Photic induction of Fos in the SCN of African mole-rats: responses to increasing irradiance.

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Abstract

African mole-rats (family Bathyergidae) are strictly subterranean rodent species that are rarely exposed to environmental light. Morphological and physiological adaptations to the underground environment include a severely reduced eye size and regressed visual system. Responses of the circadian system to light, however, appear to be intact since mole-rats are able to entrain their circadian activity rhythms to the light-dark cycle and light induces Fos expression in the SCN. Social organization varies from solitary species to highly elaborated eusocial structures, characterized by a distinct division of labour and in which one reproductive female regulates the behavior and reproductive physiology of other individuals in the colony. We studied light-induced Fos expression in the SCN to increasing light intensities in four mole-rat species, ranging from strictly solitary to highly social. In the solitary Cape mole-rat, light induces significant Fos expression in the SCN and the number of Fos-immunopositive cells increases with increasing light intensity. In contrast, Fos induction in the SCN of social species was slightly greater than but was not statistically different from the dark control animals as is typical of most rodents. One species showed a trend for an increase in expression with increased light while a second species showed no trend in expression. In the naked mole-rat, Fos expression appeared higher in the dark controls than in the animals exposed to light, although these differences in Fos expression were not significant. These results suggest a gradient in the sensitivity of the circadian system to light in mole-rats, with a higher percentage of individuals that are unresponsive to light in correlation with the degree of sociality. In highly social species such as the naked mole-rat that live in a relatively stable subterranean milieu in terms of food availability, temperature, constant darkness and, that is devoid of 24-hr cyclic cues from the environment, the temporal coordination of rest-wake activities may be dependent on social interactions and social status rather than on photic regulation of the circadian timing system.

Introduction

The visual systems of subterranean mammals have received an increasing amount of attention over recent years (Cooper *et al.* 1993a,b, Němec*et al.* 2004, 2007, 2008). Different species of subterranean mammals show a large degree of diversity in the development of their visual systems ranging from species with severe visual regression and a complete lack of visual abilities to other species that possess visual capabilities equal to those of certain aboveground rodents (Němec*et al.* 2007, 2008).

Eye size appears to be a good predictor of visual capabilities. The Eurasian mole-rat, *Spalax*spp, possesses minute subcutaneous eyes and lacks any image forming abilities (Haim*et al.* 1983, Necker *et al.* 1992, Cooper *et al*., 1993a, b). However, large-eyed fossorial species such as ctenomyids and pocket gophers have reasonable visual acuity and are capable of detecting moving objects (Němec*et al.* 2007). African mole-rats from the family Bathyergidae fall somewhere between these two extremes, with some variation in eye size and visual abilities among the different species. African mole-rats have small superficial eyes and are able to distinguish between light and dark (Wegner *et al*. 2006). However, severe regression of midbrain structures impairs coordination of visuomotor reflexes (see Němec*et al.* 2004,Němec*et al.* 2007).

Despite the regression of the visual structures in African mole-rats, a functional circadian system is conserved. All species thus far investigated display locomotor activity rhythms that can be entrained to light (Hart *et al.* 2004, Oosthuizen*et al.* 2003, Riccio and Goldman 2000a, Schöttner*et al.* 2006, Vasicek*et al.* 2005a). In addition, melatonin secretion (Gutjahr*et al.* 2004, Hart *et al.* 2004; Richter *et al.* 2003, Vasicek*et al.* 2005b) and body temperature (Lovegrove and Muir 1996, Riccio& Goldman 2000b) also show rhythmic cycles and light can suppress melatonin secretion in the blind mole-rat (Zubidat*et al.* 2009).

The circadian system enables animals to anticipate cyclical environmental events, which is an important adaptation for survival. The suprachiasmatic nucleus (SCN) is the central circadian pacemaker that is responsible for the generation of endogenous biological rhythms (Morin 1994). For appropriate function, the phase of the circadian clock must be synchronized with the external environment (Aschoff& Pohl 1978). The daily light dark cycle is the primary zeitgeber to which endogenous circadian rhythms entrain. In mole-rats, the SCN is of normal size, receives a bilateral innervation from the retina, contains typical neuropeptide expressing cells and expresses Fos in response to light stimulation (Cooper *et al*. 1993a, b, Crish*et al.* 2006, Negroni*et al.* 1997, Negroni*et al*. 2003, Němec*et al.* 2004, Němec*et al.* 2007; Oosthuizen*et al*. 2005, Vuillez*et al*. 1994,). However, the photic induction of Fos in SCN and light entrainment of circadian activity rhythms appears to qualitatively differ in certain solitary and social species of mole-rats. In the solitary Cape mole-rat, as in other rodents, Fos is expressed in the SCN in response to light during the subjective night but not during the subjective day. However in certain social species a clear phase dependence of Fos expression was not observed (Oosthuizen*et al*. 2005).

