ELSEVIER

Neurobiology of Aging 33 (2012) 383–392

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Impact of age and retinal degeneration on the light input to circadian brain structures

Daniela Lupi[a], Ma'ayan Semo[b], Russell G. Foster[a,*]

[a] University of Oxford, Circadian and Visual Neuroscience Group, Nuffield Laboratory of Ophthalmology, Level 5 and 6, West Wing, The John Radcliffe Hospital, Headley Way, Headington, Oxford OX3 9DU, UK

[b] Ocular Biology and Therapeutics, Institute of Ophthalmology, University College London, 11–43 Bath Street, London EC1V 9EL, UK

Abstract

Aging causes anatomical and functional changes in visual and circadian systems. In wild type mice rods, cones, and photosensitive retinal ganglion cells (pRGCs) decline with age. In *rd/rd cl* mice, the early loss of rods and cones is followed by protracted transneuronal loss of inner retinal neurons as well as the pRGCs. Here we use Fos induction to study the light input pathway to the suprachiasmatic nuclei (SCN), the intergeniculate leaflets (IGL) and ventral lateral geniculate nuclei (vLGN) of old (~700 days) and young (~150 days) wild type and *rd/rd cl* mice. Cholera toxin tracing was used in parallel to study the anatomy of this pathway. We find that aging rather than retinal degeneration is a more important factor in reducing light input to the SCN, causing both a reduction in Fos expression and retinal afferents. Furthermore, we show light-induced Fos within the vLGN and IGL is predominantly subserved by rods and cones, and once again aging reduces the amplitude of this response.

Keywords: Circadian rhythms; Immediate early gene; Melanopsin; Neuronal tracing; Photoreceptors; Retinohypothalamic tract

Photoreception in rodents is mediated by rods, cones, and melanopsin-based photosensitive retinal ganglion cells (pRGCs) (Hattar et al., 2003; Lucas et al., 2003; Panda et al., 2003; Sekaran et al., 2003). The pRGCs alone are capable of mediating circadian photoentrainment and the phase shifting effect of single pulses of light on activity onset delivered to animals maintained under constant darkness (Barnard et al., 2004; Freedman et al., 1999), and multiple other irradiance detection tasks including the suppression of pineal melatonin (Lucas et al., 1999), pupil constriction (Lucas et al., 2001), negative masking behavior (Thompson et al., 2008) and the modulation of sleep (Altimus et al., 2008; Lupi et al., 2008).

Aging affects both the retina and circadian system. In the retina there are age related neuronal reductions, particularly in the rods, cones, and retinal ganglion cells (RGCs) (Cavallotti et al., 2001; Danias et al., 2003; Gao and Hollyfield, 1992; Katz and Robison, 1986). In the circadian system, both the amplitude of the pacemaker and its responses to light have been shown to be reduced in humans (Czeisler et al., 1991; Mirmiran et al., 1992), hamsters (Davis and Viswanathan, 1998; Penev et al., 1997; Zee et al., 1992; Zhang et al., 1996), rats (Sutin et al., 1993) and mice (Aujard et al., 2001; Benloucif et al., 1997; Valentinuzzi et al., 1997; Welsh et al., 1986). Here we use a mouse model (*rd/rd cl*) (Freedman et al., 1999; Lucas et al., 1999) to assess the relative importance of age versus retinal degeneration on the responsiveness of the circadian system to light. In old *rd/rd cl* mice the complete loss of the outer retina is followed by a more protracted decline of the inner retina that starts at approximately 9 months of age. By 18–24 months the inner nuclear layer is considerably thinner and in many regions totally absent. Our previous studies demonstrated that there is an age related decline of melanopsin and Thy-1 expression in wild type and *rd/rd cl* mice (Semo et al., 2003b), both genotypes showing an ~40%

* Corresponding author at: University of Oxford, Circadian and Visual Neuroscience Group, Nuffield Laboratory of Ophthalmology, Level 5 and 6, West Wing, The John Radcliffe Hospital, Headley Way, Headington, Oxford OX3 9DU, UK. Tel.: +44(0)1865 234792.

E-mail address: russell.foster@eye.ox.ac.uk.

0197-4580/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2010.03.006

reduction in melanopsin pRGCs (Semo et al., 2003a). Here we study the impact of the *rd/rd cl* lesion on the light-induced gene expression (*c-fos*) within, and the projections to, the master circadian pacemaker (suprachiasmatic nuclei/SCN) in aging congenic *rd/rd cl* and wild type mice. The immediate early gene *c-Fos* provides a powerful marker of photic signaling to the SCN. In rodents, pulses of light during the night results in Fos induction in the retinorecipient region of the SCN (Chambille et al., 1993; Earnest et al., 1990; Rea, 1989; Rusak et al., 1990; Schwartz et al., 2000). Moreover, the numbers of Fos positive neurons (Beaule and Amir, 1999), and the overall levels of *c-fos* and Fos expression in the SCN is broadly correlated with the effects of light on circadian behavior (Dkhissi-Benyahya et al., 2000; Kornhauser et al., 1990; Lupi et al., 1999).

In addition to the SCN, we have used light-induced Fos and tract-tracing to assess the impact of age and retinal degeneration on two other retinorecipient structures that receive afferents from pRGCs; the intergeniculate leaflets (IGL) and the ventral lateral geniculate nuclei (vLGN) (Gooley et al., 2001; Gooley et al., 2003; Hattar et al., 2002; Hattar et al., 2006; Morin et al., 2003). The IGL of the lateral geniculate complex receives direct bilateral retinal input from RGCs (Hickey and Spear, 1976; Ling et al., 1998; Morin and Blanchard, 1997; Morin et al., 1992; Muscat et al., 2003; Pickard, 1982) that are collateral to those that project to the SCN (Pickard, 1985). In contrast to the SCN, the relationship between light and Fos induction within the IGL has received relatively little attention and remains poorly understood (Lupi et al., 1999; Peters et al., 1996). Through its projections to the SCN, the IGL is a functional contributor to circadian rhythmicity by its modification of circadian responses to both photic and nonphotic cues (Harrington and Rusak, 1986; Janik and Mrosovsky, 1994; Johnson et al., 1989; Morin and Pace, 2002; Mrosovsky, 1996; Pickard et al., 1987; Reebs and Mrosovsky, 1989). The vLGN is another component of the visual system that has been shown to project bilaterally via the optic tracts to the SCN (Legg, 1979; Pickard, 1982; Ribak and Peters, 1975; Swanson et al., 1974). While the vLGN shows up-regulation of Fos in response to light, any role it may have in circadian organization remains unresolved (Prichard et al., 2002; Rusak et al., 1990).

1. Methods

1.1. Animals

Congenic wild type and *rd/rd cl* C3H/He mice (described in Lucas et al., 1999) were maintained at 22 °C, 50% humidity in a 12 : 12 h light/dark (12 : 12 LD) cycle. Food and water available *ad libitum*. All procedures were conducted according to the Home Office (UK) regulations, under the Animals (Scientific Procedures) Act of 1986.

