Frontiers in Neuroendocrinology 37 (2015) 13–28

Contents lists available at ScienceDirect

Frontiers in Neuroendocrinology

journal homepage: www.elsevier.com/locate/yfrne

Review

The hypothalamic photoreceptors regulating seasonal reproduction in birds: A prime role for VA opsin

José M. García-Fernández [a], Rafael Cernuda-Cernuda [a], Wayne I.L. Davies [b], Jessica Rodgers [c], Michael Turton [c], Stuart N. Peirson [c], Brian K. Follett [d], Stephanie Halford [e], Steven Hughes [c], Mark W. Hankins [c], Russell G. Foster [c,*]

[a] Departamento de Morfología y Biología Celular, Universidad de Oviedo, INEUROPA (Instituto de Neurociencias del Principado de Asturias), Spain
[b] School of Animal Biology and University of Western Australia Oceans Institute, University of Western Australia, Perth, Western Australia, Australia
[c] Sleep and Circadian Neuroscience Institute, Nuffield Laboratory of Ophthalmology, Nuffield Department of Clinical Neurosciences, University of Oxford, Headley Way, Oxford OX3 9DU, United Kingdom
[d] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom.eNuffield Laboratory of Ophthalmology, Nuffield Department of Clinical Neurosciences, University of Oxford, Headley Way, Oxford OX3 9DU, United Kingdom
[e] Nuffield Laboratory of Ophthalmology, Nuffield Department of Clinical Neurosciences, University of Oxford, Headley Way, Oxford OX3 9DU, United Kingdom

ARTICLE INFO

Article history:
Available online 22 November 2014

Keywords:
Arginine-vasotocin (AVT)
Birds
Gonadotropin-releasing hormone (GnRH)
Hypothalamus
Neurosecretory
Opsin
Photoperiodism
Photopigment
Seasonal reproduction
VA opsin

ABSTRACT

Extraretinal photoreceptors located within the medio-basal hypothalamus regulate the photoperiodic control of seasonal reproduction in birds. An action spectrum for this response describes an opsin photopigment with a λ_{max} of ~492 nm. Beyond this however, the specific identity of the photopigment remains unresolved. Several candidates have emerged including rod-opsin; melanopsin (OPN4); neuropsin (OPN5); and vertebrate ancient (VA) opsin. These contenders are evaluated against key criteria used routinely in photobiology to link orphan photopigments to specific biological responses. To date, only VA opsin can easily satisfy all criteria and we propose that this photopigment represents the prime candidate for encoding daylength and driving seasonal breeding in birds. We also show that VA opsin is co-expressed with both gonadotropin-releasing hormone (GnRH) and arginine-vasotocin (AVT) neurons. These new data suggest that GnRH and AVT neurosecretory pathways are endogenously photosensitive and that our current understanding of how these systems are regulated will require substantial revision.

1. Introduction

Reproduction and other seasonal phenomena in temperate birds are profoundly influenced by changes in photoperiod. The mechanisms by which photoperiodic responses influence seasonal reproductive physiology in birds are complex (Sharp and Blache, 2003; Follett and Nicholls, 1984; Sharp, 1993; Dawson et al., 2001; Dawson and Sharp, 2007). However, as a minimum the ability to generate a photoperiodic response requires: (i) photoreceptors to detect the light, (ii) a clock or timer to measure photoperiod duration and (iii) an effector system to activate reproductive and allied physiological responses. There is strong evidence that photoreceptors may exist in several regions of the avian brain, including the pineal gland and septal regions of the telencephalon (Homma et al., 1979; Wang and Wingfield, 2011; Nakane and Yoshimura, 2010). However, there is compelling evidence that all of the components required to generate a photoperiodic response in birds reside within the medio-basal hypothalamus (MBH). To date, the identity of the photopigment(s) in the MBH that mediate these responses, and the cellular pathways involved remain to be determined. In this review we shall focus on the evidence for the MBH as the central site for photoperiodic responses, and discuss the current evidence for and against the involvement of specific classes of opsin photopigments.

