Neuroscience 133 (2005) 555–560

FOS EXPRESSION IN THE SUPRACHIASMATIC NUCLEUS IN RESPONSE TO LIGHT STIMULATION IN A SOLITARY AND SOCIAL SPECIES OF AFRICAN MOLE-RAT (FAMILY BATHYERGIDAE)

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Abstract—Mole-rats are strictly subterranean rodents that are rarely exposed to environmental light. They are well adapted to their environment and have reduced eyes and a severely regressed visual system. It has been shown, however, that mole-rats do exhibit endogenous circadian rhythms that can be entrained, suggesting an intact and functional circadian system. To determine whether light is the entraining agent in these animals, Fos expression in response to light pulses at different circadian times was investigated to obtain phase response curves. Light is integrated effectively in the suprachiasmatic nucleus of the Cape mole-rat (*Georychus capensis*), and Fos expression is gated according to the phase of the circadian clock. The Fos response in the Cape mole-rat was comparable to that of aboveground rodents. In contrast, the highveld mole-rat (*Cryptomys hottentotus pretoriae*) was less sensitive to light and did not show a selective Fos response according to the phase of the circadian cycle. Social species appear to be less sensitive to light than their solitary counterparts, which compares well with results from locomotor activity studies.

Key words: SCN, subterranean, phase response curve.

Light enters the circadian system through the eye and is subsequently captured by photoreceptors in the retina where it is translated into a neural signal and transmitted to the retinal ganglion cells (RGC). The RGC axons in turn form the retinohypothalamic tract that projects to the suprachiasmatic nucleus (SCN) (Moore, 1995).

In mammals, the SCN is the site of the endogenous circadian pacemaker. It is located in the anterior hypothalamus and is responsible for the generation and maintenance of a wide range of physiological, behavioral and biochemical rhythms (Moore, 1983). Although the circadian pacemaker has its own innate timing, it has to be synchronized with the external environment to provide the organism with temporal information (Aschoff and Pohl, 1978). Light is the most prominent environmental cue that organisms use to entrain the period of their endogenous rhythms (Amir and Stewart, 1998). Although the circadian clock is relatively well characterized, the precise cellular mechanisms are unknown. Therefore rhythmic gene expression in the SCN is a good marker of the phase of the circadian clock.

The induction of the immediate early gene *c-fos*, which codes for the protein Fos, is believed to be related to the daily phase shifts required for entrainment to the external environment. Fos is induced transiently in the SCN in response to light during the same period that light induces phase shifts in behavior (Kornhauser et al., 1992). Furthermore, behavioral phase shifts and Fos induction are reciprocal to the number of photons received by the retina (Dkhissi-Benyahya et al., 2000). Fos is thus a suitable marker of light activated neuronal activity in the SCN.

Different temporal niches that animals utilize influence the amount of light that they are exposed to. Mole-rats are rarely, if ever, exposed to light since they are strictly subterranean (Bennett and Faulkes, 2000; Nevo, 1979). Mole-rats possess microphthalmic eyes and a severely regressed visual system. The eyes of the blind mole-rat (family Spalacidae) are minute and s.c. The retina appears to be structurally normal but less organized than that of sighted rodents, and contains functional visual pigments. However, the number of RGC is severely reduced, the optic tract is degenerate, structures involved in image formation are severely reduced and receive sparse innervation from the retina (Cooper et al., 1993). Interestingly, the circadian system does not appear to be affected by this degeneration, as the SCN receives a significant retinal projection and the total number of RGC projecting to the SCN is similar to that of other above ground rodents (Cooper et al., 1993).

The eyes of the African mole-rat (family Bathyergidae) are not as rudimentary as those of the blind mole-rat. They possess small, superficial eyes approximately double the size of that of the blind mole-rat. Structurally, the retina is well organized it is rod dominated but contains a surprisingly large proportion of cones. The cones consist of almost 10% of the photoreceptors in the retina, which proportionally corresponds to diurnal and crepuscular species (Peichl et al., 2004).

Although the morphological structure of the retina appears to be normal and functional photo pigments are present in the photoreceptors, a dramatic reduction in RGC suggests a possible loss in visual function (Cernuda-Cernuda et al., 2003).