1.2. Fos induction

Quantitative analysis of Fos induction in brain nuclei was undertaken in both old mice (610–847 d old; wild type pulsed n = 6; wild type sham n = 4; *rd/rd cl* pulsed n = 7; *rd/rd cl* sham n = 6) and in young mice (100–200 d; wild type pulsed n = 5; wild type sham n = 5; *rd/rd cl* pulsed n = 3; *rd/rd cl* sham n = 3). Mice were entrained for at least 10 d to a 12 : 12 LD, lights on at 4:00 and off 16:00. On the day of the light pulse the animals remained in darkness. At 20:00 (4 h after the lights would normally have been turned off) mice were transferred to the light pulse equipment under infrared illumination and exposed to a 15 min pulse of light (λ_{max} at 505 nm at an irradiance of 8.0 $\mu W/cm^2$ measured using an optical power meter, Macam Photometrics). Sham pulsed mice were handled in a similar manner but no light pulse was given. Following the light pulse mice were returned to their cages. Ninety min after the beginning of light treatment animals were deeply anesthetized with sodium pentobarbitone (60 mg/kg), perfused transcardially with warm (32 °C) 0.9% NaCl, followed by 300 mL of cold 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.4. The brain was removed and processed as described in Section 1.4.

1.3. Neuronal tracing

Neuronal tracing was carried out in old and young wild type and *rd/rd cl* mice (n = 3 young wild type, n = 3 young *rd/rd cl*, n = 2 old wild type and n = 2 old *rd/rd cl*). Mice were anaesthetized with ketamine hydrochloride, 60 mg kg^{-1} and xylazine, 7 mg kg^{-1} and a topical analgesic ophthalmic solution (Proparacaine parachloride). Cholera toxin Subunit B tracer (CTB, List Biological, Campbell, CA) (1% diluted in sterile distilled water) was injected into the vitreous chamber of one eye with the aid of a micropipette (50 mm tip) sealed to the needle of a 5 μL Hamilton syringe. Twenty-four hours later, the mice were perfused transcardially as described in Section 1.2.

1.4. Tissue preparation and immunohistochemistry

Following perfusion, brains were removed, postfixed overnight in the same fixative at 4 °C, and cryo-protected by immersion in a solution of 30% sucrose in PBS overnight at 4 °C, then stored in PBS-A (0.01 M PB, 0.9% NaCl, 0.1% NaN_3 0.1%). Following cryo-protection in 30% sucrose, serial coronal brain sections (40 μm) containing the SCN, IGL, and vLGN were cut from each brain on a freezing microtome. Sections were processed for Fos or CTB immunohistochemistry depending on previous treatment (light induction or intraocular injection). They were incubated in 50% ethanol, 0.9% NaCl, and 0.05% H_2O_2 to block endogenous peroxidase at 4 °C for 1 h. Then they were washed in PBS (0.01 M PB, 0.9% NaCl) and blocked in 1% normal serum (goat or rabbit according to the primary antibody host) in PBST-A (0.1 M PB, 0.9% NaCl, 0.3% Triton-X 100, NaN_3 0.1%) for 60 min at 4 °C followed by incubation

in primary antibody rabbit anti-Fos (Ab-5 Oncogene Research Products Calbiochem) at a final dilution of 1 : 20,000 or goat anti-CTB (List Biological, Campbell, CA) at a dilution of 1 : 10,000 at 4 °C for 72 h. The secondary antibody binding and avidin biotin amplification was carried out using the Vectastain ABC Elite kit (PK-6101 for Fos or PK-6105 for CTB). Brain sections were then washed in Tris buffer 0.05M, pH 7.4 twice for 10 min and then transferred to chilled 4 °C Tris buffer containing 0.02% 3, -3′ Diaminobenzidine, 0.5% nickel ammonium sulfate (DAB-Ni) and 0.001% H_2O_2. The development of the Chromagen was kept the same for all brain slices. The slices were thoroughly washed with Tris buffer (two times and left overnight at 4 °C). Then they were mounted on chrome alum and gelatin-coated slides, air dried, dehydrated in a series of alcohols, cleared in xylene, and coverslipped with DePeX. Control slices where the primary or secondary antibodies were replaced with normal serum did not show any label.

1.5. Image analysis

All the brain sections through the SCN, IGL, and vLGN were analyzed using a computerized image analysis system. They were examined under a Zeiss Axioplan 2 microscope and images captured with a Spot Digital Camera (Diagnostic Instruments). In our study, the computerized image analysis software Image-Pro Plus (Media Cybernetics) was used to determine the integral optical density (IOD) of the labeled brain sections. The IOD represents the integral sum of the surface area of single pixels multiplied by their correspondent optical density values. This method has been used widely to measure levels of Fos in the SCN (Barnard et al., 2004; Dkhissi-Benyahya et al., 2000; Lupi et al., 1999; Lupi et al., 2006; Sekaran et al., 2005) and takes into account not only the total area of Fos positive nuclei but also the density of the label (Rieux et al., 2002). For the purposes of standardization each brain area was processed in parallel. The IOD values were summed for paired nuclei and in all sections, rostral to caudal in which the nuclei could be identified.

1.6. Statistics

For the Fos induction studies we used three way ANOVA for three factors (pulse, genotype, and age), carried out using StatView SE+ Graphics, v1.0.3 (SAS Institute, Inc, Cary, USA), followed by Bonferroni's multiple comparison test for *post hoc* comparisons. For neuronal tracing the average and standard deviations for densitometry measurements are quoted in the results, the number of animals precluded statistical analysis of these data.

2. Results

2.1. SCN: light activation and retinal afferents

Light-induced Fos occurs in the SCN of both wild type and *rd/rd cl* mice at both young and old ages (Fig. 1). Analysis of all the IOD data of Fos immunoreactivity in the SCN by three way ANOVA (factors: pulsing, genotype and age) shows: (1) there is a significant effect of light ($F_{1,29} = 108.3$, $p < 0.0001$), a light pulse causes a substantial increase in Fos immunoreactivity; (2) a significant effect of age ($F_{1,29} = 20.15$, $p < 0.0001$), older animals show attenuated Fos levels and (3) an interaction between light pulsing and age ($F_{1,29} = 21.58$, $p < 0.0001$) the amplitude of light induced Fos is reduced in old animals. In both young wild type and *rd/rd cl* mice there is a ~25 fold induction of Fos after a light pulse. Fos is also induced by light in old wild type and *rd/rd cl* mice but the magnitude of induction is attenuated to ~9 fold (for *post hoc* comparisons see Table 1). Interestingly there is no significant difference in the amplitude of Fos induction between genotypes (Fig. 2).

We have also investigated the extent of retinal projections to the SCN in young versus old *rd/rd cl* and wild type mice. Representative sections are shown in Fig. 1I–L. Retinal projections to the SCN show a similar symmetrical bilateral distribution for both *rd/rd cl* and wild type mice. Significantly, labeling appears reduced in old animals, and again is similar for both genotypes. Densitometry values indicate that the projection is similar between young wild type (average CTB IOD 413,905 ± SD 35,474) and young *rd/rd cl* (average CTB IOD 319,483 ± SD 11,938). In old wild type and *rd/rd cl* these values are reduced (wild type 159,408 ± SD 30,873 and *rd/rd cl* 269,764 ± SD 8,313).

2.2. IGL: light activation and retinal afferents

Analysis of the Fos IOD in the paired IGL nuclei by three way ANOVA shows: (1) a significant effect of light ($F_{1,32} = 18.01$, $p < 0.0002$) resulting in an increase in Fos; (2) a significant effect of age ($F_{1,32} = 10.52$ $p < 0.003$) with attenuated Fos levels in old animals and (3) a significant effect of genotype ($F_{1,32} = 4.50$, $p < 0.04$), the *rd/rd cl* show slightly lower levels of Fos (Figs 3 and 4A). *Post hoc* tests (Table 1) indicate that there is significant light induction of Fos in the IGL of young wild types ($p < 0.05$), and that this is reduced in old animals ($p < 0.05$) such that there is not a significant light induction of Fos in the IGL of old light pulsed wild type animals compared with old sham pulsed wild types. Although the level of Fos in the IGL is higher in the *rd/rd cl* following light administration (in both young and old) this is not to a significant level.