During the 1970s lesions within the avian MBH were shown to block the photoperiodic response (Davies and Follett, 1975a,b). A decade later electrical stimulation of this region of the brain was shown to induce the release of luteinizing hormone (LH) (Konishi et al., 1986), and more recently, the isolated MBH of the quail maintained *in vitro* could be stimulated to secrete GnRH in response to a long photoperiod (Perera and Follett, 1992). The use of immunocytochemical approaches in the 1980s allowed an anatomical description of output pathways including the gonadotropin-releasing hormone (GnRH) system. Neurosecretory GnRH perikarya reside within the anterior and medial hypothalamus

* Corresponding author.
E-mail address: russell.foster@eye.ox.ac.uk (R.G. Foster).

http://dx.doi.org/10.1016/j.yfrne.2014.11.001
0091-3022/

and project to the median eminence (ME) (Dawson et al., 2001; Foster et al., 1987). Changes in photoperiod have also been shown to alter the pattern of Fos expression within the MBH and ME, reinforcing roles for these regions in transducing photoperiod into an endocrine response (Meddle and Follett, 1997). The expression of multiple clock genes within both the MBH and the suprachiasmatic nuclei (SCN) has suggested a location within the hypothalamus for the photoperiodic clock (Yasuo et al., 2003). In parallel with these findings, It has been known for many years that thyroid hormones play a critically important role in regulating the avian photoperiodic response (Follett and Nicholls, 1985) but a series of recent studies by Yoshimura and colleagues have placed these observations into a physiological context suggesting that long daylengths regulate thyroid hormone metabolism within the MBH itself (Nakao et al., 2008; Yoshimura et al., 2003).

Collectively these discoveries have been assembled into a working model for photoperiodic time measurement. In brief, photoperiod is detected by uncharacterized hypothalamic photoreceptors and daylength is measured using a molecular clock. Long daylengths stimulate cells (thyrotrophs) in the pars tuberalis (PT) of the anterior pituitary to release thyrotrophin (TSH) which in turn stimulates ependymal cells (tanycytes) lining the third ventricle. The tanycytes express a type 2 deiodinase (*DIO2*) enzyme which converts the prohormone thyroxine (T_4) to bioactive triodothyronine (T_3). T_3 then somehow stimulates neurosecretory GnRH release from terminals within the ME. GnRH is released into the portal blood supply and reaches the anterior pituitary where it stimulates the secretion of the pituitary reproductive hormones including LH and FSH (follicle stimulating hormone). These hormones travel via the general circulation to the reproductive organs to regulate the production of the steroid hormones which include testosterone and estrogen. This description, however, lacks any molecular or cellular detail regarding the nature of the photoreceptors within the MBH which detect photoperiod (Hazlerigg and Loudon, 2008).

In both birds and mammals the final links in the photoperiodic pathway appear broadly conserved with altered daylength changing TSH secretion from the PT. Rising TSH stimulates DIO2 activity and the conversion of T4 to T3, which in turn regulates GnRH secretion (Hanon et al., 2008). The differences between the two vertebrate groups occur at the level of photoperiodic detection. Birds use deep brain photoreceptors (DBPs) whilst mammals have lost all extraretinal photoreceptors, including DBPs, and employ a much less direct photosensory pathway (Hankins et al., 2014). In mammals photoperiod is detected by photosensitive retinal ganglion cells (pRGCs) which, in-turn, alter the pattern of neural activity within the suprachiasmatic nuclei (SCN) (Hankins et al., 2008). The SCN, via a multi-synaptic pathway to the superior cervical ganglion, regulates the sympathetic input to the pineal gland and hence melatonin synthesis and release. Melatonin release from the pineal mirrors the night length. The PT of mammals possesses a very high density of melatonin receptors and the duration of melatonin release from the pineal regulates TSH release from thyrotroph cells within the PT (Hazlerigg and Loudon, 2008). Quite why and when the birds and mammals diverged in their photoperiodic detection mechanisms is unclear but a partial explanation may relate to the early evolutionary history of mammals and their passage through a "nocturnal bottleneck". Modern mammals appear to have been derived from nocturnal insectivorous or omnivorous animals about 100 million years ago (Young, 1962). Whilst deep brain photoreceptors would have been adequate for monitoring photoperiod in diurnal species (from fish to birds), they probably lacked the sensitivity to detect light reliably in a nocturnal mammal emerging from its burrow in late twilight. Perhaps deep brain photoreceptors were lost in the early mammals when they moved into the nocturnal realm during the Triassic (Gerkema et al., 2013).

