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Light-induced Fos expression in the suprachiasmatic nucleus of the four-striped field mouse, *Rhabdomys pumilio*: A southern African diurnal rodent

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Abstract

Previous studies have suggested that nocturnal and diurnal species of rodents differ in their circadian responses to light including phase shifts and early gene expression. *Rhabdomys pumilio*, the four-striped field mouse, is diurnal both in nature and in the laboratory. We studied in this species the response of the suprachiasmatic nucleus (SCN) to light stimuli at different time periods using light-induced expression of Fos as marker of neuronal activity. Fos induction in the SCN was investigated using immunohistochemistry and quantitative image analysis. The animals were exposed to a 15 min light pulse with monochromatic green light at different circadian times throughout a 24-h cycle. Animals maintained in constant darkness served as controls. *R. pumilio* exhibited an endogenous Fos rhythm in the SCN during constant darkness with highest expression during the subjective day at circadian time (CT) 2 and CT10. Photic stimulation resulted in significant Fos induction in the SCN at CT6, CT14, CT18 and CT22, compared to controls kept in constant darkness, with a peak of expression at CT22, i.e. during late subjective night, mainly due to expression in the ventral SCN. In tract tracing experiments based on the use of cholera toxin subunit B, we found that retinal fibres innervate mainly the contralateral ventral SCN. The intergeniculate leaflet received bilateral retinal innervation with overlap between ipsilateral and contralateral fibres. Altogether the data show that the rodent *R. pumilio* is a unique diurnal model for chronobiological studies.

Keywords: Visual system; Retina; Phase shift; *c-Fos*; Circadian rhythm

1. Introduction

The suprachiasmatic nucleus (SCN), the site of the primary circadian pacemaker in mammals [5,20,23,25], is located in the anterior hypothalamus dorsal to the optic chiasm. Light induces in the SCN a rapid and transient expression of *c-Fos*, a molecular marker of neuronal activity and the amplitude of Fos protein synthesis has been shown to be proportional to the total number of photons in the light stimulus [8]. Fos protein synthesis has also been shown to be phase-dependent in nocturnal rodents and this phase-dependence is correlated with that for light-induced phase shifts of locomotor activity [2–4,8,21,24].

The pattern of Fos protein expression in nocturnal animals is well elucidated, whereas information on the relationships between the molecular mechanisms, clock genes or Fos expression and behavioural phase shifts in diurnal animals is rather limited. Honma and Honma [13] and Abe et al. [1] found that photic induction of Fos in the diurnal chipmunk, *Eutamias asiaticus*, does not strictly correspond with the times at which light can induce behavioural phase shifts, since Fos is expressed during both the subjective night and the subjective day, while behavioural phase shifts occur only during subjective night. In *Octodon degus*, in which phase shifts occur at circadian time (CT) 16 and CT4, there is an increase in Fos expression at CT16

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(subjective night) in the ventrolateral SCN but a decrease in Fos expression in the dorso-medial SCN at CT4 [16,17]. The diurnal *Arvicanthis niloticus* more closely resembles nocturnal species, since the increase in Fos expression during the subjective night corresponds to the times when light also elicits a phase shift [19]. Surprisingly, in primates, the most diurnal of all mammals, no information on photic-induced phase shifts is available. In contrast, the human phase response curve (PRC) differs from all nocturnal species in that light can induce a shift throughout the 24-h circadian cycle, whereas the rodent PRC shows a "dead zone" in mid-subjective day during which the system is unresponsive to light [15].

To further clarify the pattern of photic responses to light in diurnal mammals, we here analysed Fos expression in the SCN of *Rhabdomys pumilio*, a rodent with diurnal behaviour in the field and when exposed to different light regimes [26]. The retina of *Rhabdomys* contains roughly 40% cones which is an extremely high percentage for a murine rodent (H.M. Cooper, unpublished observations). Additionally, since photic induction of Fos occurs mainly in the ventrolateral part of the SCN that receives retinal afferents [2,8,28], cholera toxin subunit B (CTb) was injected in the eye of *R. pumilio* to define the pattern of retinal innervation in the SCN and in other visual structures in this species.