The retinal projections to the IGL have an asymmetrical distribution with a different contralateral/ipsilateral component. In both *rd/rd cl* and wild type young and old there is a higher contralateral component than ipsilateral (Fig. 5). The sum of the IOD values for CTB from both the ipsi- and contralateral contribution to the IGL shows a marked reduction with aging in the wild type animals (wild type young 175,232 ± 22,559 vs. wild type old 80,462 ± 17,899). The values from the *rd/rd cl* IGL also show a slight reduction with aging (*rd/rd cl* young 156,044 ± 33,280 vs. *rd/rd cl* old 150,201 ± 28,666).

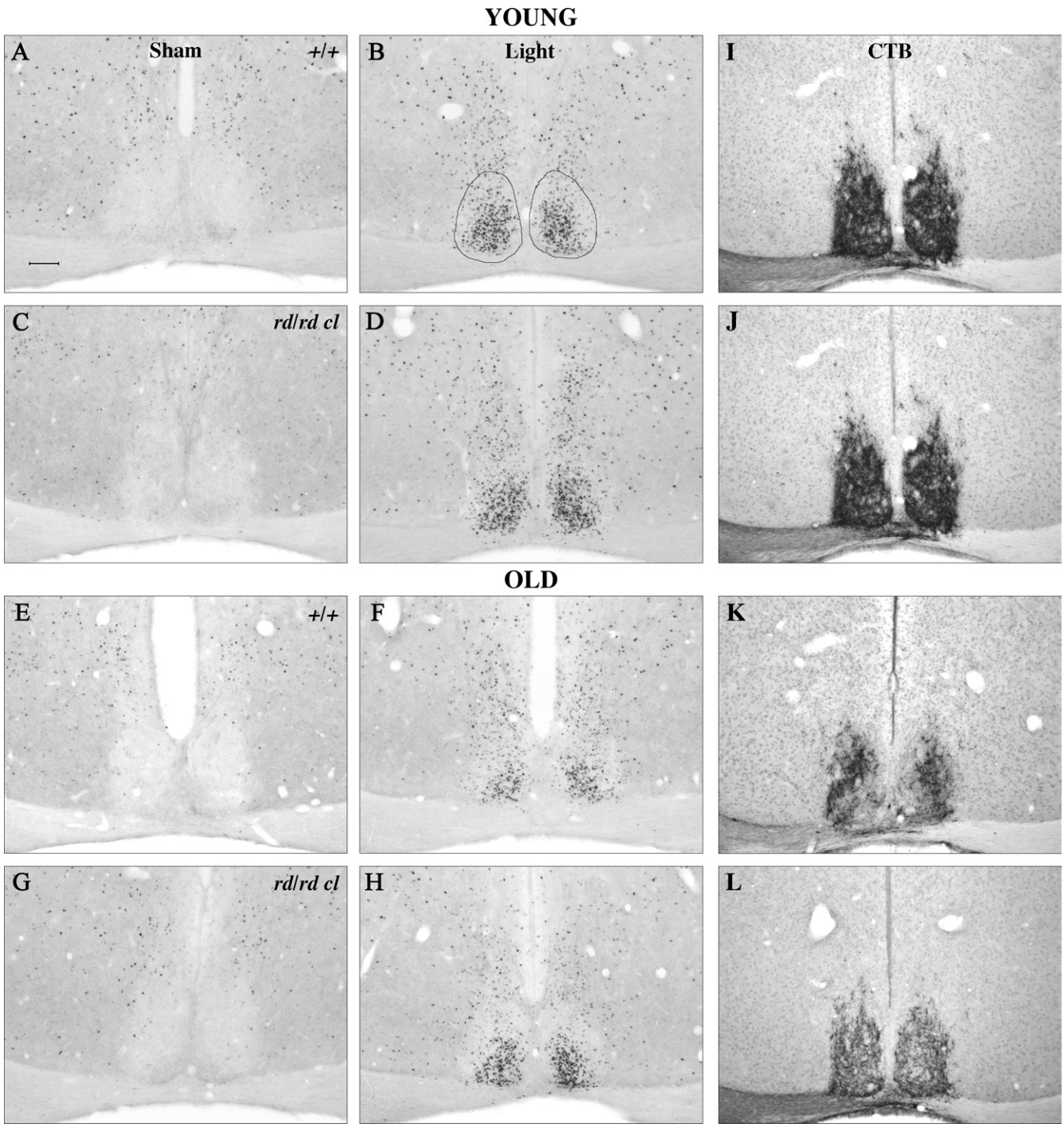

Fig. 1. Light induced Fos and cholera toxin B (CTB) immunoreactivity in the suprachiasmatic nuclei (SCN) of young and old, wild type, and *rd/rd cl* mice. Representative images of Fos immunoreactivity are shown in A–H. Images A, C, E, and G are from animals that received no light (sham pulse) while those in B, D, F, and H received a pulse of light. SCN from young wild type mice are shown in (A) and (B), and young *rd/rd cl* in (C) and (D), those from old wild type mice are shown in (E) and (F), and old *rd/rd cl* in (G) and (H). Examples of regions of interest analyzed for integrated optical density of Fos in the SCN are shown in (B). I–L representative images of CTB immunoreactivity following unilateral intravitreal injection of CTB. SCN from (I) wild type young, (J) *rd/rd cl* young, (K) wild type old and (L) *rd/rd cl* old mice. Scale bar for all images in (A) 100 μm.

2.3. vLGN: light activation and retinal afferents

Three way ANOVA on IOD data from the vLGN shows a significant effect of light only ($F_{1,27} = 27.76$, $p < 0.0001$), with no significant effects of either age or genotype (Figs 3 and 4B). *Post hoc* testing (Table 1) indicates that there is a significant light induction of Fos in the young wild type animals ($p < 0.001$) and that the magnitude of this induction is reduced in old wild types ($p < 0.05$). Three way

Table 1
Post hoc Bonferroni comparisons for IOD of Fos in the SCN, IGL and vLGN

Post hoc comparison	SCN *p*-values	IGL *p*-values	vLGN *p*-values
WT young light v. WT young sham	$p < 0.001$	$p < 0.05$	$p < 0.001$
RC young light v. RC young sham	$p < 0.001$	NS	NS
WT old light v. WT old sham	$p < 0.05$	NS	NS
RC old light v. RC old sham	$p < 0.05$	NS	NS
WT young light v. WT old light	$p < 0.01$	$p < 0.05$	$p < 0.05$
RC young light v. RC old light	$p < 0.001$	NS	NS
WT young light v. RC young light	NS	NS	NS
WT old light v. RC old light	NS	NS	NS

Abbreviations: IOD, integrated optical density; IGL, intergeniculate leaflets; NS, not significant; SCN, suprachiasmatic nuclei; RC, *rd/rd cl*; vLGN, ventral lateral geniculate nuclei; WT, wild type.

ANOVA does not describe any genotype differences, however at the *post hoc* level we have been unable to detect a significant light induction of Fos in the vLGN of *rd/rd cl*, the IOD levels do indicate a subtle light induced increase in Fos compared with sham pulsed *rd/rd cl* (Fig. 4B).

Retinal projections to the vLGN again have an assymetrical distribution with a higher contralateral than ipsilateral component (Fig. 5). The IOD values for CTB in the vLGN (ipsi- and contralateral) are markedly reduced with age in the wild type animals (wild type young 761,795 ± 114,706 vs. wild type old 468,464 ± 98,007). There is a slight reduction in the IOD values for the *rd/rd cl* projection but again this is not as marked as for the wild types (*rd/rd cl* young 798,531 ± 118,799 vs. *rd/rd cl* old 668,448 ± 49,229).