The optic nerve is severely reduced compared with other rodents (Cernuda-Cernuda et al., 2003; Nemec et

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Abbreviations: CT, circadian time; IOD, integral optical density; PFA, paraformaldehyde; RGC, retinal ganglion cells; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

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doi:10.1016/j.neuroscience.2005.01.017

Table 1. Number of animals in each experimental group

	CT4	CT10	CT16	CT22
G. capensis	Stim $n = 3$	Stim $n = 3$	Stim $n = 3$	Stim $n = 3$
	DC $n = 1$	DC $n = 1$	DC $n = 2$	DC $n = 2$
Chottentotus pretoriae	Stim $n = 3$	Stim $n = 3$	Stim $n = 3$	Stim $n = 3$
	DC $n = 2$	DC $n = 2$	DC $n = 2$	DC $n = 2$

Stim, stimulated animals. DC, dark controls.

al., 2004; Omlin, 1997). Viral and CT-HRP tracing studies indicate a well-developed SCN with significant bilateral retinal projections, whereas the intergeniculate leaflet is present but very small. Most other visual nuclei were reduced in size and receive sparser retinal innervation that is almost exclusively contralateral (Negroni et al., 2003; Nemec et al., 2004). However, the reduction of the visual structures was not as severe as in the blind mole-rat and the possibility that certain visual functions are retained, is not completely excluded (Nemec et al., 2004).

The aim of this study was to determine whether light is transmitted effectively to the SCN in mole-rats and if so whether the response to light is gated according to the phase of the circadian clock. This experiment also allows for exploring intrinsic differences between species with different life history traits, e.g. solitary and social.

Phase response curves were generated in two species of African mole-rats by presenting light pulses at four different circadian time (CT) periods, then determining the Fos expression in the SCN at each period.

EXPERIMENTAL PROCEDURES

Eighteen Cape mole-rats, *Georychus capensis* captured in Darling, Cape Town (33°56′S, 18°29′E) and 20 highveld mole-rats, *Cryptomys hottentotus pretoriae* captured in the suburbs of Pretoria (25°45′S, 28°14′E), were entrained to a 12L:12D light cycle for at least 3 weeks prior to the experiment, where Zeitgeber time (ZT)0–ZT12 corresponded to lights on and ZT12 to ZT24 to lights off. On the day of the experiment, the lights remained off following the end of the previous LD cycle and the animals were kept in total darkness so that ZT0 was used as the beginning of subjective day, CT0 (Cooper et al., 1998). The period of CT0–CT12 corresponded to subjective day and the period CT12–CT24 to subjective night. Animals were exposed to a 15 min monochromatic light pulse (1.0×10^{15} photons $cm^{-2}s^{-1}$) at different CTs (CT4, CT10, CT16 and CT22; Table 1). Animals of both species were perfused 60 min after the beginning of the light pulse. Dark control animals were not exposed to light.

Animals were deeply anesthetized with an overdose of fluorothane anesthetic. Once the animals had died they were perfused intracardially with 0.9% saline at 37 °C, followed by 4% paraformaldehyde (PFA; Saarchem, Johannesburg, South Africa) in a 0.1 M phosphate buffer (pH 7.4; Sigma, St. Louis, MO 63178, USA) at 4 °C. The brains were stored in 2% PFA until further treatment. Prior to sectioning, brains were placed in 30% sucrose for cryoprotection. Forty micrometer thick coronal sections were cut on a freezing microtome. Sections were inspected under a microscope to determine whether the SCN was present. The SCN-containing sections were divided into two series and one of the series was processed (±six to 10 sections per animal).

Endogenous peroxidase was suppressed using an alcohol–saline–H_2O_2 solution. The sections were briefly rinsed in phosphate-buffered saline and incubated in normal goat serum for an hour after which the sections were incubated in Fos primary antibody (rabbit polyclonal anti-c-fos; Oncogene Science; PC05) for 3 days at 4 °C (dilution 1:10,000). After a brief rinse, sections were incubated in secondary biotinylated antibody for 2 h (dilution 1:200; Ab-5 rabbit antiserum; Oncogene Research Products; Calbiochem, La Jolla, CA, USA). Final amplification was done with the aid of an avidin–biotin peroxidase complex. Following incubation in diaminobenzidine with ammonium nickel sulfate, the presence of Fos containing cell bodies was visualized with H_2O_2. All experiments conformed to the local and international guidelines on the ethical use of animals. Experiments were approved by the ethics committee of the University of Pretoria, South Africa, under the animal ethics clearance number E030110/002. The number of animals used in the experiment was limited to the absolute minimum, and all efforts were made to minimize their suffering.