R. pumilio forms one of the few diurnal rodent divisions in Africa and this is the first study to characterise Fos expression in the SCN as well as retinal projections to the SCN and intergeniculate leaflet (IGL). The four-striped field mouse, *R. pumilio*, has a discontinuous distribution from southern Africa (South Africa, Angola, Botswana, Zimbabwe and Malawi) to East Africa (Kenya, Tanzania and Uganda) [27]. They live aboveground in heavily vegetated areas and their nest sites characteristically have runways, which lead from one bush to another. *R. pumilio* has a highly structured social system based upon a dominance hierarchy. They are grey-brown in colour, with four distinctive black stripes on their back, are approximately 10–14 cm in length and weigh 40–70 g. Small mammals can be aged using body mass, pelage characteristics, as well as body and tail length. Body mass was used to age *R. pumilio* according to the table constructed by (P.M. Brooks, University of Pretoria, personal communication).

2. Materials and methods

2.1. Experiment 1: Fos induction

2.1.1. Photic stimulation and Fos immunohistochemistry

The mice used in the experiments were live trapped (following ethical regulations) with Sherman traps in the Cape Flats Nature Reserve, Bellville in Cape Town, South Africa. Only animals 10 weeks or older were used for experimentation. 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 the Western Cape. The number of animals used in the experiment was limited to the absolute minimum and all efforts were made to minimise their suffering.

Forty-eight male *R. pumilio* housed individually in plastic cages (60 cm × 45 cm × 35 cm) were entrained to a 12-h light:12-h dark (LD) cycle for 3 weeks in a room illuminated with fluorescent lights of approximately 500 lx. The mice were maintained under complete darkness during the dark phase of the 12-h light:12-h dark LD cycle prior to experimentation.

When an animal is entrained to an environmental factor (*Zeitgeber*) such as the LD cycle, the clock time is referred to as *Zeitgeber* time (ZT), whereas in constant conditions the behavioural rhythm is under the control of the endogenous circadian clock and the time reference is referred to as CT. In our experiments, we used an Aschoff type 2 protocol, in which the lights remained off after the last dark phase preceding the day of light exposures. In this way, the animals are maintained in constant darkness and the time that the light would have normally been turned on is CT0 (beginning of subjective day) instead of ZT0 beginning of real day) (for more details see ref. [7]).

Five animals per time point were pulsed with monochromatic green light (10^{14} photons/cm^2/s) for 15 min at CT2, 6, 10, 14, 18 and 23. The animals were perfused 60 min after the end of the light pulse. Three additional mice per time point were used as controls in darkness and were not exposed to light.

The mice were sacrificed by an overdose of halothane anaesthetic in a jar filled with halothane vapour and subsequently injected intramuscularly with 0.3 ml sodium pentobarbital. Once no movement was visible, they were perfused intracardially with warmed (37 °C), 0.9% saline followed by Zamboni fixative (0.1M sodium phosphate, 2% paraformaldehyde and 15% picric acid). The brains were postfixed for 24 h at 4 °C in the fixative and subsequently placed in 30% sucrose for cryoprotection. Coronal sections (40 μm-thick) were subsequently cut on a freezing microtome. Every second section through the rostrocaudal extent of the SCN was collected and subsequently processed for Fos protein immunohistochemistry. Sections from all experimental and control animals were reacted simultaneously to assure identical treatment necessary for the subsequent quantitative image analysis [22]. Endogenous peroxidase activity was suppressed using 50% alcohol in saline with 0.03% H_2O_2. The sections were briefly rinsed in 0.01 M phosphate-buffered saline, pH 7.4 and incubated overnight in PBSTA (0.01 M phosphate buffer, pH 7.4, 0.9% saline, 0.3% Triton-X and 0.1% sodium azide) containing 1.5% normal goat serum. Subsequently, the sections were incubated in anti-Fos primary antibody (rabbit polyclonal anti-*c-Fos*, Oncogene Research Products, Calbiochem, La Jolla, CA; dilution: 1:3000) for 3 days at 4 °C. Sections were rinsed twice in PBST for 10 min and incubated in a secondary biotinylated antibody (Ab-5 rabbit antiserum, Oncogene Research Products; dilution 1/100) for 2 h at room temperature. Immunoreactivity was visualised using a Vectastain ABC Elite kit (PK-6100, Vector Laboratories, Burlingame, CA) followed by one rinse in PBST and two rinses in 0.05 M Tris buffer, pH 7.6. Horseradish peroxidase reaction product was visualised by incubation in 0.2% 3.3′-diaminobenzidine with 0.5% ammonium nickel sulphate and 0.015% hydrogen peroxide in Tris buffer. After two rinses in Tris buffer, sections were mounted on gelatinised slides and coverslipped with Depex.