In rodents, behavioural phase shifts and Fos expression in the SCN increase with increases in light irradiance and duration of exposure, and these increases are directly proportional to the number of photons in the light stimulus (Nelson and Takahashi 1991, Dkhissi-Benyahya*et al.* 2000). The difference in light responsiveness of the SCN between solitary and social species raises the question as to whether social species may require higher light intensities to elicit a response. To further investigate the light sensitivity of the SCN in African mole-rats, four species of mole-rat with varying levels of social organization (solitary, social and eusocial) and eye development were exposed to light pulses of increasing intensity.

Material and Methods

In this study, one solitary species, the Cape mole-rat (*Georychuscapensis*; n=15) and three social species, the common mole-rat (*Cryptomyshottentotushottentotus*; n=25), the Damaraland mole-rat (*Fukomysdamarensis*; n=13) and the naked mole-rat (*Heterocephalusglaber*; n=12) were examined for Fos expression in the SCN. The Cape and common mole-rats were captured in Darling, Western Cape (33^{o}22'S 15^{o}25'E), and the Damaraland mole-rats were captured in Hotazel, Northern Cape (27^{o}58'S 17^{o}41'E). The naked mole-rats were offspring that had been derived from individuals captured at Archers Post and Lerata water hole in northern Kenya (0^{o}38'N 37^{o}40'E). Sample sizes were rather small as certain species are difficult to obtain.

While in the laboratory, animals were housed individuallyin plastic basins containing a thin layer ofwood shavings and were fed on sweet potato, carrots, gem squash and apples. All animals were maintained on a 12L:12D light cycle for two weeks at 25ºC. Light was provided by fluorescent tubes (500 lux, OSRAM lumilux plus). Procedures on live animals followed guidelines approved by Chronobiology International (Portaluppi*et al*. 2008), and were approved by the Animal Use and Care Committee of the University of Pretoria (No. 000418-006). For administration of the light pulses, the animals were removed from their home cage and exposed to monochromatic light in a specifically designed light pulse chamber (Rieux*et al.* 2002). Light pulses of increasing irradiance to investigate the resulting Fos response in the SCN in an Ascoff type II protocol. On the day of the experiment, the lights remained off following the end of the previous light cycle, such that ZT0 corresponded to CT0 (Circadian Time 0), the beginning of the subjective day. Animals were exposed to a 15-minute monochromatic light pulse (500 nm,

half bandwidth=10 nm) at CT16, corresponding to the time point that elicited the greatest response in previous studies (Oosthuizen*et al*. 2005) and in nocturnal rodents in general. The experimental groups were exposed to light pulse intensities ranging from 3.1 x 10^8 photons.cm^{-2}.s^{-1} to 3.1 x 10^{14} photons.cm^{-2}.s^{-1}. Animals that served as dark controls received an identical treatment, but were not exposed to light.

After light exposure the mole-rats were returned to darkness and subsequently sacrificed one hour after the beginning of light stimulation. Using a red headlamp in darkness, animals were deeply anaesthetized with an overdose of fluorothane anaesthetic. Once breathing had ceased, animals were transferred to a dimly lit room and perfused intracardially with 0.9% saline at 37^{o}C, followed by 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4, Sigma) at 4^{o}C. The brains were stored in 2% PFA until further treatment. Prior to sectioning, brains were placed in 30% sucrose until saturated for cryoprotection. 40μm thick coronal sections were cut on a freezing microtome. Every second section of the SCN (6-10 sections per animal) was processed for Fos immunohistochemistry.