Image analysis

The optical density of immuno-reacted cells in the SCN was assessed using a Leitz microscope equipped with a CCD camera (Photonic Science), according to Rieux et al., 2002. An image analysis program (Visiolab 1000; Biocom, Les Ulis, France) was used to determine the integral optical density (IOD) of each SCN section. This value takes into account the surface area of the label as well as the intensity of the label. The threshold well above the background level was determined visually for each animal before commencing analysis, and was kept constant. The IOD of all sections containing the SCN was summed; thus the measure represents the total IOD for the structure. According to Rieux et al. (2002), this method of quantifying Fos expression is the most objective.

Statistical analysis

As a result of the small sample size, the non-parametric Kruskal-Wallis ANOVA was employed to compare the different time periods, as well as stimulated and dark control animals. When the Fos expression levels were significantly different according to the Kruskal-Wallis ANOVA, the Mann-Whitney *U* test was performed to determine which groups were significantly different from each other. Statistical significance was maintained at $P<0.05$.

RESULTS

The Cape mole-rat (*G. capensis*)

Dark control animals did not have any noticeable Fos induction during any of the experimental time points. 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 light stimulated 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 that obtained 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 ($P=0.080$). A Mann Whitney *U* test was performed on the CT16 stimulated group and the dark control, which confirmed this result ($P=0.083$; Fig. 1).

By clumping the experimental groups for the subjective day (CT4 and CT10) and the two subjective night groups (CT16 and CT22) together, a significant difference was

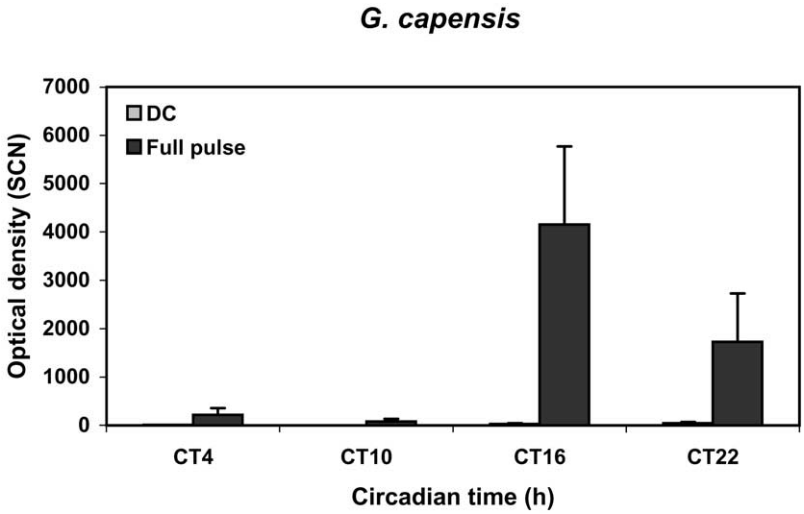

Fig. 1. Fos expression in response to a light pulse in the SCN of *G. capensis* at different CTs.

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 values for subjective night were markedly higher than those for subjective day (Mann-Whitney *U* test, subjective day/dark control: $P=0.046$, subjective night/dark control $P=0.011$; Fig. 2).

Highveld mole-rat (*C. hottentotus pretoriae*)

Over the whole 24-h 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 data sets. In fact, the dark control Fos expression is slightly higher than in the stimulated animals at CT 10 and CT16. The Fos expression in the experimental animals did not differ statistically from the dark control animals during either the subjective day or night (Kruskal-Wallis ANOVA, $P=0.333$; Fig. 3).

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 additive groups (Mann Whitney *U* test, $P=0.63$; Fig. 4).

DISCUSSION

In mammals, photoreceptive pigments have only been identified in the eye. The question has arisen as to whether mole-rats can perceive changes in light, and to what extent, since they possess microphthalmic eyes, and a regressed visual system. The small eye size necessarily results in a reduction of the absolute number of photoreceptors (Peichl et al., 2004). However, the proportion of cones in the retina is surprisingly high (approximately