2.1.2. Image analysis

The optical density of the immunohistochemical label was assessed using a computerised image analysis software (Visiolab, Biocom, Les Ulis, France) connected to a microscope (Aristoplan, Leica) via a cooled digital camera (Photonic Science). The software analyses the gray level (GL) of every pixel of the digitised image and the GL values can vary from zero (black) to 255 (white). Integral optical density (IOD) is equal to the negative log of GL_{object} divided by GL_{max}, where GL_{max} is the mean gray level of a reference region with maximal transmittance. The measure of IOD affords the most objective measurement of Fos expression in the SCN since various parameters, such as conditions of illumination and threshold may be maintained constant throughout the analysis [22]. Fos expression was measured in both the left and right SCN (five to seven sections per animal) along the rostrocaudal axis in both the ventral and dorsal regions of the SCN. The limits of the SCN were visible in the non-counterstained Fos-reacted sections as a region of higher optical density due to the high cell packing in the SCN and were delimited by a thin (50 μm) cell free band formed by the surrounding fibre shell. These features were obvious during the image analysis quantification of Fos label. The ventral sub-region within the SCN corresponded to the area defined by the retinal innervation. The dorsal subregion lies above this ventral area.

2.1.3. Statistical analysis

The data were statistically analysed using the Kruskal–Wallis one-way analysis of variance (ANOVA) to determine the effect of time on Fos expression in

the light-stimulated and dark controls, respectively. Since data were not normally distributed, the non-parametric alternative to the one-way independent-samples ANOVA was used. Thereafter, the *post hoc* unpaired *t*-test was performed to determine which groups were significantly different from one another. Light-stimulated animals were compared to the dark controls for each time point using the Mann–Whitney *U*-test for unpaired data. Statistical significance was maintained at $p < 0.05$.

2.2. Experiment 2: study of retinal projections

Three *R. pumilio* received an intraocular injection of 0.5–1.0 μl of 0.2% cholera toxin subunit B (CTb). The animals were anaesthetised with halothane vapour and ketamine (30 μl, injected intramuscularly). The pupil of the right eye was dilated with atropine. A small hole was made with a sharpened pipette near the corneal–scleral margin and the CTb solution was injected into the vitreous using a 50 μl tipped glass pipette sealed to the needle of a Hamilton syringe. The animals were perfused 48 h later. Anaesthesia, brain sections and fixation methods were similar as for Experiment 1. Brain sections were collected from the rostral region of the SCN to the caudal part of the superior colliculus. The CTb labelling was visualised using immunohistochemistry, for which one every second section was processed. The primary antibody used was rabbit polyclonal anti-CTb (Sigma, St. Louis, MO; dilution: 1:3000) in 1.5% normal goat serum in PBSTA. The secondary antibody comprised anti-rabbit biotinylated antibody, diluted 1:200/18 ml PBST. The remainder of the immunoistochemical procedure was identical to that described above.

3. Results

3.1. Fos expression in the SCN

In the study of Fos expression in the SCN, control mice kept in darkness showed an endogenous rhythm of Fos expression ($H = 39.45$, d.f. 5, $p < 0.0001$, Kruskal–Wallis test, Fig. 1) with two peaks, at CT2 and CT10, respectively. The quantitative analysis showed that Fos expression at CT2 was significantly different compared to expression at CT6 (d.f. 82; $t = 2.53$; $p < 0.01$), CT18 (d.f. 74; $t = 2.24$; $p < 0.02$) and CT22 (d.f. 76; $t = 2.32$; $p < 0.02$, Fig. 1). In addition, Fos expression at CT10 was significantly higher compared to expression at CT6 (d.f. 84; $t = 3.55$; $p = 0.0006$), CT14 (d.f. 80; $t = 3.14$; $p < 0.016$), CT18 (d.f. 76; $t = 3.14$; $p = 0.0024$) and CT22 ($t = 3.15$; $p = 0.0023$), as calculated using the unpaired *t*-test (Fig. 1).