Endogenous peroxidase was suppressed using an alcohol-saline-H_2O_2 solution. Sections were incubated in normal goat serum (1 hr), rinsed in phosphate buffer and then incubated in Fos primary antibody (dilution 1:10 000; rabbit polyclonal anti-c-fos, Oncogene Science, #PC05) for three days at 4^{o}C. Following the primary antibody, sections were rinsed and incubated in a secondary biotinylated antibody for two hours (dilution 1:200; Ab-5 rabbit antiserum, Oncogene Research Products, Calbiochem, La Jolla, CA). Final amplification of the Fos protein was performed with the aid of an avidin-biotin peroxidase complex. The presence of Fos containing cell bodies was visualized with diaminobenzidine (DAB) with ammonium nickel sulphate and 0.005% H_2O_2.

Analysis of Fosimmunolabel

The optical density of immuno-reacted cell bodies in the SCN was assessed using aAristoplan microscope (Leica) equipped with a CCD camera (Photonic Science). An image analysis program (Visiolab 1000, Biocom, LesUlis, France) was used to determine the integral optical density (IOD) of each SCN section. This value takes into account the surface area as well as the intensity of the labelled neurons (Rieux*et al.* 2002). The IOD of all sections containing the SCN were summed, thus the measure represents the total IOD for the structure.

Statistical analysis

Due to the small sample size and non-normal distribution, non-parametric statistical analyses (Mann Whitney U-test and Kruskal-Wallis ANOVA) were employed to compare the dark control and light stimulated groups. A Kruskal-Wallis ANOVA was used to compare all the stimulated groups with one another, whereas the Mann Whitney U-test was applied to compare the dark control with each of the stimulated groups. Statistical significance was maintained at $p<0.05$. Errors are expressed as SEM.

Results

In the Cape mole-rat, the level of Fos expression in the SCN of the dark control animals was extremely low compared to background. In the two groups exposed to 3.1×10^{12} and 3.1×10^{14} photons/cm^2/sec, Fos expression in the SCN was significantly higher than the dark control animals (Kruskal-Wallis ANOVA, $X^2 = 6.29, p=0.04$). Animals stimulated with the higher

light intensity showed a significantly greater amount of Fos expression in the SCN compared to the group exposed to two log units lower light intensity (Mann Whitney U-test, n_1=6, n_2=6, Z=-2.56, p=0.008; Fig. 1A, Fig. 2). There is a clear increasing trend of responsiveness with increasing light intensity (R^2=0.97).

The basal level of Fos expression in the common mole-rat in darkness was relatively high compared to background and slightly higher than in the Cape mole-rat. Light exposure, however, did not lead to significant Fos expression in the SCN compared to the dark control animals. There was no significant difference between the groups of light stimulated animals (Kruskal-Wallis ANOVA, X^2 =7.77,p=0.10). Fos expression in the SCN in response to increasing light intensities was highly variable within and between each experimental group. There was no significant difference in Fos expression between any of the different stimulated groups (Kruskal-Wallis ANOVA, X^2 =4.0,p=0.26; Fig. 1B, Fig. 2). There was only a weak positive trend in the regression for Fos induction (R^2 =0.20)

In the Damaraland mole-rat, Fos expression in the SCN of the dark control animals was relatively low. However none of the stimulated groups showed significantly higher Fos expression than the dark control group (Kruskal-Wallis ANOVA,X^2 =3.28, p=0.35). Visually, Fos expression in the SCN of light stimulated groups increased with light intensity as reflected by an increased trend in responses to light (R^2 =0.96). However, due to the variation in responses within each group including certain individuals that appeared not to respond to light stimulation, there was no significant difference in the Fos expression in the SCN between the groups (Kruskal-Wallis ANOVA, X^2 =2.44, p=0.89; Fig. 1C, Fig. 2).

In the naked mole-rat Fos expression was low in all experimental groups. The highest level was seen in one of the dark control animals. There was no significant difference between

the dark control group and any of the light stimulated groups (Kruskal-Wallis ANOVA,X^2 =3.0, p=0.39) or between the different light stimulated groups (Kruskal-Wallis ANOVA, X^2 =2.13, p=0.09; Fig. 1D, Fig. 2). Most individuals appeared to be non-responders to the light stimulation with even a loose negative trend in responses to increasing light (R^2 =0.45).