In the following sections of this review we shall focus on the evidence for the MBH as the central site for photoperiodic responses, and discuss the current evidence for and against the involvement of specific classes of opsin photopigments.

2. The hunt for the deep brain photoreceptor (DBP) of birds

The existence of DBPs in birds was first shown by Benoit in the 1930s. Glass rods were placed within the hypothalamus of ducks which allowed this region of the brain to be illuminated with artificial daylengths. Spring-like daylengths stimulated testicular growth whilst short winter photoperiods had no effect upon reproduction (Benoit, 1935a,b). Refinements of this technique during the 1970s and 1980s (Oliver et al., 1977; Yokoyama et al., 1978) confirmed that birds utilize photoreceptors somewhere within the MBH. The avian skull and brain are remarkably permeable to light and although sunlight is scattered and absorbed by the overlying tissues, surprisingly large numbers of photons penetrate deep into the brain to stimulate these encephalic receptors (Foster and Follett, 1985). In 1985 an attempt was made to define the precise molecular identity of the photopigment by undertaking an action spectrum for photoperiodic induction in the Japanese quail. From this it was concluded that the most likely candidate was an opsin/vitamin A based photopigment with a wavelength of maximum sensitivity (λ_{max}) around 492 nm (Foster and Follett, 1985; Foster et al., 1985).

The opsin/vitamin A photopigments constitute the dominant light sensing molecules across the animal kingdom and their basic biochemistry is fairly well understood. The opsin protein is linked to a light-sensitive retinoid chromophore based upon vitamin A. The first stage of light detection is the absorption of a photon by the 11-*cis* isoform of the chromophore and its isomerisation to the all-*trans* configuration, which results in a conformational change in the opsin protein. This conformational change subsequently permits the binding of a G protein and the activation of a complex biochemical phototransduction cascade (Foster and Hankins, 2007).

In the mid-1980s the only known opsin/vitamin A photopigments of the vertebrates were the rod and cone opsins of rod and cone photoreceptors of the retina, and so the assumption from the action spectrum results was that the deep brain photopigment was avian rod opsin, even though the λ_{max} of avian rhodopsin is closer to 505 nm, rather than 492 nm (Bowmaker, 1977; Bowmaker and Knowles, 1977). A study by Silver and colleagues supported a rod opsin based photopigment system. They used an antibody raised to the amino terminal of rod-opsin (RET-P1) to label a population of cerebrospinal fluid (CSF) contacting neurons within the septal and tuberal areas of the brain of the ring dove (Silver et al., 1988). However, the authors were cautious about the interpretation of their findings because the RET-P1 antibody labeled multiple proteins on immunoblot analysis and none were of the expected molecular weight, suggesting that the immunolabeling might arise from a cross-reaction with a non-rod opsin protein. This was subsequently confirmed by the absence of staining in brain regions with the 1D4 antibody, which detects a highly conserved C-terminal rod opsin sequence motif. Similar results have also been reported in pigeon deep-brain regions, where staining was observed with a rod opsin amino terminal specific antibody but was absent when investigated with a C-terminal antibody (Wada et al., 1998). Although there have been more recent reports of rod opsin labeling within the avian hypothalamus e.g. (Wang and Wingfield, 2011), multiple independent studies have also been unable to detect labeling of any rod opsin positive neurons within

the avian brain using antibodies raised against a range of vertebrate rod opsins (Foster et al., 1987; Vigh and Vigh-Teichmann, 1988). However, despite the inconsistent nature of these results, the findings with RET-P1 are still often cited as evidence that the avian DBP is based upon rod opsin. Viewed today, however, these data suggest that the proteins detected in these studies using anti-rod opsin antibodies probably share some similarity to the amino terminal sequence of rod opsin but that the DBP is almost certainly not based upon the opsin found in retinal rods.