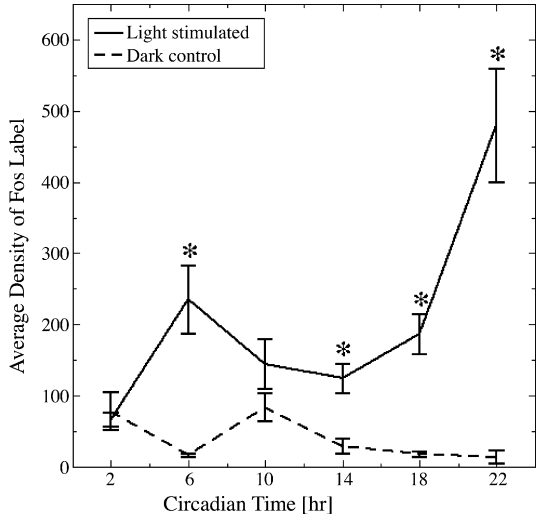

Fig. 1. Average immunostaining intensity (density, as evaluated in integral optical density units) of Fos in the SCN of *Rhabdomys pumilio* kept in total darkness ($n = 3$) or given a 15 min light pulse at CT2, CT6, CT10, CT14, CT18 and CT22 ($n = 5$ at each time point). Endogenous Fos expression at CT2 and CT10 is significantly elevated compared to the other circadian time points. The highest induction of Fos by light occurs during the late subjective night (CT22). The values are presented as mean ± standard error of the mean. $^{*}p < 0.05$ relative to dark controls (unpaired Mann–Whitney *U*-test).

Comparison of Fos expression in light-stimulated and dark control mice using the Mann–Whitney *U*-test showed a significant difference in expression at CT6 ($Z = 5.87$; $p < 0.0001$; $n_1 = 5$; $n_2 = 3$), CT14 ($Z = -4.51$; $p < 0.0001$; $n_1 = 5$; $n_2 = 3$), CT18 ($Z = 4.07$; $p < 0.0001$; $n_1 = 5$; $n_2 = 3$) and CT22 ($Z = 8.1$; $p < 0.0001$; $n_1 = 5$; $n_2 = 3$), but no significant difference at CT2 ($Z = 1.60$; $p = 0.11$; $n_1 = 5$; $n_2 = 3$) and CT10 ($Z = -0.59$; $p = 0.55$; $n_1 = 5$; $n_2 = 3$, Fig. 1). The highest induction of Fos expression occurred at CT22.

Fig. 2 illustrates the pattern of Fos immunoreactivity in sections of the SCN, showing induction of Fos in the ventral and dorsal regions of the SCN and at different circadian time points in the light-stimulated mice compared to the basal expression detected in control mice kept in darkness. Fos expression occurred mainly in the ventral SCN, thus dominating the pattern of Fos expression of the entire SCN (Figs. 2 and 3a). In the dorsal SCN, Fos expression in light-stimulated *R. pumilio* was completely different to that in the ventral SCN (Fig. 3b). Fos expression in the dorsal SCN was significantly different in both light-stimulated and dark control mice at CT22, compared to other time points (Fig. 3b). In addition to the ventral versus dorsal difference in Fos expression, there was also a difference in the rostrocaudal distribution, with an increase in Fos labelling in the mid-caudal region of the SCN (Fig. 4).

3.2. Retinal afferents

The retinal tract tracing experiments using CTb showed that the retina of *R. pumilio* projects to all image forming and non-image forming components of the visual system. The SCN of both sides were innervated by retinal fibres, with a slightly denser innervation contralateral to the injected eye, although this was not quantified (Fig. 5).

The CTb labelling indicated that retinal afferents were mainly distributed in the ventral SCN. The retinal fibres were relatively dense in the rostral and caudal SCN, but the pattern of innervation was unusual because rostrally the innervation was predominantly ipsilateral, whereas caudally the innervation was predominantly contralateral.

We observed labelling of retinal fibres in several structures of the thalamus and in the tectum (Fig. 6). In *R. pumilio*, as in other species, the IGL innervation was formed by a thin horizontal band of retinal fibres, located between the dorsal lateral geniculate nucleus (dLGN) and the ventral lateral geniculate nucleus (vLGN). In the present tract tracing experiments, both the dLGN and vLGN received innervation almost exclusively contralateral to the injected eye, whereas innervation of the IGL was nearly bilateral and showed a complementary spatial overlap in the distribution of retinal label. There was intense labelling contralateral to the injected eye in both the dLGN and the superior colliculus.