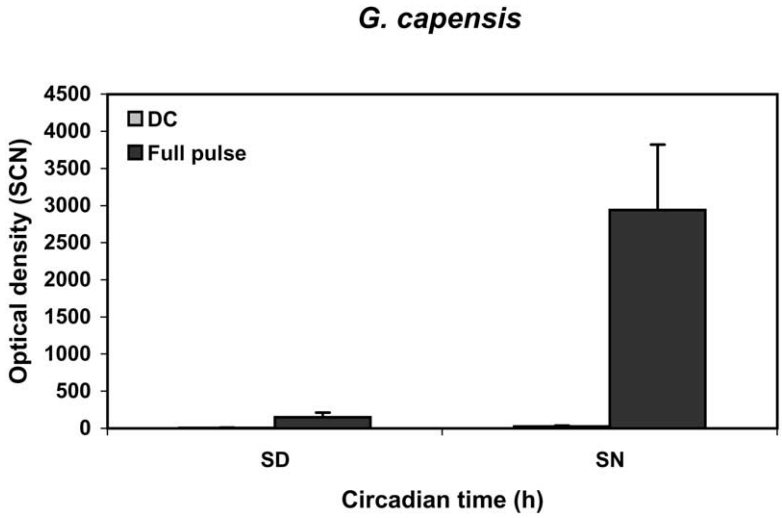

Fig. 2. Fos expression in response to a light pulse in the SCN of *G. capensis* during subjective day and subjective night.

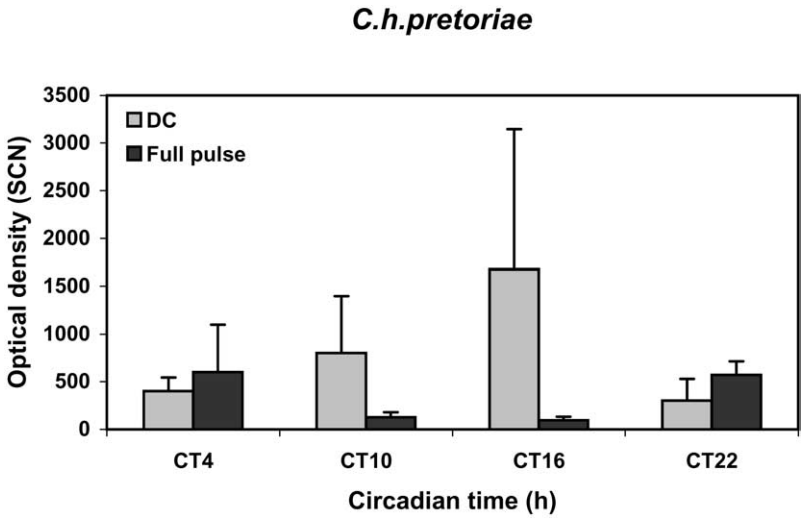

Fig. 3. Fos expression in response to a light pulse in the SCN of *C. hottentotus pretoriae* at different CTs.

10%), and corresponds to cone densities of the mouse, a nocturnal rodent (Peichl et al., 2004).

Despite the fact that mole-rats are not often exposed to light, it has been shown that they do indeed have endogenous rhythms that can be entrained by light (Lovegrove et al., 1995; Tobler et al., 1998; Riccio and Goldman, 2000; Oosthuizen et al., 2003).

Fos expression in rodents is dependent on the phase of the circadian clock. In both nocturnal and diurnal rodents, photic induction of Fos is low during subjective day and higher during the subjective night (Rose et al., 1999). 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 *G. capensis* displays sensitivity toward light at different times in the day. The Cape mole-rat is a nocturnal species (Lovegrove et al., 1995; Oosthuizen et al., 2003), and the results of Fos expression in response to light obtained from this experiment correspond to those of other rodents. Very little Fos expression occurs during the subjective day, whereas elevated levels of Fos expression occurs during the subjective night. The number of Fos immunoreactive cells in the SCN was extremely low compared with other rodents. Fos is only expressed during certain times of the circadian cycle and this can be interpreted as the circadian clock gating the circadian response of Fos expression according to the phase of the clock. These findings are in accord with the early immediate gene expression of another strictly subterranean and solitary mole-rat, *Spalax ehrenbergi* that displays a similar Fos induction pattern (Tobler et al., 1998).

The pattern of Fos expression in the SCN of these solitary, subterranean rodent moles corresponds to that of classical aboveground rodent models such as rats (Rusak et al., 1992) and hamsters (Kornhauser et al., 1990). Thus Fos expression in *G. capensis* is comparable to that of other rodents, although expressed at a lower intensity.