Discussion

Mole-rats are strictly subterranean and are nearly never exposed to the natural light/dark cycle (Bennett &Faulkes 2000). Subjected to an environment devoid of light, evolution of the visual system has led to severe regression in eye size and visual structures of the brain with the exception of the SCN (Cooper *et al*. 1993a,b, Němec *et al.* 2007). Individuals of all mole-rat species investigated to date express light entrainable circadian activity rhythms as well as light induced Fos expression in the SCN (Vuillez*et al.* 1994, Tobler*et al*. 1998, Riccio& Goldman 2000a, Oosthuizen*et al*. 2003, 2005, Hart *et al*. 2004, Schöttner*et al*. 2006, Vasicek*et al*. 2005a).

African mole-rats display a gradient of sociality with species ranging from strictly solitary, to social and a highly evolved eusocial colony organisation (Jarvis & Bennett, 1991). Of the social mole-rat species, two (Damaraland and naked) are classified as eusocial. Eusociality is a term used for the highest level of social organisation and defined by three characteristics: a reproductive division of labour, overlapping generations and cooperative care of the young. Other social mole-rat species display some of these characteristics but not all. Also, the eusocial species typically have larger colony sizes than the other social species (Faulkes*et al.* 1997). In social and eusocial species, a single sexually mature reproductive female and several

reproductive males are present, while all other individuals are workers in the colony. Subordinate, non-breeding animals in a colony are reproductively suppressed. Reproductive suppression amongstsubordinate animals is achieved through either behavioural or physiological interactions or a combination ofthe two. Behavioural suppression mostly occurs in the more loosely social species and entails interference with breeding attemptsof subordinate animals by dominant animals or the subordinate individualsthat refrain from breeding to avoid inbreeding (Snowdon 1996). Alternatively, in eusocial species, reproduction is physiologically interrupted and in extreme cases, reproductioncan be completely suppressed by blocking ovulation (Abbott 1987, Bennett *etal*.1999). Eusocial species normally have larger colony sizes and like other eusocial animals have a distinct division of labour in the colony (Bennett &Faulkes 2000).

This gradient from solitary to social species appears to parallel an inverse gradient of responsiveness of the mole-rat circadian system to light. Behaviourally, solitary animals display more robust locomotor activity rhythms than social species (Hart *et al.* 2004, Lovegrove*et al*. 1993, 1995, Oosthuizen*et al.* 2003, Riccio*et al*. 2000a, Schöttner*et al*. 2006, Vasicek*et al*. 2005a). These studies and the present report indicate that the gradient in responsiveness between solitary and social species is also expressed in terms of the number of individuals in a given species that express distinct rhythms of activity or light induced Fos expression (Fig. 3). In the solitary Cape mole-rat, Fos in the SCN is expressed differentially according to the time of light exposure, similar to that of other normally sighted rodents (Oosthuizen*et al.* 2005). In contrast, certain social species appear to be less responsive to light and show no indication of a gated phase response of Fos over the circadian cycle (Oelschläger*et al*. 2000, Oosthuizen*et al*. 2005). These same species also show no significant increase of Fos induction with irradiance and/or a higher proportion of animals that appear non-responsive to light.

The solitary Cape mole-rat exhibits an extremely low basal level of Fos in the SCN in constant darkness. Fos expression in the SCN of the solitary Cape mole-rat increases with increasing light intensity, similar to findings in other rodents. Another solitary mole-rat species, *Spalaxehrenbergi*, which has subcutaneous eyes, nevertheless shows physiological responsiveness at unexpectedly low threshold irradiances (Zubidat*et al.* 2009). In contrast with the solitary species, none of the social species of mole-rats show a significant increase in Fos expression even when relatively high light intensities are used (10^{14} photons/cm^2/sec) that, in other rodents such as mice and hamsters, cause saturating responses (Kornhauser*et al*. 1992, Dkhissi-Benyahya*et al.* 2000, Rieux*et al.* 2002).

Three trends however, can be distinguished in the social mole-rat species. The Damaraland mole-rat shows a clear tendency towards an increase in the response, although even at the highest irradiance Fos expression is not significantly different from the dark control. In the common mole-rat, light exposure leads little or no increase in the response in relation to light intensity. The naked mole-rat shows consistently low levels of Fos expression in all cases and a slightly negative trend in response to higher light levels. The lack of significance in the response to increasing light appears to be mainly related to the high degree of variability of the responses (partly due to the relatively small sample sizes) and the presence of individuals that are non-light responsive (Fig. 3). Individual variations in response to light are not unique to mole-rats, as "non-responders" (no light-induced shift response) have also been described in diurnal chipmunks (Honma&Honma, 1999) and certain individual Degus show phase advances but not phase delays in response to a light pulse (Lee &Labyak 1997).