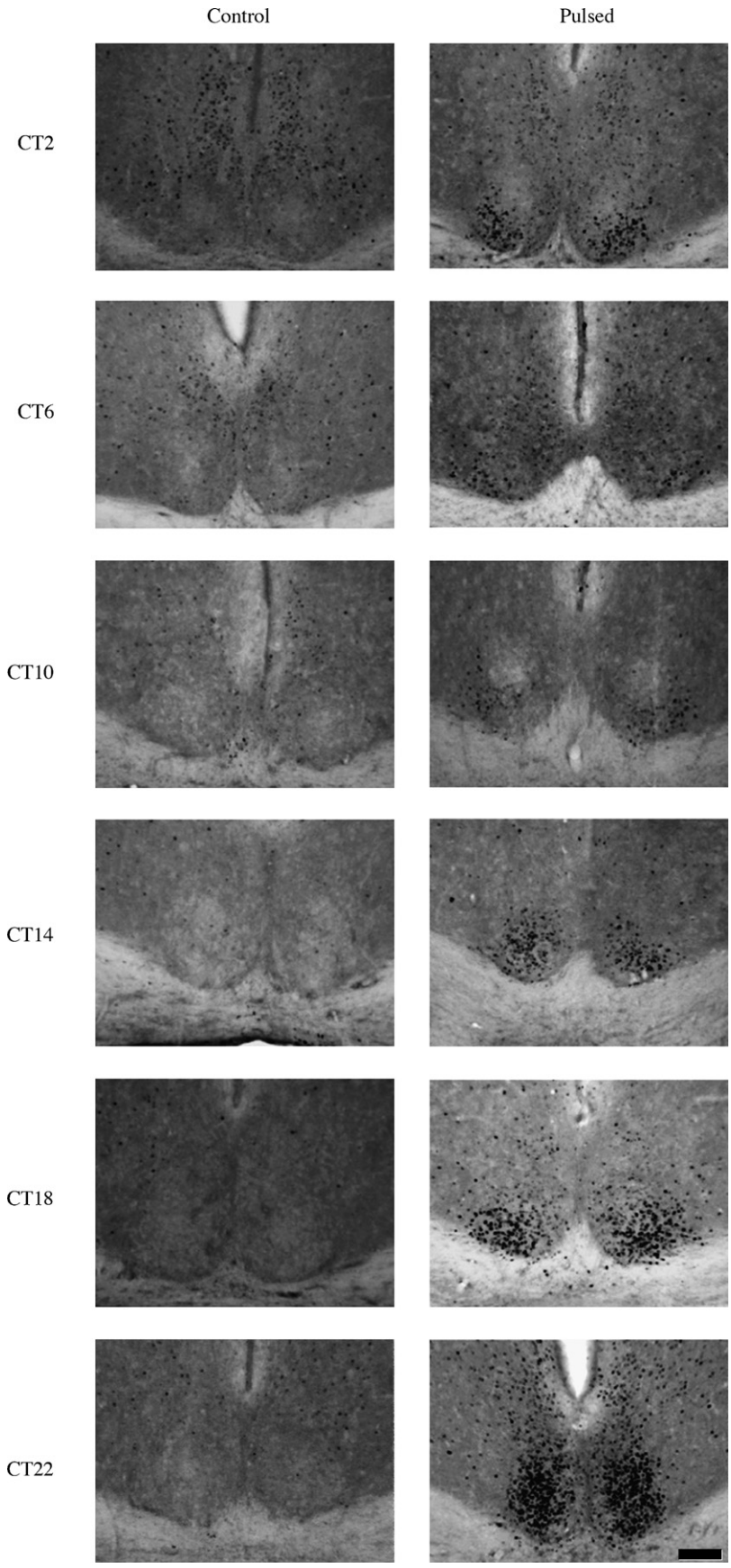

Fig. 2. Coronal sections through the SCN of *Rhabdomys pumilio* kept in constant darkness (dark controls) and exposed to a 15 min light pulse (pulsed) at CT2, CT6, CT10, CT14, CT18 and CT22. Fos immunoreactivity appears as black staining of cell nuclei. Scale bar (lower right panel) = 0.2 mm.