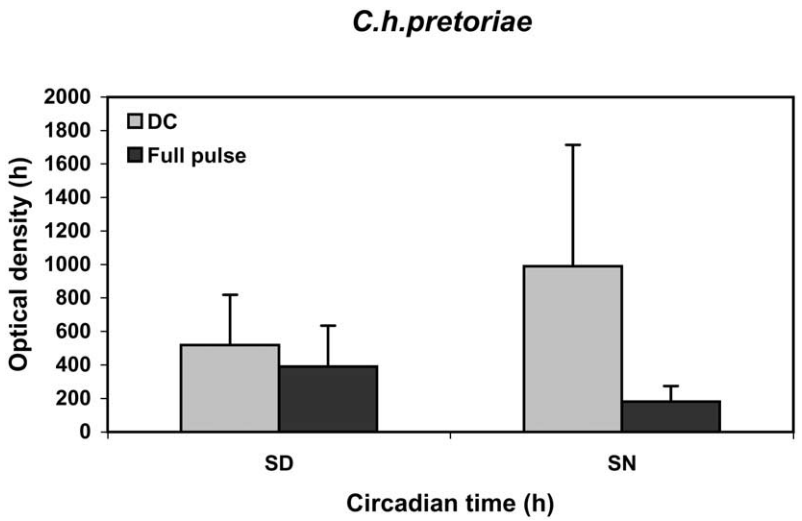

Fig. 4. Fos expression in response to a light pulse in the SCN of *C. hottentotus pretoriae* during subjective day and subjective night.

In contrast, the highveld mole-rat does not appear to be as sensitive to light as its solitary counterpart. The data obtained from this experiment showed no significant difference between stimulated and dark control groups, while the basal Fos expression was rather high compared with the solitary species. Accordingly, there was no indication of differential sensitivity to light at different times of the circadian cycle.

A similar trend is seen in other social species, the Zambian mole-rat also have a high basal Fos expression, and large intra-specific variation when light stimulated (Oelschläger et al., 2000). Also, the common mole-rat, Damaraland mole-rat and the naked mole-rat display no significant differences between dark control and stimulated groups at any of the CTs (Negroni, 1998). However, when comparing the summated values for the subjective day and night, Fos expression in the dark control animals was significantly different from the stimulated animals in the common and Damaraland mole-rats (Negroni, 1998).

The lack of response to light in *C. hottentotus pretoriae* can be attributed to a number of factors. 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, detailed studies on the retina indicate that there are well-developed photoreceptors and functionally visual pigments (Cernuda-Cernuda et al., 2003; Peichl et al., 2004). A more plausible alternative could be that the light pulse presented was either lacking in duration or intensity.

Thus, light information evidently reaches the SCN of *G. capensis* and is sufficient for the induction of Fos protein. In addition the Fos expression is gated according to the phase of the clock; although statistical analyses of the individual groups do not reflect a significant difference from the dark control group, this may be due to the small sample sizes.

It does not appear as if the SCN of *C. hottentotus pretoriae* effectively integrates light information in response to 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.

These findings support previous studies investigating locomotory activity rhythms and the rate of entrainment in solitary and social mole-rats (Oosthuizen et al., 2003). Fos induction over the circadian cycle of the solitary species investigated, was comparable to that of the solitary mole-rat, *S. ehrenbergi*, as well as aboveground rodents. In contrast, it appears as if the circadian clock of social species does not integrate light as efficiently. It is possible that the small sample sizes could influence the results obtained; however, since locomotor activity studies (Oosthuizen et al., 2003) provide similar results, it is unlikely.

The presence of circadian and more specifically circannual rhythms could be of greater importance for seasonally breeding, solitary species than for aseasonal, social species. Solitary species are highly aggressive toward conspecifics during the non-breeding season, while females allow males into their tunnels during the breeding season (Bennett and Jarvis, 1988). Therefore they need to be synchronized to the annual environmental cycles to anticipate the breeding season.

In contrast, the majority of the social species breed non-seasonally, and breeding partners are already at hand. It is therefore not cardinal for the survival of the more social species to retain a functional mechanism to keep track of the external environment, and could explain the higher degree of degeneration of the circadian system.

The presence of a time sense and ability to respond to changes in light could suggest that bathyergids were once surface dwelling species, relying on chronobiological rhythms for survival. Over evolutionary time, the adoption of a strictly fossorial lifestyle may have alleviated the dependence on light changes in the regulation of activity. Seasonal timing is still of significant value for seasonally breeding species, while it is redundant for aseasonal and social species. This would offer an explanation for the present day scenario where circadian rhythmicity appears not to be essential for the survival of mole-rats.

Acknowledgments—Eugene Oosthuizen is thanked for help with trapping mole-rats and Naura Chounlamountri is thanked for technical assistance in the laboratory. This research has been conducted with support of a South African (NRF)/French Ministry partnership grant. We acknowledge a grant from the National Research Foundation, South Africa, and the University of Pretoria to N.C.B. to collect the mole-rats.

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(Accepted 10 January 2005)
(Available online 4 May 2005)