3. Discussion

In the present study we have used a mouse model (*rd/rd cl*) to assess the relative importance of age versus retinal degeneration on light-induced Fos within specific retinorecipient areas (SCN, IGL, and vLGN) of the brain. Fos expression has been used widely as a marker for neuronal activation in response to various stimuli including light (Dkhissi-Benyahya et al., 2000; Kornhauser et al., 1992). In parallel, we have employed CTB as an anterograde tracer to label these retinal target areas (Angelucci et al., 1996; Mikkelsen, 1992; Reiner et al., 1996).

3.1. Light activation in the SCN following retinal degeneration and aging

Previously, levels of Fos expression in the SCN have been shown to correlate with light intensity and the magnitude of circadian phase shifts (Dkhissi-Benyahya et al., 2000; Kornhauser et al., 1990; Lupi et al., 1999). In the present study, we show light induction of Fos in both young *rd/rd cl and wild type animals* and we also show that the levels of Fos in the SCN of both genotypes are statistically indistinguishable, indicating that at the cellular level, the light input to the SCN in *rd/rd cl* mice is unaffected by outer retinal cell loss. Thus, melanopsin-based pRGCs alone are able to induce normal levels of Fos. These findings are consistent with our previous results showing that the circadian behavior of mice lacking rods and cones and that of wild type mice is broadly similar (Barnard et al., 2004; Freedman et al., 1999; Lucas et al., 1999; Semo et al., 2003b). Indeed the magnitudes of phase shifts to a light pulse of 505-nm wavelength (as used here) are not significantly different between wild type and *rd/rd cl* mice, suggesting compensatory mechanisms of the pRGC system in the absence of outer retinal photoreception (Semo et al., 2003b). The rods and cones, however, do provide light information to the SCN. For example, in the absence of melanopsin ($Opn4^{-/-}$ mice) rods and cones can partially compensate for the loss of functional pRGCs, showing attenuated phase shifts and Fos induction of ~40% (Hattar et al., 2003; Panda et al., 2002; Ruby et al., 2002). In view of this input from the rods and cones it is perhaps surprising that their loss does not appear to attenuate light-induced Fos in the SCN of *rd/rd cl* mice.

In aged wild type and *rd/rd cl* mice, SCN neurons still show significant light-induced Fos, but there is a marked reduction in levels compared with younger animals of both genotypes. We have shown previously that aged *rd/rd cl* and wild type mice have significantly fewer melanopsin positive RGCs than young mice, but that the numbers of melanopsin cells are not significantly different between the two genotypes (Semo et al., 2003a; Semo et al., 2003b). This attenuation in

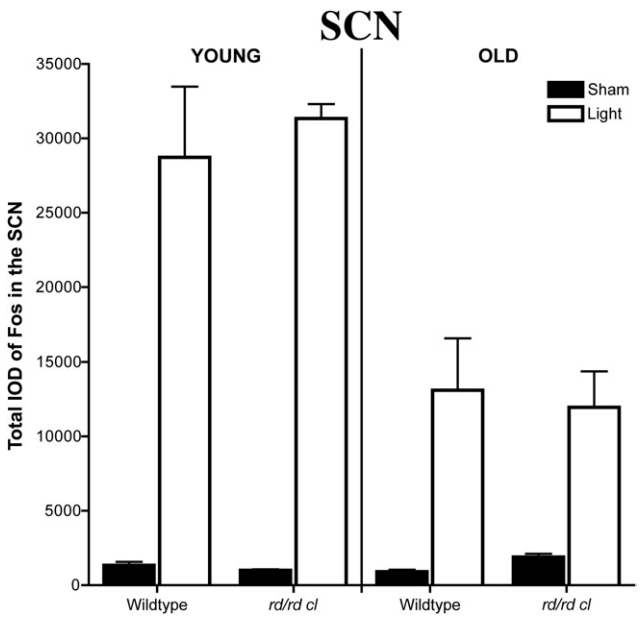

Fig. 2. The total integrated optical density (IOD) for Fos immunoreactivity measured in the paired nuclei of the suprachiasmatic nuclei (SCN) from sham and light pulsed, old and young, wild type and *rd/rd cl* mice. Fos is significantly induced by light in both the *rd/rd cl* and wild type (*post hoc* $p < 0.05$). The magnitude of this induction is equivalent in both genotypes and is reduced in the old mice by ~58%.