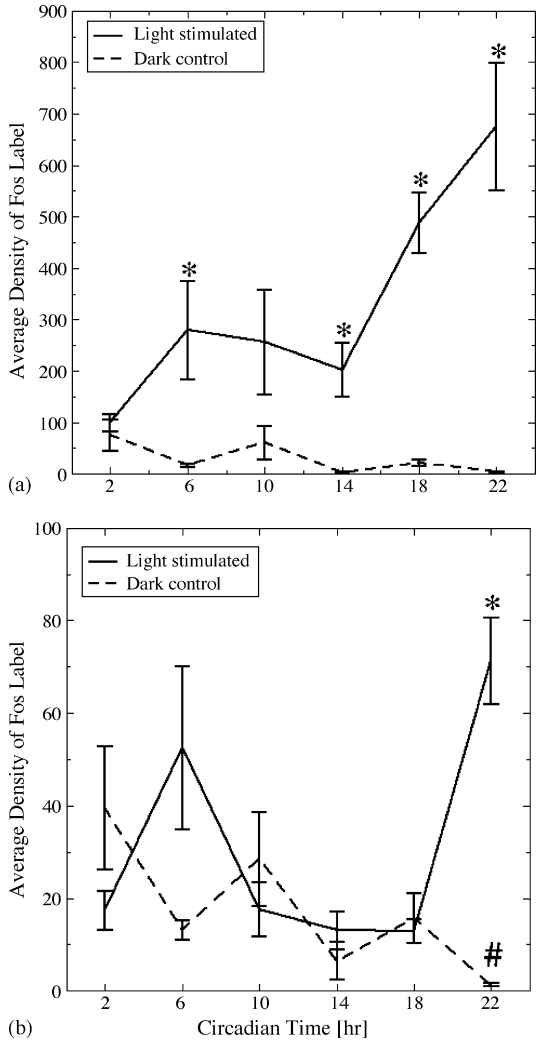

Fig. 3. Evaluation of average Fos labelling in the (a) ventral SCN and (b) dorsal SCN of *Rhabdomys pumilio* given a light pulse (light stimulated, $n = 5$) and *Rhabdomys pumilio* kept in constant darkness (dark control, $n = 3$). The values are presented as mean ± standard error of the mean. $^{*}p < 0.05$ relative to dark control, $^{\#}p < 0.05$ relative to dark control at CT2 (unpaired Mann–Whitney *U*-test).

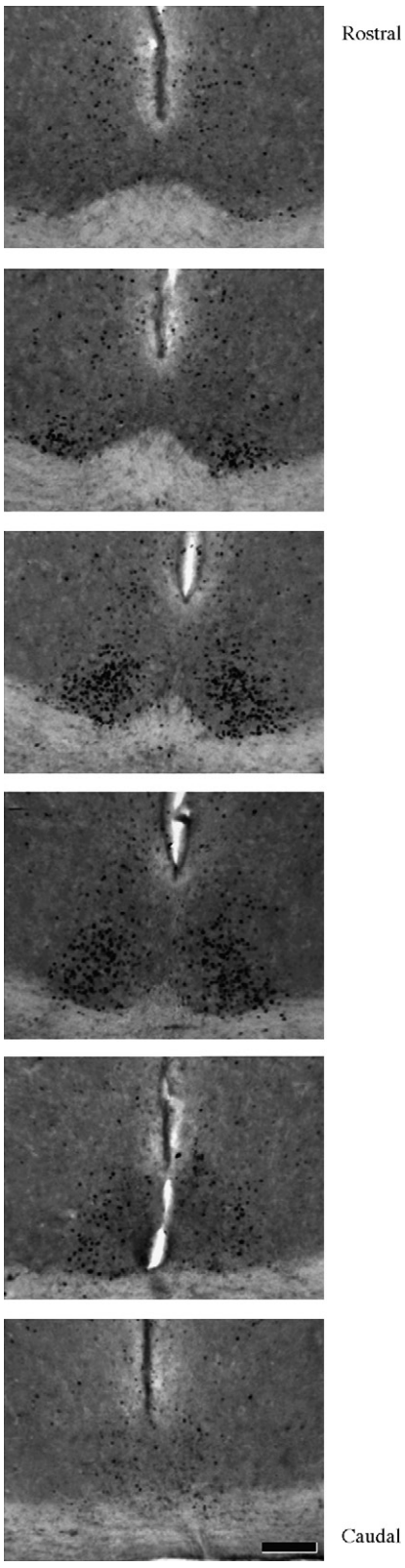

Fig. 4. Rostrocaudal coronal sections through the SCN of *Rhabdomys pumilio* exposed to 15 min of monochromatic light. Note that Fos expression increased from rostral to caudal in the SCN. Scale bar = 0.2 mm.

4. Discussion

In nocturnal rodents, Fos is typically induced by photic stimulation in the ventral SCN during the subjective night but not during the subjective day [11]. In the absence of photic stimulation, the ventral SCN typically shows little or no Fos expression, whereas the dorso-medial SCN expresses an endogenous rhythm of Fos synthesis, with an increase at the end of subjective night.

The present findings show that *R. pumilio*, an African murid rodent which shows diurnal behaviour both in nature and in the laboratory, exhibits an endogenous rhythm of Fos expression with increases in the expression at CT2, 2 h after the beginning of subjective day and at CT10, 2 h before the end of subjective day. This correlates well with the activity patterns of *R. pumilio*, investigated in a previous study [26], which showed intense bouts of activity at these periods. Fos expression occurred both in the ventral and dorsal SCN.

