output, or alternatively a high threshold for response to light. The results of the previous chapter imply a relatively high threshold for photic response, reinforcing this hypothesis.

Colonies of *C. damarensis* have been found to show good entrainment to LD cycles, apparently better than animals housed singly. This can be ascribed to either an individual animal behaving unnaturally when separated from its colony, or that there is a component of social entrainment. Social entrainment is difficult to define, but in this case it seems to be the more likely explanation for the difference in results.

In all the species studied to date, relatively large intra-individual variability was observed, some animals entrained very well to the light regimes, some showed weak entrainment whereas others did not entrain their activity to the light cycles at all. The phase angle of activity also differed considerably between animals. Some animals became active immediately after the change of lights and remained active until the lights changed again, while others became active only later and concentrated their activity at the end of the 12-hour period. This large variation in the phase angle, as well as the number of arrhythmic or weakly entrained animals indicates that the circadian clock is not very strongly coupled to the photic input in this species. About fifty percent of *S. ehrenbergi* entrained to temperature rhythms (Goldman *et al.* 1997), suggesting that non-photic cues may play an important supporting role in entrainment of these subterranean species.

In *G. capensis*, activity was shifted according to changes in the light cycles almost immediately in the majority of the experimental animals and activity was adjusted to light regimes of different durations, implying that light is effectively perceived and integrated by the circadian pacemaker. Both social species also altered their activity according to an inverse in the light cycle, however, in contrast to the solitary species, this change took place over the course of several days.

Social species exhibit a markedly poorer response to photic stimulation when compared to solitary species. It can thus be inferred that besides a weak link between the clock and the output of behavioural activity, integration of light information in the pacemaker does not take place properly or alternatively the clock mechanism itself is degenerate.

c) Constant conditions

Animals that readily entrained to the first light cycle displayed endogenous rhythmicity, the rhythm started to drift from the same phase angle that the animal was entrained to. This indicates that these animals were entrained to the light cycle, and that the behavioural response to the light cycles were not merely masked. Clear endogenous rhythms were, however, not obtained from all the animals, thus it is still possible that some animals may have masked responses. The period of constant conditions were too short to make conclusive statements other than that some of the mole rats did display an endogenous rhythm.

Both social species exhibited endogenous rhythms, the number of animals being generally comparable to the percentage of entraining animals in each species. The existence of endogenous rhythms in DD of these species suggests a functional circadian clock. Some of the animals did not show any entrainment to LD cycles but did exhibit endogenous rhythms under constant conditions, suggesting that light integration in the circadian clock may not take place properly. Generally rhythms seem to disappear rapidly under constant light conditions. Very few animals showed traces of rhythmic behaviour under this cycle.

In comparison to other rodents, mole-rats have a relatively high threshold for response to light. Under normal circumstances these animals spend nearly all of their time underground and are not exposed to light for extended periods. It has been suggested that the abnormal 12-hour or

longer light periods of laboratory experiments may cause retinal damage and result in reduced photosensitivity (Ricchio & Goldman 2000).

Seasonal breeding may provide an explanation for the gradient in sensitivity encountered in this family. Solitary species are highly xenophobic towards other animals outside the breeding season. As external cues have to provide an indication of the start of breeding season in order for individuals to search for breeding partners, it is essential for this species to be able to perceive at least seasonal changes in the environment. *G. capensis* is sighted aboveground more often than the two social species (N.C. Bennett *pers.comm.*).

Likewise, *C. h. pretoriae* is a seasonal breeder, but unlike *G. capensis*, it does not need to look for breeding partners as they occur within the same colony. Thus being able to perceive seasonal changes in the environment still has a functional role in this species. In contrast with the other species, *C. damarensis* breeds aseasonally, and this species would therefore be expected to show less response to light than species that breeds seasonally. This species has, however, been found to exhibit seasonal moulting patterns.

In conclusion:

- All three species of mole rat show daily entrainment of locomotor activity to external light stimulation, confirming that light reaches the SCN and is integrated.
- The ability of these species to adapt their activity according to shifts in the LD cycle demonstrates that these species do entrain to light and not to some other cue.
- Likewise, under conditions of constant darkness, endogenous rhythms are present in all three species, providing evidence for a functional circadian clock.
- A gradient of photic responsiveness is evident, the solitary species being more sensitive to light than the social species. This is supported by the higher percentage of entrainment as well as the more concentrated activity patterns in the solitary *Georychus capensis*, likely indicating better integration of photic information by the circadian clock.