The variability and/or lack of a relation between Fos expression and light exposure may be related to a weak coupling of the circadian pacemaker to the initial entraining LD cycle prior

to the light pulse at CT16. Previous studies in a social species of the highveld mole-rat have shown a weak entrainment of circadian locomotor activity rhythms by light and the lack of a differential expression of Fos in response to light exposure during the subjective day and subjective night (Oosthuizen*et al.* 2003, 2005). In the present Ascoff type II protocol, a lack, or weak, phase coupling of the circadian pacemaker may result in an insufficient alignment of the pacemaker to the previous LD cycle in certain individuals.

Photic information transmitted from the retina influences the circadian pacemaker in the SCN of mole-rats, as most species display circadian rhythms and show gene expression in response to light stimulation (Lovegrove*et al*. 1993, 1995, Oelschläger*et al*. 2000, Riccio& Goldman 2000a, Oosthuizen*et al*. 2003, 2005). In certain social species, this photic information may be insufficient to entrain the circadian clock or to induce significant Fos expression. The naked mole-rat is an extreme case and expresses very low levels of Fos regardless of the intensity of the light pulse. It is debatable whether this species shows a response to light stimulation in the SCN at all, since Fos has a basal level of expression in the SCN that is independent of light. This is in agreement with observations that only a few individuals of naked mole-rats exhibit circadian rhythms in the presence of a light cycle (Riccio& Goldman 2000a). Naked mole-rats are at the pinnacle of mammalian sociality and it may be argued that in the subterranean niche, devoid of rhythmic cues from the environment (including light), temporal physiology in this species is regulated independently of a circadian rhythm. Naked mole-rats show many unusual features for a mammal, including a lack of homoeothermic regulation of body temperature and the determination of reproductive function and morphological development through social interaction. In naked mole-rats, reproductive status is strictly

controlled by the single dominant breeding female, with breeding occurring throughout the year (Bennett &Faulkes 2000).

In contrast, all solitary species have a seasonal period of breeding which may provide a selective pressure for the conservation of the circadian system. Young are born at a time of the year which is optimal for their survival, therefore the adults require a temporal measure of environmental time to anticipate the breeding season. These cues may be provided by light directly and/or indirectly by food availability and temperature. Solitary mole-rats are occasionally seen aboveground and an intact circadian system may ensure that their infrequent ventures aboveground occur at a time when their potential predators are least active. It may well be that this species is more dependent on light cues than their social counterparts who rarely, if ever are found on the surface.

Adaptation to the unique conditions of the subterranean environment has led to a number of regressive features in mole-rats that have shaped the evolution of genes controlling eye development, metabolism and circadian rhythms. The blind mole-rat has a truncatedαB-crystallin promoter that lacks one or more critical DNA regulatory elements needed for lens expression (Li*et al*. 2007). Mole-rats have also lost significant long-wavelength (LW) cone opsin (Peichl*et al*. 2004) or short-wavelength (SW) cone opsin expression (David-Gray *et al.* 1999), while melanopsin expression in retinal ganglion cells is conserved (Hannibal *et al*. 2002). Clock genes of mole-rat species have also been shown to have numerous deletions, substitutions and polymorphisms (Avivi*et al*. 2001, 2002, 2004). Although the circadian cycling of clock genes (*Clock/Bmal1, Per, Cry*) and light induction of *Per1/Per2* appears to be conserved, certain gene substitutions can lead to a reduction of transcriptional activity of essential clock components (*Clock/Bmal1*; Avivi*et al.* 2001). Circadian and other natural clock-like endogenous rhythms

have evolved to anticipate regular temporal changes in the environment and there is evidence from Drosophila that thermal conditions may have contributed to variation in the Thr-Gly repeat of *Per* genes (Kyriacou*et al.* 2008). The relaxed selection pressures in the thermally stable, lightless and predictable subterranean niche may be a driving factor affecting clock genes and rhythmic functions that may in turn affect other complex social and reproductive behaviors (Sandrelli*et al*. 2008).