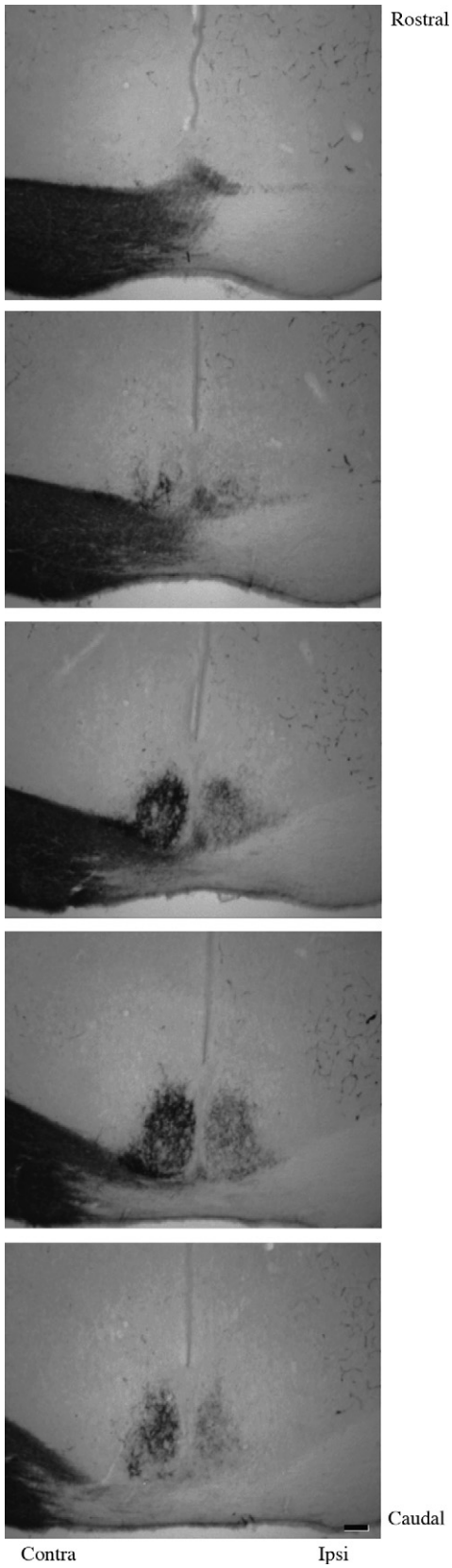

Fig. 5. The plate illustrates the retinal projections to the SCN of *Rhabdomys pumilio* along the rostrocaudal axis. Note that retinal afferents terminate bilaterally, with a slightly contralateral prevalence and with a distribution mainly in the ventral SCN. Scale bar = 0.2 mm.