CHAPTER 5: DISCUSSION

DISCUSSION

1. Retinal projections of several species of mole-rat

Previous studies on fossorial mammals have demonstrated varying degrees of ocular regression and consequently a correlated reduction in retinal ganglion cells projecting to various visual and circadian structures in the brain. The proportional retinal input to the visual and circadian systems of animals that are not exclusively subterranean, is not significantly different from that of aboveground mammals, although the projections are reduced in size (Herbin *et al.* 1994). In contrast, strictly subterranean mammals, such as the blind mole-rat *Spalax ehrenbergi*, exhibit a modified proportional distribution of the retinal ganglion cells to the two innervated systems (Cooper *et al.* 1993a). Similar results were found in the present study in the giant Zambian mole-rat, *Cryptomys mechowi*. Projections to the visual system were severely regressed and ipsilateral projections to these structures were mostly absent. However, the circadian system received a relatively dense bilateral innervation, with a more prominent ipsilateral projection.

In general, albino animals have smaller retinal projections than pigmented animals, especially to the ipsilateral side, the projections are very sparse or absent. Retinal projections in the albino highveld mole-rat, *Cryptomys hottentotus pretoriae*, closely resembled those seen in the pigmented giant Zambian mole-rat. In contrast, the innervation pattern of the albino Damaraland mole-rat, *Cryptomys damarensis*, was totally different from the other two species.

Thus, it appears if the classical visual system of mole-rats is redundant, and consequently the components involved with the visual system suffer a severe evolutionary regression. In parallel with this regression, the retinal projections are downscaled or absent. In contrast, the circadian system is proportionally expanded, which supports the hypothesis that it may be essential for some aspect of survival.

2. Comparison between different mole-rat species and other rodents

The results of the experiments in chapters 3 and 4 provide evidence that in most African mole-rat species a percentage of individuals are indeed photosensitive, they possess a functional circadian clock and are able to entrain their activity to external light stimuli. Animals also display endogenous rhythms in constant dark conditions.

Fos expression and behavioural activity patterns of solitary mole-rat species are found to be similar to that observed in other aboveground rodents. Fos induction in the SCN of both aboveground rodents and mole-rats are gated according to the phase of the circadian clock. However, immunohistochemical staining of Fos expression in the SCN is less dense than in other rodent species, and a smaller proportion of mole-rats entrain well to light stimulation. In comparison to rodents such as mice or hamsters, a smaller percentage of mole-rats entrain well to light cycles, but in most cases the expressed rhythms are clear and shifts to a delayed or advanced light cycle occur rapidly.

The different species of solitary mole-rats, *G. capensis* and *S. ehrenbergi* appear to display very similar patterns of Fos expression and locomotor activity. In response to light, *G. capensis*, the species investigated in this thesis, and *S. ehrenbergi*, investigated by other research groups, both express circadian rhythmicity in locomotor activity rhythms. In most cases activity is relatively restricted to either the light or dark phase of the light cycle, depending on whether the individual expresses a nocturnal or diurnal pattern. Most animals exhibit a free-running period of close to 24h. In addition, both species exhibit Fos expression gated by the circadian clock - in the SCN Fos can only be induced during the subjective night (Tobler *et al.* 1998).

Generally social mole-rat species are found to be less sensitive to light than their solitary counterparts. This pattern is reflected in both the Fos expression in the SCN and locomotor activity patterns in response to light. Fos protein is expressed in the SCN of all the species investigated, but less densely than what is seen in the solitary species. In contrast with solitary species, photic induction of Fos in the SCN does not appear to be gated according to the circadian clock in the social species. Although both the social species investigated exhibited circadian rhythmicity in LD cycles, a smaller proportion of animals entrained to light cycles. The activity was not as confined to a certain phase of the light cycle as is the case in the solitary species.

Fos expression is not always correlated with locomotor activity patterns. Some individuals of the social species exhibit clear behavioural activity rhythms while Fos expression was low and did not show gating according to the circadian clock. Experimental results suggest that increased light intensity and prolonged pulse duration rendered a higher Fos expression in the SCN, it could be possible that the circadian clock of these social species requires a longer integration time for light information. The 12-hour light stimulation received during behavioural activity pattern experiments provides sufficient integration time for the circadian clock to express clearly visible activity rhythms. Taken together, it is obvious that light is integrated in the SCN of all Bathyergid species and can effectively be displayed as locomotor and other rhythms. However, a clear decrease can be seen in the sensitivity of the circadian clock as the level of sociality increases.