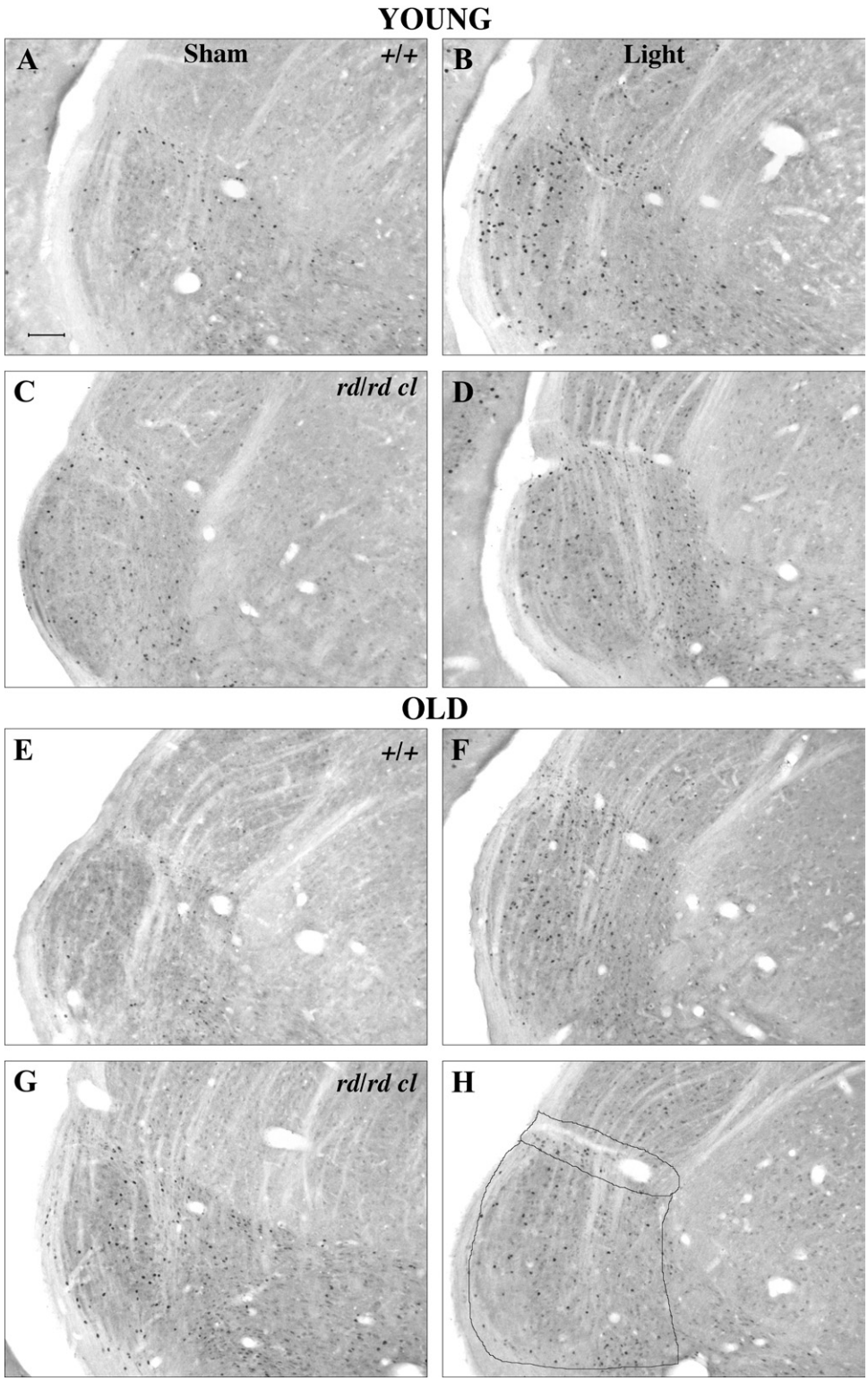

Fig. 3. Light induced Fos immunoreactivity in the intergeniculate leaflets (IGL) and ventral lateral geniculate nuclei (vLGN) of young and old wild type and *rd/rd cl* mice. The images A, C, E, and G are from animals that received no light (sham pulse) while those in B, D, F, and H received a pulse of light. IGL/vLGN from young wild type mice are shown in (A) and (B), and young *rd/rd cl* in (C) and (D), while those from old wild type mice are shown in (E) and (F), and old *rd/rd cl* in (G) and (H). The smaller dashed area in (H) marks an example of the region of interest that would be anlaysed for Fos in the IGL, while the larger area defines the region of interest for the vLGN. Scale bar 100 μm.

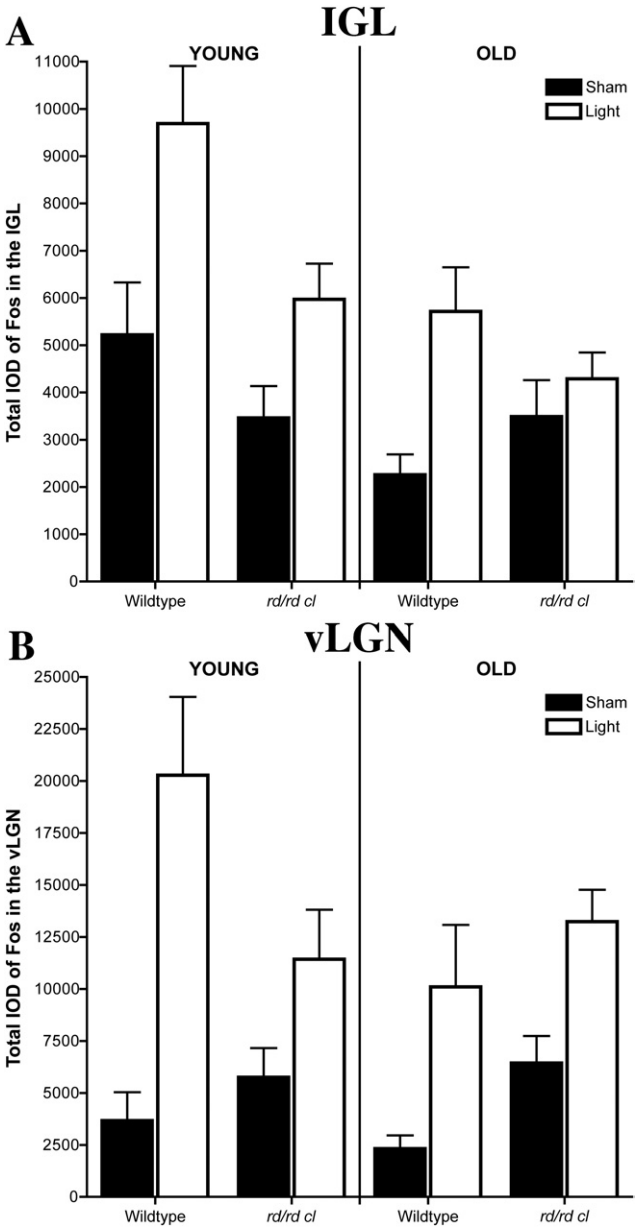

Fig. 4. The total integrated optical density (IOD) for Fos immunoreactivity measured in the paired nuclei of the (A) intergeniculate leaflets (IGL) and (B) ventral lateral geniculate nuclei (vLGN) from sham and light pulsed, old and young, wild type and *rd/rd cl* mice. In the IGL and vLGN there is a significant light induction of Fos only in wild type animals and the magnitude of this induction is reduced in old animals. There is no statistically significant light induction of Fos in the *rd/rd cl*.

RGCs may well account for the reduction in Fos expression we observe within the SCN of aged mice and is consistent with our observation of an ~40% decrease in retinal afferents in these mice (Fig. 1). Since we did not find differences in melanopsin numbers between the genotypes, our result showing that the aged *rd/rd cl* and wild type have a similar magnitude of Fos induction within the SCN is also consistent. Studies in humans also suggest age-related changes in the pRGC system. In a recent paper, lipofuscin deposits have been reported within melanopsin RGCs of aging individuals (Vugler et al., 2007). Although loss of melanopsin pRGCs seems to be the most parsimonious explanation for the reduction in Fos within the SCN, we cannot entirely exclude the existence of other causes. For example, a reduction in light transmission through the lens has been reported in old hamsters (Zhang et al., 1998) and humans (Cuthbertson et al., 2009). It is also possible that there might be age related impairments in the physiological responses of the SCN neurons perhaps by changes in neurotransmitter release and/or a reduction in neurotransmitter receptors (Aujard et al., 2001; Tamaru et al., 1991).

3.2. Light activation in the IGL and vLGN following retinal degeneration and aging

In the IGL and vLGN, in contrast to the SCN, we observe differences in Fos expression between young *rd/rd cl* and wild type mice. Wild type animals show a marked induction of Fos in the IGL and vLGN in response to light, while *rd/rd cl* mice show only a slight elevation of Fos in response to light and this fails to reach statistical significance when compared with sham treated controls. This suggests strongly that rod and cone photoreceptors play a more dominant role in light activation of the IGL and vLGN. In view of the different sensory tasks mediated by rods/cones and pRGCs, these results are not too surprising (Hankins et al., 2008), reflecting their respective roles as image and irradiance detectors. These findings are similar to those in another visually impaired mouse ($Rho^{-/-}$ $Cnga3^{-/-}$) which lacks functional rods and cones, where light stimuli failed to elicit Fos induction in the IGL (Barnard et al., 2004). Old *rd/rd cl* and wild type mice fail to show any significant light-induced Fos within the IGL and vLGN. We suggest this lack of Fos expression in the old wild type, compared with young animals, is related to a loss of rod/cone input. Our neuronal tracing suggests a reduction of fiber density of retinal afferents to the IGL/vLGN in aged wild type mice and correlates well with previously reported results showing a reduction of rods and cones in the aged human and rodent retina (Gao and Hollyfield, 1992; Katz and Robison, 1986).