The entire field of vertebrate photoreception changed from the mid 1990s when an increasing number of new opsin gene families were discovered (for review see Hankins et al., 2014; Peirson et al., 2009), and this prompted researchers to entertain the possibility that the avian DBP or DBPs might be based upon novel opsins. Three candidates emerged and have been considered seriously: melanopsin (OPN4) (Chaurasia et al., 2005); neuropsin (OPN5) (Nakane et al., 2010); and vertebrate ancient (VA) opsin (Halford et al., 2009). Traditionally, a number of criteria need to be satisfied before assigning a particular function to a candidate photopigment (opsin) gene. In the case of the DBP these criteria should include:

(i) The candidate mRNA transcript and protein should be expressed unambiguously in photoreceptor cells or areas that are known to be photosensitive.
(ii) The biochemistry of the molecule must be capable of signaling light.
(iii) The photopigment should exhibit a characteristic opsin/vitamin A absorption spectrum with a spectral maxima (λ_{max}) that matches the action spectrum for the appropriate biological response.
(iv) The absorbance characteristics of the photopigment should be appropriately matched to the spectral qualities of the available light.

Based upon these criteria (Foster and Helfrich-Forster, 2001; Davies et al., 2012), the DBPs of birds should utilize a photopigment located within the MBH (see Section 1); be based upon an opsin/vitamin A-based photopigment with a λ_{max} of ~492 nm (Foster et al., 1985); and this λ_{max} should maximize photon capture within the spectrally filtered light environment of the hypothalamus (Foster and Follett, 1985). The extent to which melanopsin (OPN4), neuropsin (OPN5) and VA opsin fulfill these criteria is assessed below.

3. Avian melanopsin (OPN4)

Melanopsin was isolated originally from the photosensitive melanophores of *Xenopus* by Provencio and colleagues (Provencio et al., 1998). Subsequently, orthologs of melanopsin were identified in mammals and shown to be expressed in a subset of retinal ganglion cells (Provencio et al., 2000, 2002), which have been shown to be endogenously photosensitive in rats (Berson et al., 2002), mice (Sekaran et al., 2003) and primates (Dacey et al., 2005). Melanopsin genes were then identified in a range of species including zebrafish (Bellingham et al., 2002) and the chicken (Chaurasia et al., 2005). Subsequently, two melanopsin genes, *OPN4M* (mammal-like) and *OPN4X* (*xenopus*-like), were described in non-mammalian vertebrates, including chickens (Hankins et al., 2014; Bellingham et al., 2006). Initially melanopsin was considered as a very attractive candidate for the DBP based upon its well described function as the "third" ocular photoreceptor regulating non-image forming light detection tasks in mammals (Hankins et al., 2008; Freedman et al., 1999; Peirson and Foster, 2006). However, avian OPN4 fails to fulfill several of the key criteria listed above. Using *in situ* hybridization techniques chicken *OPN4* mRNA was shown to be expressed within the retina, pineal and brain of 1 day-old chicks but its expression was confined to the lateral septal organ and habenular nuclei, with no expression in the hypothalamus (Chaurasia et al., 2005). This is an important finding because all the evidence suggests that the components of the avian photoperiodic response (photoreceptor, timer and effectors) are confined to the MBH (although this does not exclude potential roles for DBPs located in other brain regions – see later discussions). Furthermore, the absorption spectra λ_{max} for cOPN4X and cOPN4 M are between 476 and 484 nm (Torii et al., 2007), which is somewhat shorter than the predicted λ_{max} for the opsin photopigment mediating the avian photoperiodic response at ~492 nm (Foster et al., 1985). On the basis of these available data alone (see below), a role for OPN4 in photoperiodic detection in the chicken should not be excluded, but it seems unlikely that this photopigment plays a dominant role in mediating seasonal reproduction in this species. This may not be the case in all birds, of course. The picture is complicated because in contrast to the chicken, the turkey shows expression of *OPN4X* mRNA and OPN4X protein within dopamine-expressing neurons of the premammillary nucleus (PMN) (Kang et al., 2010; Kosonsiriluk et al., 2013). Although hypothalamic, the PMN technically lays outside the MBH. As a result the expression pattern of OPN4 does not "fit" the predicted location of the DBPs particularly well, but as the PMN is adjacent to the MBH, this pattern of expression should not on its own be used to exclude OPN4 as the photopigment regulating photoperiodic phenomena in this species.