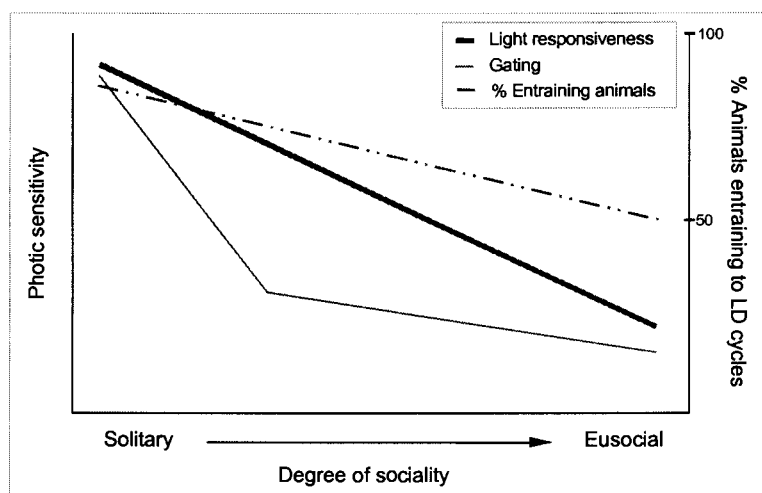


Figure 5.1: Schematic representation of the decrease in sensitivity of the circadian clock in mole-rats.

3. Diurnality and nocturnality in mole-rats

While the advantage of selective nocturnality and diurnality is obvious, it is unknown where in the circadian system it is determined whether an animal is nocturnal or diurnal. It has been shown that animals displaying diurnal activity patterns can be induced to shift their activity temporally to exhibit nocturnal activity. In some cases the presence of a running wheel can cause

animals to invert their activity (Kas & Edgar 1999, Mahoney *et al.* 2001). This implies that within one animal, the possibility of nocturnality and diurnality occurs. The clock function is determined genetically, as can be seen in animals with gene mutations altering rhythm lengths, suggesting that the circadian clock alone does not determine the degree of nocturnality or diurnality.

Mole-rats as well as some other rodents can display multiple chronotypes (diurnal and nocturnal animals within the same species), however one chronotype usually occurs more frequently than the other. The presence of diurnal and nocturnal animals within the same species could suggest a weak coupling between the mechanism that determine temporal activity and the output as activity rhythms.

Despite the 180° difference in temporal distribution of activity in diurnal and nocturnal animals, a number of attributes are similar in both groups. Neuronal and metabolic activity patterns of the SCN are similar in nocturnal and diurnal animals (Nunez *et al.* 1999), as are the general pattern of photic PRC's of nocturnal and diurnal species (Pohl 1985, Mahoney *et al.* 2001). Neuronal activity in hypothalamic target areas just outside the SCN is 180° out of phase in diurnal and nocturnal animals (Nunez *et al.* 1999). This suggests that the temporal distribution of activity is determined downstream from the central circadian pacemaker in the SCN, somewhere between the pacemaker and its effector mechanisms.

Some mole-rat species exhibit diurnal activity, (for example *S. ehrenbergi* and *C. damarensis*) (Goldman *et al.* 1997), whereas others display distinct nocturnal activity, as can be seen in *G. capensis*, and most of the other species of *Cryptomys* studied (Lovegrove & Papenfus 1995). As a result of their protected habitat, there is not a great deal of pressure for distinct nocturnality or diurnality as a result of predation. Likewise, there is no significant competition between species for resources. If activity patterns were linked to specific habitat types, one would expect that desert inhabiting species such as *C. damarensis* and *S. ehrenbergi* would rather benefit from nocturnal activity, avoiding the heat of the day during the summer, but this is not the case. Thus, discrimination between nocturnal and diurnal activity patterns does not seem to have a selective advantage in mole-rats.

It is possible that temperature plays an important role in the temporal distribution of activity of mole-rats in field conditions. The experiments in this thesis were executed at 26°C,

whereas in natural conditions temperatures vary largely over the course of the seasons. Therefore it is likely that activity is related to surface and burrow temperatures.