3.3. Overall summary and conclusions

We show a generalized loss of retinal afferents (~40% to the SCN) and a reduction in light activation in both normal and retinally degenerate mice (*rd/rd cl*) (20–27 months of age). Previous studies in aging wild type mice have shown a steady decrease in RGC numbers, declining by ~41% at 18 months of age (Neufeld and Gachie, 2003). Outer retinal degeneration appears to accelerate RGC loss in *rd/rd* mice as they show ~20% fewer RGCs at 11–12 months compared to wild types (Wang et al., 2000). What is perhaps surprising in the present study is that retinal afferent loss is not enhanced by rod and cone loss in the aged *rd/rd cl* mouse. Indeed, at this advanced age it appears that the afferents are

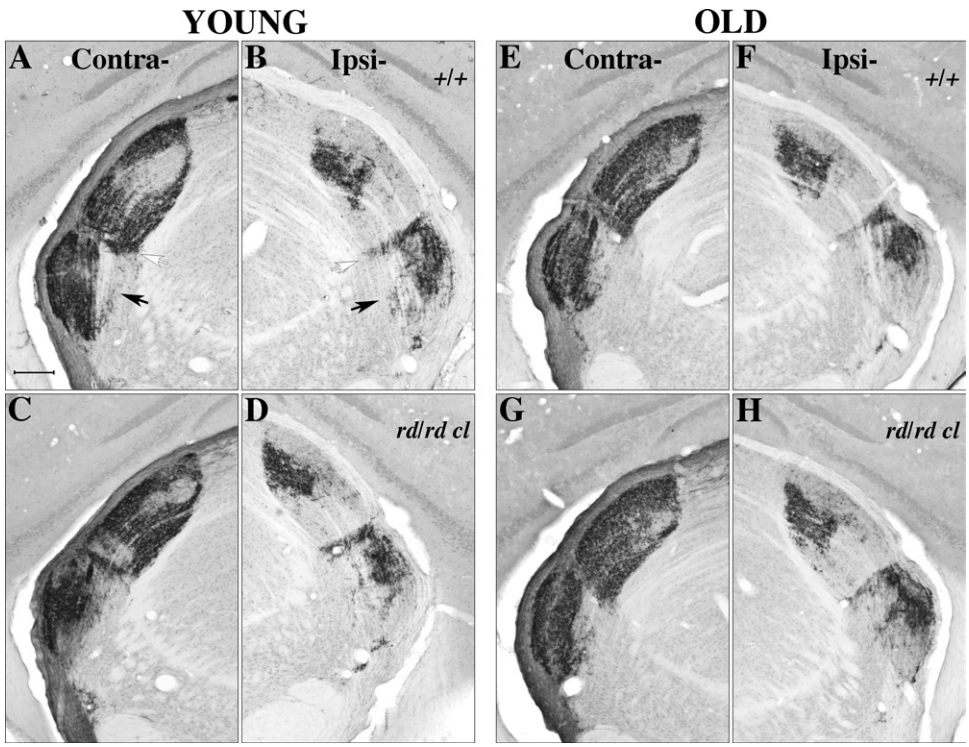

Fig. 5. Cholera toxin B (CTB) immunoreactivity in the intergeniculate leaflets (IGL) and ventral lateral geniculate nuclei (vLGN) of young and old wild type and *rd/rd cl* mice after unilateral intravitreal injection of CTB. The images A, C, E, and G are contralateral to the injected eye while those in B, D, F, and H are ipsilateral. Projections to the IGL/vLGN in young wild type mice are shown in (A) and (B), and young *rd/rd cl* in (C) and (D), while those from old wild type mice are shown in (E) and (F), and old *rd/rd cl* in (G) and (H). Black arrows indicate the vLGN and white arrows the IGL. Scale bar 200 μm.

preserved to the same level as wild types. Our data indicate that the extensive loss of RGCs over the life span of the mouse is not related to the loss of outer retinal photoreceptors and other factors must be involved, such as nutritional intake, light levels, and/or strain specific diseases (Danias et al., 2003; Neufeld and Gachie, 2003). Our results might also reflect the fact that we have focused on regions that receive inputs from melanopsin pRGCs, and these neurons may be somewhat more resistant to axotomy induced cell death (Robinson and Madison, 2004), that may be occurring in the dystrophic retina (Wang et al., 2000).

In summary, our data allow us to conclude that advanced age is a more important factor than retinal degeneration in reducing the level of light activation in the SCN. Our study provides important anatomical correlates to recent studies showing the therapeutic benefit of providing increased light to elderly people for the improvement of circadian rhythm deficits (Dowling et al., 2008; Lieverse et al., 2008; Riemersma-van der Lek et al., 2008).

Disclosure statement

The authors report no actual or potential conflicts of interest.

Acknowledgements

The first two authors contributed equally to this work. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), the Wellcome Trust, and a BBSRC studentship to MS. The authors would like to thank Prof. Robert J. Lucas for technical assistance and Stuart N. Peirson for constructive comments during preparation of this manuscript.

References

Altimus, C.M., Guler, A.D., Villa, K.L., McNeill, D.S., Legates, T.A., Hattar, S., 2008. Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. Proc. Natl. Acad. Sci. USA 105, 19998–20003.

Angelucci, A., Clasca, F., Sur, M., 1996. Anterograde axonal tracing with the subunit B of cholera toxin: a highly sensitive immunohistochemical protocol for revealing fine axonal morphology in adult and neonatal brains. J. Neurosci. Methods 65, 101–112.

Aujard, F., Herzog, E.D., Block, G.D., 2001. Circadian rhythms in firing rate of individual suprachiasmatic nucleus neurons from adult and middle-aged mice. Neuroscience 106, 255–261.

Barnard, A.R., Appleford, J.M., Sekaran, S., Chinthapalli, K., Jenkins, A., Seeliger, M., Biel, M., Humphries, P., Douglas, R.H., Wenzel, A., Foster, R.G., Hankins, M.W., Lucas, R.J., 2004. Residual photosensitivity in mice lacking both rod opsin and cone photoreceptor cyclic nucleotide gated channel 3 alpha subunit. Vis. Neurosci. 21, 675–683.

Beaule, C., Amir, S., 1999. Photic entrainment and induction of immediate-early genes within the rat circadian system. Brain Res. 821, 95–100.

Benloucif, S., Masana, M.I., Dubocovich, M.L., 1997. Responsiveness to melatonin and its receptor expression in the aging circadian clock of mice. Am. J. Physiol. 273, R1855–R1860.

Cavallotti, C., Artico, M., Pescosolido, N., Feher, J., 2001. Age-related changes in rat retina. Jpn. J. Ophthalmol. 45, 68–75.

Chambille, I., Doyle, S., Serviere, J., 1993. Photic induction and circadian expression of Fos-like protein. Immunohistochemical study in the retina and suprachiasmatic nuclei of hamster. Brain Res. 612, 138–150.

Cuthbertson, F.M., Peirson, S.N., Wulff, K., Foster, R.G., Downes, S.M., 2009. Blue light-filtering intraocular lenses: review of potential benefits and side effects. J. Cataract Refract. Surg. 35, 1281–1297.

Czeisler, C.A., Chiasera, A.J., Duffy, J.F., 1991. Research on sleep, circadian rhythms and aging: applications to manned spaceflight. Exp. Gerontol. 26, 217–232.

Danias, J., Lee, K.C., Zamora, M.F., Chen, B., Shen, F., Filippopoulos, T., Su, Y., Goldblum, D., Podos, S.M., Mittag, T., 2003. Quantitative analysis of retinal ganglion cell (RGC) loss in aging DBA/2NNia glaucomatous mice: comparison with RGC loss in aging C57/BL6 mice. Invest. Ophthalmol. Vis. Sci. 44, 5151–5162.

Davis, F.C., Viswanathan, N., 1998. Stability of circadian timing with age in Syrian hamsters. Am. J. Physiol. 275, R960–R968.

Dkhissi-Benyahya, O., Sicard, B., Cooper, H.M., 2000. Effects of irradiance and stimulus duration on early gene expression (Fos) in the suprachiasmatic nucleus: temporal summation and reciprocity. J. Neurosci. 20, 7790–7797.