African mole-rats may thus represent a cline of adaptations of the visual system, with a general trend of regression of both vision and photic sensitivity of the circadian system. The trend in the evolution of mole-rat species towards increased confinement in a relatively stable subterranean habitat and the development of unique and rigid social systems may be associated with a decreased utility of sensitivity of the circadian system to light in this particular environmental context.

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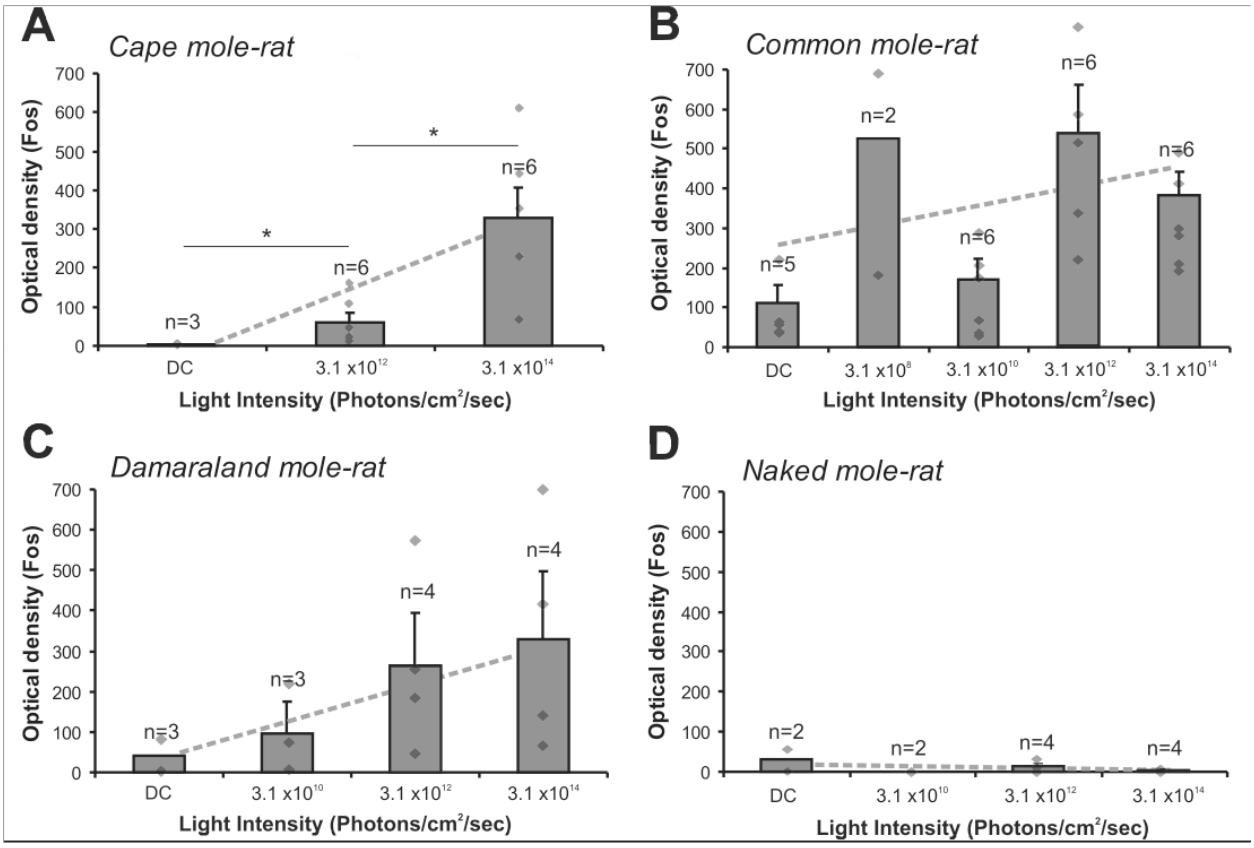

Figure 1. Quantity of Fos expression (expressed as optical density) in the SCN of the different mole rat species in response to increasing irradiance. Individual values are indicated as a scatter plot overlay. (A) Solitary Cape mole-rat (*Georychuscapensis*). (B) Social common mole-rat (*Cryptomyshottentotus*), (C) eusocialDamaraland mole-rat (*Cryptomysdamarensis*), (D) eusocial naked mole-rat (*Heterocephalusglaber*). Significant differences in the amounts of Fos expression were only observed in the Cape mole-rat (*>0.05). The gray dashed lines illustrate the linear regressions for the trends in dose-responses (A, R^2=0.97; B, R^2 =0.20; C, R^2 =0.96; D, R^2 =0.45).