Why some species of mole-rats should be nocturnal whereas others are diurnal at a given temperature, remains a mystery. Assuming that the different species have been exposed to the same type of evolutionary pressures, there seems to be no obvious reason for this temporal distribution of activity.

4. Significance of the circadian system for mole-rats

Circadian rhythms are proposed to have developed as a selective adaptation allowing animals to anticipate predictable changes in the environment associated with the day/night cycle. Subterranean animals, as a result of their protected habitat, are subjected to very little variation of the environment. The selection pressure for circadian rhythms is therefore minimal. Still most fossorial species have retained the ability to entrain to environmental changes and furthermore it appears that light is still the primary zeitgeber used as more animals are able to entrain to light cycles than temperature cycles.

However, with increasing sociality, a decreasing percentage of animals entrain their activity to light cycles and overall there is a trend for a reduction in sensitivity of the circadian system. A possible explanation for this gradient might be found in the life history of these animals.

Time keeping on a daily basis may not be as important, but on a longer time scale it would be important to detect seasonal changes, particularly in solitary species and some social species (*C. h. hottentotus*, *C. h. pretoriae*) which are seasonal breeders. Social species also already have breeding partners living together in the same colony, but solitary animals are highly aggressive towards conspecifics outside the breeding season. These animals would therefore need some mechanism for measuring day length to indicate the onset of the breeding season. It is thus not unreasonable to assume a functional circadian system is of importance for the survival of these species.

Considering that eusocial mole-rats are non-seasonal breeders, and breeding partners do not have to be searched for (except non-reproductives that disperse for new mates), it could

suggest that entrainment is not of cardinal importance for the more social mole-rats. It could therefore be argued that the circadian system is of less importance for these species and this could explain the higher degree of degeneration of the circadian system.

In conclusion, the presence of a time sense and the ability to respond to light changes, could suggest that bathyergids may once have been surface dwelling creatures or at least semi-fossorial in nature, relying more heavily on chronobiological rhythms for survival. Over evolutionary time, the subsequent adoption of a strictly subterranean lifestyle, particularly in the more social species, may have negated the use of only light changes in the regulation of activity. Seasonal timing is still of significant value for seasonally breeding species, whereas this property is redundant in eusocial and aseasonally breeding species. This would have resulted in the present day scenario where circadian rhythmicity appears not to be essential for the survival of mole-rats, offering an explanation for the regressive features of the circadian system in this unusual family.



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APPENDIX A

APPENDIX A

This appendix contains tables for chapter 3:

CT time	CT4	CT10	CT16	CT22
Stimulated animals (# of animals)	218.43±138.55 (n=3)	79.78±56.68 (n=3)	4153.64±1616.72 (n=3)	1727.63±1002.38 (n=3)
Dark control animals (# of animals)	11.36 (n=1)	0.77 (n=1)	28.64±16.69 (n=2)	45.76±22.88 (n=2)

Table 3.2: Variation of the total optical density (S.E.) of label in the SCN of *Georychus capensis* during different times of the circadian cycle.

CT time	Subjective day	Subjective night
Stimulated animals (# of animals)	149.1033±62.84 (n=6)	2940.635±881.33 (n=6)
Dark control animals (# of animals)	6.2±5.43 (n=2)	25.7575±11.68 (n=4)

Table 3.3: Variation in the optical density (S.E.) of Fos expression in the SCN of *Georychus capensis* comparing additive subjective day values with the additive subjective night values.

CT time	CT4	CT10	CT16	CT22
Stimulated animals (# of animals)	601.02±498.14 (n=3)	127.54±52.51 (n=6)	95.02±38.30 (n=6)	571.12±143.06 (n=6)
Dark control animals (# of animals)	400.93±143.67 (n=4)	802.23±594.99 (n=2)	1677.51±1468.09 (n=2)	302.60±226.04 (n=2)

Table 3.4: Variation of the mean optical density (S.E.) of label in the SCN of *Cryptomys h. pretoriae* during different times of the circadian cycle.

CT time	Subjective day	Subjective night
Stimulated animals (# of animals)	390.7±244.3 (n=6)	181.82±92.21 (n=6)
Dark control animals (# of animals)	519.52±299.36 (n=4)	990.05±724.75 (n=4)

Table 3.5: Variation in the optical density (\pm S.E.) of Fos expression in the SCN of *Cryptomys h. pretoriae* comparing additive subjective day values with the additive subjective night values.