Dowling, G.A., Burr, R.L., Van Someren, E.J., Hubbard, E.M., Luxenberg, J.S., Mastick, J., Cooper, B.A., 2008. Melatonin and bright-light treatment for rest-activity disruption in institutionalized patients with Alzheimer's disease. J. Am. Geriatr. Soc. 56, 239–246.

Earnest, D.J., Iadarola, M., Yeh, H.H., Olschowka, J.A., 1990. Photic regulation of c-fos expression in neural components governing the entrainment of circadian rhythms. Exp. Neurol. 109, 353–361.

Freedman, M.S., Lucas, R.J., Soni, B., von Schantz, M., Munoz, M., David-Gray, Z., Foster, R., 1999. Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science 284, 502–504.

Gao, H., Hollyfield, J.G., 1992. Aging of the human retina. Differential loss of neurons and retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 33, 1–17.

Gooley, J.J., Lu, J., Chou, T.C., Scammell, T.E., Saper, C.B., 2001. Melanopsin in cells of origin of the retinohypothalamic tract. Nat. Neurosci Dec;4(12):1165.

Gooley, J.J., Lu, J., Fischer, D., Saper, C.B., 2003. A broad role for melanopsin in nonvisual photoreception. J. Neurosci. 23, 7093–7106.

Hankins, M.W., Peirson, S.N., Foster, R.G., 2008. Melanopsin: an exciting photopigment. Trends Neurosci. 31, 27–36.

Harrington, M.E., Rusak, B., 1986. Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. J. Biol. Rhythms 1, 309–325.

Hattar, S., Kumar, M., Park, A., Tong, P., Tung, J., Yau, K.W., Berson, D.M., 2006. Central projections of melanopsin-expressing retinal ganglion cells in the mouse. J. Comp. Neurol. 497, 326–349.

Hattar, S., Liao, H.W., Takao, M., Berson, D.M., Yau, K.W., 2002. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295, 1065–1070.

Hattar, S., Lucas, R.J., Mrosovsky, N., Thompson, S., Douglas, R.H., Hankins, M.W., Lem, J., Biel, M., Hofmann, F., Foster, R.G., Yau, K.W., 2003. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature 424, 75–81.

Hickey, T.L., Spear, P.D., 1976. Retinogeniculate projections in hooded and albino rats: an autoradiographic study. Exp. Brain Res. 24, 523–529.

Janik, D., Mrosovsky, N., 1994. Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms. Brain Res. 651, 174–182.

Johnson, R.F., Moore, R.Y., Morin, L.P., 1989. Lateral geniculate lesions alter circadian activity rhythms in the hamster. Brain Res. Bull. 22, 411–422.

Katz, M.L., Robison, W.G., Jr, 1986. Evidence of cell loss from the rat retina during senescence. Exp. Eye Res. 42, 293–304.

Kornhauser, J.M., Nelson, D.E., Mayo, K.E., Takahashi, J.S., 1990. Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. Neuron 5, 127–134.

Kornhauser, J.M., Nelson, D.E., Mayo, K.E., Takahashi, J.S., 1992. Regulation of June-B messenger RNA and AP-1 activity by light and a circadian clock. Science 255, 1581–1584.

Legg, C.R., 1979. An autoradiographic study of the efferent projections of the ventral lateral geniculate nucleus of the hooded rat. Brain Res. 170, 349–352.

Lieverse, R., Nielen, M.M., Veltman, D.J., Uitdehaag, B.M., van Someren, E.J., Smit, J.H., Hoogendijk, W.J., 2008. Bright light in elderly subjects with nonseasonal major depressive disorder: a double blind randomised clinical trial using early morning bright blue light comparing dim red light treatment. Trials 9, 48.

Ling, C., Schneider, G.E., Jhaveri, S., 1998. Target-specific morphology of retinal axon arbors in the adult hamster. Vis. Neurosci. 15, 559–579.

Lucas, R.J., Douglas, R.H., Foster, R.G., 2001. Characterization of an ocular photopigment capable of driving pupillary constriction in mice. Nat. Neurosci. 4, 621–626.

Lucas, R.J., Freedman, M.S., Munoz, M., Garcia-Fernandez, J.M., Foster, R.G., 1999. Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. Science 284, 505–507.

Lucas, R.J., Hattar, S., Takao, M., Berson, D.M., Foster, R.G., Yau, K.W., 2003. Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. Science 299, 245–247.

Lupi, D., Cooper, H.M., Froehlich, A., Standford, L., McCall, M.A., Foster, R.G., 1999. Transgenic ablation of rod photoreceptors alters the circadian phenotype of mice. Neuroscience 89, 363–374.

Lupi, D., Oster, H., Thompson, S., Foster, R.G., 2008. The acute light-induction of sleep is mediated by OPN4-based photoreception. Nat. Neurosci. 11, 1068–1073.

Lupi, D., Sekaran, S., Jones, S.L., Hankins, M.W., Foster, R.G., 2006. Light-evoked FOS induction within the suprachiasmatic nuclei (SCN) of melanopsin knockout (Opn4−/−) mice: a developmental study. Chronobiol. Int. 23, 167–179.

Mikkelsen, J.D., 1992. Visualization of efferent retinal projections by immunohistochemical identification of cholera toxin subunit B. Brain Res. Bulletin 28, 619–623.

Mirmiran, M., Swaab, D.F., Kok, J.H., Hofman, M.A., Witting, W., Van Gool, W.A., 1992. Circadian rhythms and the suprachiasmatic nucleus in perinatal development, aging and Alzheimer's disease. Prog. Brain Res. 93, 151–163.

Morin, L.P., Blanchard, J., Moore, R.Y., 1992. Intergeniculate leaflet and suprachiasmatic nucleus organization and connections in the golden hamster. Vis. Neurosci. 8, 219–230.

Morin, L.P., Blanchard, J.H., 1997. Neuropeptide Y and enkephalin immunoreactivity in retinorecipient nuclei of the hamster pretectum and thalamus. Vis. Neurosci. 14, 765–777.

Morin, L.P., Blanchard, J.H., Provencio, I., 2003. Retinal ganglion cell projections to the hamster suprachiasmatic nucleus, intergeniculate leaflet, and visual midbrain: bifurcation and melanopsin immunoreactivity. J. Comp. Neurol. 465, 401–416.

Morin, L.P., Pace, L., 2002. The intergeniculate leaflet, but not the visual midbrain, mediates hamster circadian rhythm response to constant light. J. Biol. Rhythms 17, 217–226.

Mrosovsky, N., 1996. Locomotor activity and non-photic influences on circadian clocks. Biol. Rev. Camb. Philos. Soc. 71, 343–372.

Muscat, L., Huberman, A.D., Jordan, C.L., Morin, L.P., 2003. Crossed and uncrossed retinal projections to the hamster circadian system. J. Comp. Neurol. 466, 513–524.

Neufeld, A.H., Gachie, E.N., 2003. The inherent, age-dependent loss of retinal ganglion cells is related to the lifespan of the species. Neurobiol. Aging 24, 167–172.

Panda, S., Provencio, I., Tu, D.C., Pires, S.S., Rollag, M.D., Castrucci, A.M., Pletcher, M.T., Sato, T.K., Wiltshire, T., Andahazy, M., Kay, S.A., Van Gelder, R.N., Hogenesch, J.B., 2003. Melanopsin is required for non-image-forming photic responses in blind mice. Science 301, 525–527.

Panda, S., Sato, T.K., Castrucci, A.M., Rollag, M.D., DeGrip, W.J., Hogenesch, J.B., Provencio, I., Kay, S.A., 2002. Melanopsin (Opn4) Requirement for Normal Light-Induced Circadian Phase Shifting. Science 298, 2213–2216.