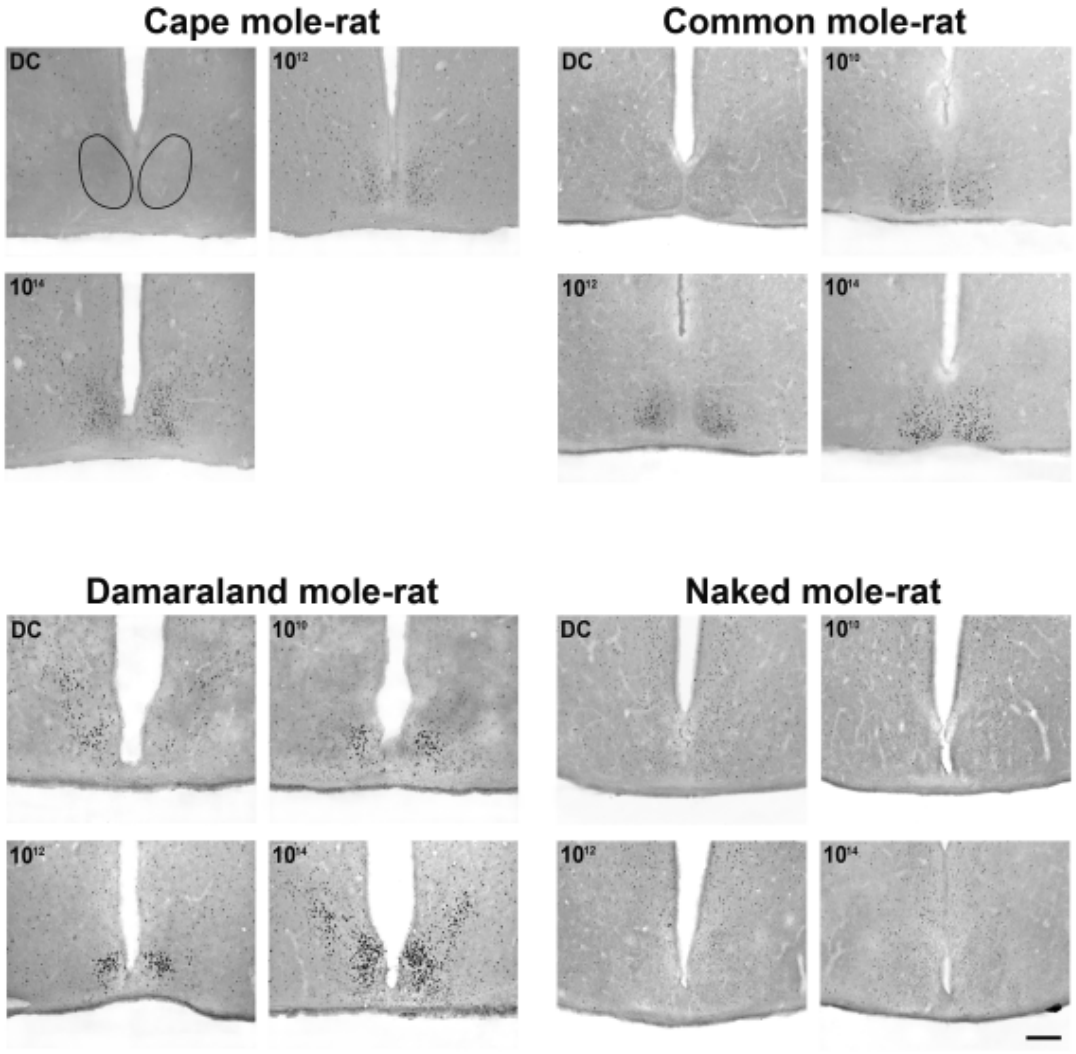

Figure 2. Fos expression in the SCN in response to light of the solitary Cape mole-rat (*Georychuscapensis*) and the social mole-rats: Common mole-rat (*Cryptomyshottentotus),*Damaraland mole-rat (*Cryptomysdamarensis*) and naked mole-rat (*Heterocephalusglabe*r). Photomicrographs were taken from light responsive animals. The outline of the SCN is indicated in the top left photomicrograph. DC=dark control. Light intensity is indicated in photons/cm^2/sec. Scale bar = 200 µm.