Irradiance Photons.cm ⁻² .s ⁻¹	D.Control (n=3)	3.1 x 10 ¹¹ (n=3)	3.1 x 10 ¹² (n=3)	3.1 x 10 ¹³ (n=3)	3.1 x 10 ¹⁴ (n=3)
Total density	2.97±0.96	124.96±46.66	30.96±12.37	502.25±115.77	267.09±102.34

Table 3.6: Variation of the optical density (\pm S.E.) of label in the SCN of *Georychus capensis* when subjected to different light irradiances.

Intensity Photons.cm ⁻² .s ⁻¹	D.control (n=5)	3.1 x 10 ⁸ (n=2)	3.1 x 10 ⁹ (n=2)	3.1 x 10 ¹⁰ (n=2)	3.1 x 10 ¹¹ (n=2)
Total density	1051.9±425.31	4207.9±2391.4	2644.69±487.3	583.3±187.2	4247.9±1057.7

Intensity Photons.cm ⁻² .s ⁻¹	3.1 x 10 ¹² (n=2)	3.1 x 10 ¹³ (n=2)	3.1 x 10 ¹⁴ (n=2)
Total density	6947.8±3260.9	4344.8±817.1	4190.3±1458.1

Table 3.7: Variation in optical density (\pm S.E.) of label in the SCN of *Cryptomys h. hottentotus* when subjected to different light intensities.

Irradiance	D.Control	3.1 x 10⁹	3.1 x 10¹⁰	3.1 x 10¹¹	3.1 x 10¹²
Photons.cm⁻².s⁻¹	(n=2)	(n=1)	(n=2)	(n=2)	(n=2)
Total density	40.8±48.2	3.1	575.6±252.8	414.2±321.3	115.2±106.7

Intensity	3.1 x 10¹³	3.1 x 10¹⁴
Photons.cm⁻².s⁻¹	(n=2)	(n=2)
Total density	100.7±76.6	560.5±380.9

Table 3.8: Variation of the optical density (±S.E.) of label in the SCN of *Cryptomys damarensis* when subjected to different light irradiances.

Intensity	D. control	3.1 x 10¹⁰	3.1 x 10¹¹	3.1 x 10¹²	3.1 x 10¹³
Photons.cm⁻².s⁻¹	(n=2)	(n=2)	(n=2)	(n=2)	(n=2)
Total density	756.7±702.7	253±39.45	153.6±66.48	405.6±221.95	160.4±129.8

Intensity	3.1 x 10¹⁴
Photons.cm⁻².s⁻¹	(n=2)
Total density	114.96±5.51

Table 3.9: Variation of the optical density (±S.E.) of label in the SCN of *Heterocephalus glaber* when subjected to different light irradiances.

Pulse duration	D.control	1.5 min	5 min	15 min	47 min
	(n=5)	(n=4)	(n=4)	(n=4)	(n=4)
Total density	1139.33±524.22	533.43±182.76	2068.62±545.27	623.86±570.37	3024.05±372.63

Pulse duration	90 min
	(n=4)
Total density	4567.93±1170.02

Table 3.10: Variation in optical density (±S.E.) of label in the SCN of *Cryptomys h. pretoriae* when subjected to different pulse durations.

Temperature	Dark control (n=2)	15°C (n=2)	27°C (n=2)
Total density	8.35±6.38	826.35±354.86	1779.57±72.45

Table 3.11: Variation in optical density (\pm S.E.) of label in the SCN of *Cryptomys darlingi* when subjected to different temperatures with constant light intensity.

Temperature	Dark control (n=3)	15°C (n=3)	27°C (n=3)
Total density	1005.95±558.5	209.3±86.96	1610.4±229.7

Table 3.12: Variation in optical density (\pm S.E.) of label in the SCN of *Cryptomys h. pretoriae* when subjected to different temperatures with constant light intensity.

Temperature	Dark control (n=2)	15°C (n=2)	27°C (n=2)
Total density	756.7±702.7	127.3±119	114.95±5.5

Table 3.13: Variation in optical density (\pm S.E.) of label in the SCN of *Heterocephalus glaber* when subjected to different temperatures with constant light irradiance.