Penev, P.D., Turek, F.W., Wallen, E.P., Zee, P.C., 1997. Aging alters the serotonergic modulation of light-induced phase advances in golden hamsters. Am. J. Physiol. 272, R509–R513.

Peters, R.V., Aronin, N., Schwartz, W.J., 1996. c-Fos expression in the rat intergeniculate leaflet: photic regulation, co-localization with Fos-B, and cellular identification. Brain Res. 728, 231–241.

Pickard, G.E., 1985. Bifurcating axons of retinal ganglion cells terminate in the hypothalamic suprachiasmatic nucleus and the intergeniculate leaflet of the thalamus. Neurosci. Lett. 55, 211–217.

Pickard, G.E., 1982. The afferent connections of the suprachiasmatic nucleus of the golden hamster with emphasis on the retinohypothalamic projection. J. Comp. Neurol. 211, 65–83.

Pickard, G.E., Ralph, M.R., Menaker, M., 1987. The intergeniculate leaflet partially mediates effects of light on circadian rhythms. J. Biol. Rhythms 2, 35–56.

Prichard, J.R., Stoffel, R.T., Quimby, D.L., Obermeyer, W.H., Benca, R.M., Behan, M., 2002. Fos immunoreactivity in rat subcortical visual shell in response to illuminance changes. Neuroscience 114, 781–793.

Rea, M.A., 1989. Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei. Brain Res. Bull. 23, 577–581.

Reebs, S.G., Mrosovsky, N., 1989. Large phase-shifts of circadian rhythms caused by induced running in a re-entrainment paradigm: the role of pulse duration and light. J. Comp. Physiol. [A] 165(6), 819–825.

Reiner, A., Zhang, D., Eldred, W.D., 1996. Use of the sensitive anterograde tracer cholera toxin fragment B reveals new details of the central retinal projections in turtles. Brain Behav. Evol. 48, 307–337.

Ribak, C.E., Peters, A., 1975. An autoradiographic study of the projections from the lateral geniculate body of the rat. Brain Res. 92, 341–368.

Riemersma-van der Lek, R.F., Swaab, D.F., Twisk, J., Hol, E.M., Hoogendijk, W.J., Van Someren, E.J., 2008. Effect of bright light and melatonin on cognitive and noncognitive function in elderly residents of group care facilities: a randomized controlled trial. J. Am. Med. Assoc. 299, 2642–2655.

Rieux, C., Carney, R., Lupi, D., Dkhissi-Benyahya, O., Jansen, K., Chounlamountri, N., Foster, R.G., Cooper, H.M., 2002. Analysis of immunohistochemical label of Fos protein in the suprachiasmatic nucleus: comparison of different methods of quantification. J. Biol. Rhythms 17, 121–136.

Robinson, G.A., Madison, R.D., 2004. Axotomized mouse retinal ganglion cells containing melanopsin show enhanced survival, but not enhanced axon regrowth into a peripheral nerve graft. Vis. Res. 44, 2667–2674.

Ruby, N.F., Brennan, T.J., Xie, X., Cao, V., Franken, P., Heller, H.C., O'Hara, B.F., 2002. Role of Melanopsin in Circadian Responses to Light. Science 298, 2211–2213.

Rusak, B., Robertson, H.A., Wisden, W., Hunt, S.P., 1990. Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. Science 248, 1237–1240.

Schwartz, W.J., Carpino A., Jr, de la Iglesia, H.O., Baler, R., Klein, D.C., Nakabeppu, Y., Aronin, N., 2000. Differential regulation of fos family genes in the ventrolateral and dorsomedial subdivisions of the rat suprachiasmatic nucleus. Neuroscience 98, 535–547.

Sekaran, S., Foster, R.G., Lucas, R.J., Hankins, M.W., 2003. Calcium imaging reveals a network of intrinsically light-sensitive inner-retinal neurons. Curr. Biol. 13, 1290–1298.

Sekaran, S., Lupi, D., Jones, S.L., Sheely, C.J., Hattar, S., Yau, K.W., Lucas, R.J., Foster, R.G., Hankins, M.W., 2005. Melanopsin-dependent photoreception provides earliest light detection in the Mammalian retina. Curr. Biol. 15, 1099–1107.

Semo, M., Lupi, D., Peirson, S.N., Butler, J.N., Foster, R.G., 2003a. Light-induced c-fos in melanopsin retinal ganglion cells of young and aged rodless/coneless (rd/rd cl) mice. Eur. J. Neurosci. 18, 3007–3017.

Semo, M., Peirson, S., Lupi, D., Lucas, R.J., Jeffery, G., Foster, R.G., 2003b. Melanopsin retinal ganglion cells and the maintenance of circadian and pupillary responses to light in aged rodless/coneless (rd/rd cl) mice. Eur. J. Neurosci. 17, 1793–1801.

Sutin, E.L., Dement, W.C., Heller, H.C., Kilduff, T.S., 1993. Light-induced gene expression in the suprachiasmatic nucleus of young and aging rats. Neurobiol. Aging 14(5), 441–446.

Swanson, L.W., Cowan, W.M., Jones, E.G., 1974. An autoradiographic study of the efferent connections of the ventral lateral geniculate nucleus in the albino rat and the cat. J. Comp. Neurol. 156, 143–163.

Tamaru, M., Yoneda, Y., Ogita, K., Shimizu, J., Nagata, Y., 1991. Age-related decreases of the N-methyl-D-aspartate receptor complex in the rat cerebral cortex and hippocampus. Brain Res. 542, 83–90.

Thompson, S., Foster, R.G., Stone, E.M., Sheffield, V.C., Mrosovsky, N., 2008. Classical and melanopsin photoreception in irradiance detection: negative masking of locomotor activity by light. Eur. J. Neurosci. 27, 1973–1979.

Valentinuzzi, V.S., Scarbrough, K., Takahashi, J.S., Turek, F.W., 1997. Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice. Am. J. Physiol. 273, R1957–R1964.

Vugler, A.A., Redgrave, P., Semo, M., Lawrence, J., Greenwood, J., Coffey, P.J., 2007. Dopamine neurones form a discrete plexus with melanopsin cells in normal and degenerating retina. Exp. Neurol. 205, 26–35.

Wang, S.M., Villegas-Pérez, M.P., Vidal-Sanz, M., Lund, R.D., 2000. Progressive optic axon dystrophy and vascular changes in rd mice. Invest. Ophthalmol. Vis. Sci. 41, 537–545.

Welsh, D.K., Richardson, G.S., Dement, W.C., 1986. Effect of age on the circadian pattern of sleep and wakefulness in the mouse. J. Gerontol. 41, 579–586.

Zee, P.C., Rosenberg, R.S., Turek, F.W., 1992. Effects of aging on entrainment and rate of resynchronization of circadian locomotor activity. Am. J. Physiol. 263, R1099–R1103.

Zhang, Y., Brainard, G.C., Zee, P.C., Pinto, L.H., Takahashi, J.S., Turek, F.W., 1998. Effects of aging on lens transmittance and retinal input to the suprachiasmatic nucleus in golden hamsters. Neurosci. Lett. 258, 167–170.

Zhang, Y., Kornhauser, J.M., Zee, P.C., Mayo, K.E., Takahashi, J.S., Turek, F.W., 1996. Effects of aging on light-induced phase-shifting of circadian behavioral rhythms, fos expression and CREB phosphorylation in the hamster suprachiasmatic nucleus. Neuroscience 70, 951–961.