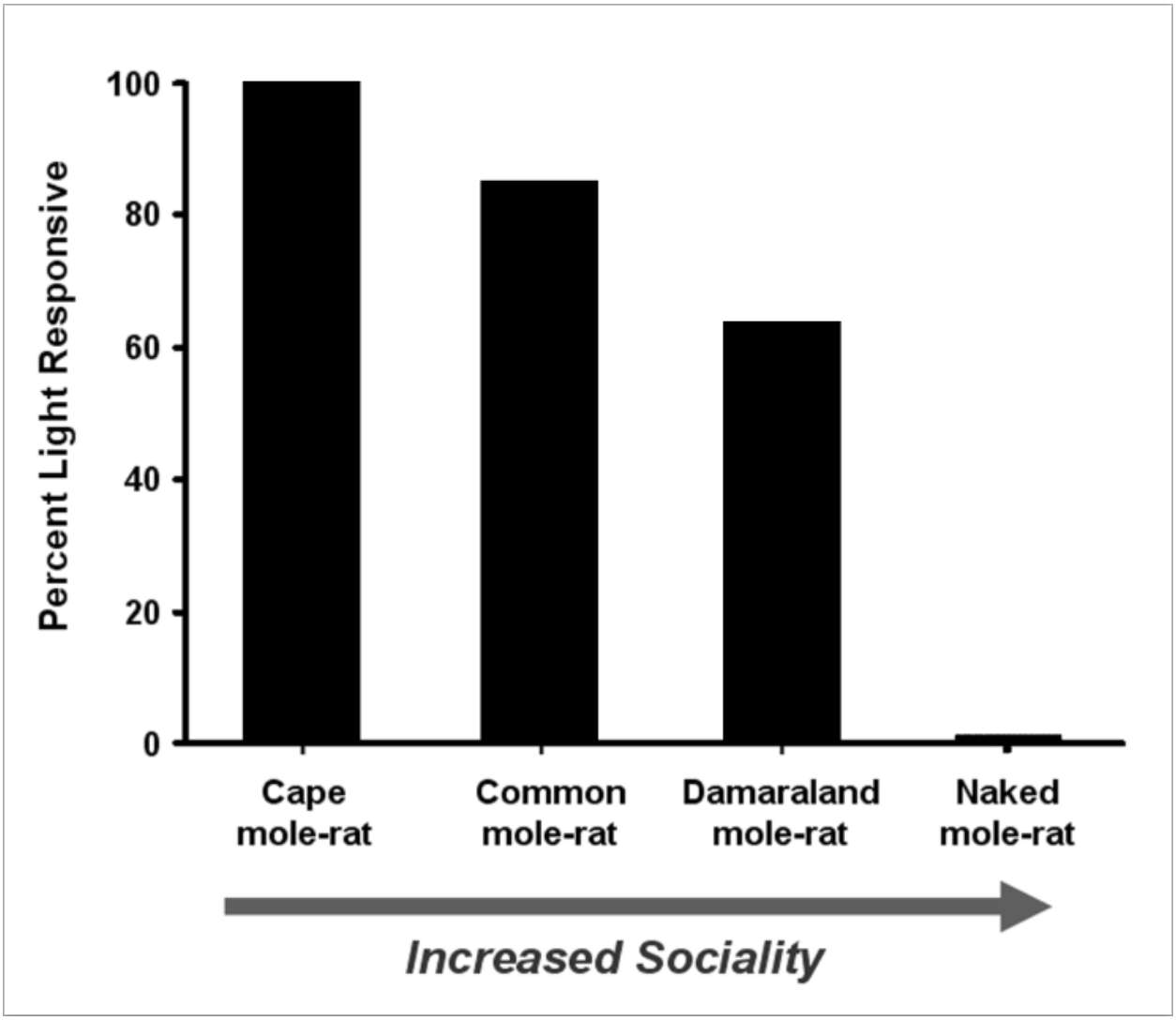

Figure 3. Percent of light responsive animals within each species of mole rats. Light responsive was defined as animals exposed to light that express Fos values greater than the dark control values plus one standard deviation. The percent of animals in the population that respond to light decreases with increased degrees of sociality from the solitary Cape mole-rat (100%) to the highly eusocial Naked mole-rat (0%).