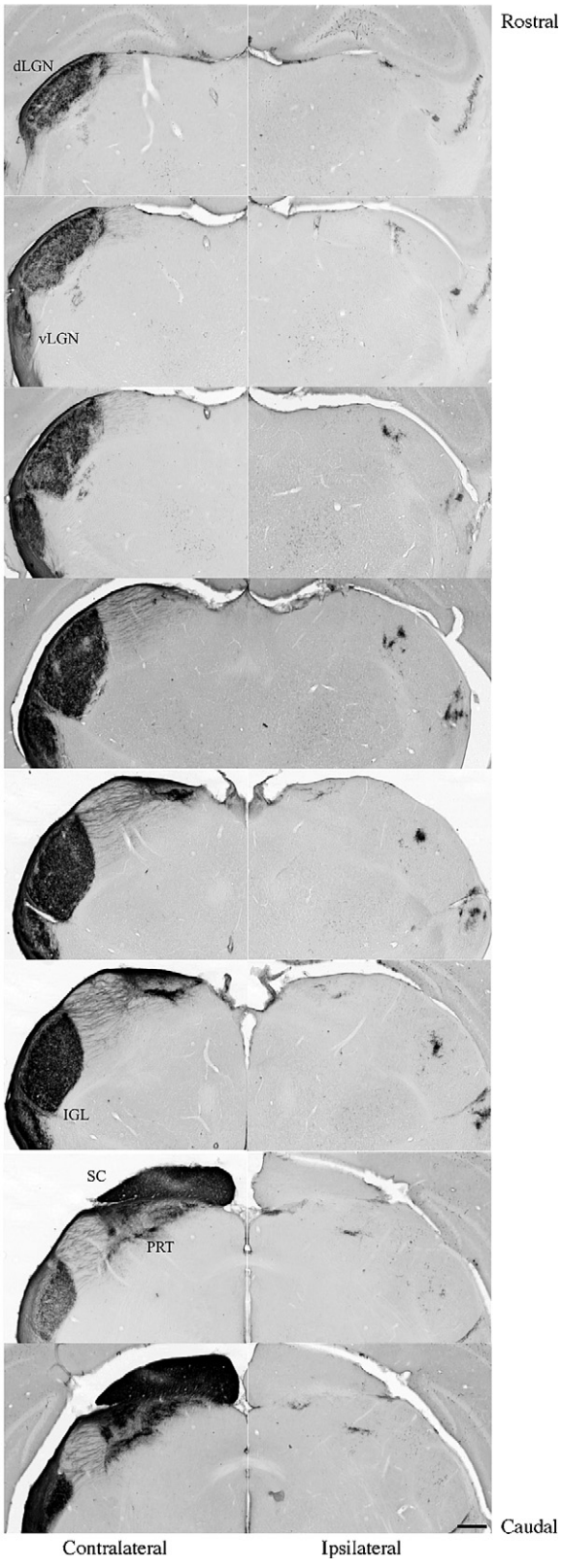

Fig. 6. The plate illustrates retinal projections in *Rhabdomys pumilio* to the dorsal lateral geniculate nucleus (dLGN), the ventral lateral geniculate nucleus (vLGN), the intergeniculate leaflet (IGL, top four pictures), the pretectum (PRT) and the superior colliculus (SC). The IGL receives a bilaterally spatially equivalent contralateral and ipsilateral innervation from the retina. Sections are ordered along the rostrocaudal axis. Scale bar (lower panel) = 0.5 mm.

Several diurnal rodents have been shown to express patterns of Fos expression that differ from that of nocturnal species. In *O. degus*, a diurnal rodent that exhibits phase shifts at both CT4 and CT16, there is a decrease in Fos expression in the dorso-medial SCN at CT4 (subjective day) and an increase in the ventrolateral SCN at CT16 (subjective night) [16,17]. In contrast, in the diurnal chipmunk *E. asiaticus*, Fos expression is photically induced both during the subjective night and the subjective day, although significant phase shifts are only observed during the subjective night [1,13]. In the diurnal *A. niloticus*, the response of the SCN to light is identical to that described in nocturnal rodents [14], with a temporal correlation between light-induced phase shifts and light-induced Fos expression in the SCN. In particular, no circadian responses to light are observed during the subjective day.

Our data indicate that the pattern of photically induced Fos expression in the SCN of *R. pumilio* more closely resembles that of the diurnal chipmunk [1] rather than *Octogon* [16]. During the subjective night, there is a gradual increase in photic induction of Fos expression until a peak at CT22 towards the end of subjective night, but there is also an increase in Fos induction during the middle of the subjective day at CT6. Following CT22 there is a sharp decline in Fos at CT2, suggesting a narrow window within which the effect of light on the phase shift would be most efficient, either late at night or early in the morning. This pattern of Fos expression relates well to the activity patterns of *R. pumilio* [26], in which the onset of locomotor activity during this time period is very robust and precise, whereas the offset of activity late in the day is more variable [26].

In most rodents, Fos induced by photic stimulation is expressed throughout the SCN but is highest in the ventral region [10,14,17,28]. The same pattern was also observed in *R. pumilio* in the present study, which correlated well with the distribution of retinal afferents. In *R. pumilio* there was an increase in Fos labelling from rostral to caudal in the SCN, with the most intense labelling occurring in the mid-caudal region of the nucleus, which corresponded to retinal input.

As for the tract tracing experiment for the study of retinal innervation, the labelling we observed in *R. pumilio* differs from that of *A. niloticus*, in which the densest terminal labelling occurs in the central portion of the SCN [13]. The present study showed that the SCN of *R. pumilio* receives bilateral retinal projections, with a slight contralateral predominance. In degus, however, the SCN receives virtually equal bilateral retinal innervation, whereas in rats the SCN receives bilateral innervation with a contralateral predominance [6,12]. Therefore, there appears to be no correlation between diurnality, nocturnality and retinal innervation of the master circadian pacemaker.

Retinal innervation of the LGN, including the IGL, was here found in *R. pumilio* to be similar to the pattern described previously in degus [9], in which there is an approximately 90% contralateral innervation, with less than 10% ipsilateral innervation. Rats, however, also have a large contralateral innervation, but have a greater proportion of retinal fibres ipsilaterally than those described in degus [18] and detected in *R. pumilio* in the present investigation.

The strict diurnality of the striped field mouse, we here investigated and the marked response in both the behavioural activity under square wave conditions [26], together with the present data on Fos expression, suggest that this diurnal animal could prove an interesting model for future research in chronobiology.

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