When comparing the differences in OPN4 distribution between the chicken and turkey, it must be stressed that data from one-day old chickens and adult turkeys are being equated, and developmental changes might account for the differing patterns. Finally, it needs to be emphasized that making generalizations regarding avian physiology is not easy. For example, there is considerable diversity in the ways birds utilize different photoreceptors and circadian pacemakers within the eye, pineal and hypothalamus to regulate circadian rhythms of activity (Cassone and Menaker, 1984). Such heterogeneity might also exist in the regulation of photoperiodic phenomena across bird species such that different photopigments in different locations might be involved. But even with these cautionary remarks, there is currently little positive data supporting a role for avian OPN4 in the avian photoperiodic response based upon the criteria outlined above.

4. Avian neuropsin (OPN5)

Neuropsin (OPN5) was identified in 2003 by Foster and colleagues using a bioinformatic approach in both mouse and human genomes (Tarttelin et al., 2003), and subsequently in all vertebrate classes. A detailed study on chicken OPN5 (cOPN5) has demonstrated that it is a UV-sensitive bi-stable photopigment (Yamashita et al., 2010). When cOPN5 is expressed in a HEK293 cell-line, this pigment forms a stable association with both 11-*cis* and all-*trans* retinal, with absorption maxima at 360 nm and 474 nm respectively. Significantly, very similar results have been observed for mouse OPN5, which also forms a bi-stable pigment with a λ_{max} at 380 nm with 11-*cis* retinal (Kojima et al., 2011). By contrast, Yoshimura and colleagues expressed quail OPN5 in *Xenopus laevis* oocytes and proposed that quail OPN5 forms a violet-sensitive pigment with a λ_{max} at 420 nm when reconstituted with 11-*cis* retinal (Nakane et al., 2010). Chicken and quail OPN5 share very high sequence identity, differing in only 7 amino acids located in the N and C terminals. Furthermore, there are no differences in any of the known opsin spectral tuning sites which might account for the reported differences between chicken and quail OPN5 λ_{max}.

As a result it is difficult to reconcile these differing spectra, other than on the basis of unidentified methodological differences.

Avian *OPN5* mRNA has been localized to the paraventricular organ (PVO) of the MBH. This localization immediately suggested that OPN5 might be the photopigment involved in the avian photoperiodic response (Nakane et al., 2010). Most recently, it has been shown that OPN5 expressing cerebrospinal fluid (CSF) contacting neurons in the PVO of the Japanese quail are directly light sensitive (Nakane et al., 2014). Furthermore, these authors show that silencing of OPN5 expression by intracerebroventricular delivery of anti-OPN5 siRNA leads to a decrease in long day induction of TSHβ under UV light in eye patched, pinealectomized quail, whilst also reducing the number of OPN5 positive cells detected within the PVO. These data provide strong evidence for a UV driven light response mediated by OPN5 that is capable of contributing to photoperiodic responses, but as with all the putative photopigments there are difficulties. Although UV light was used specifically for *in vivo* studies of TSHβ induction performed by Nakane et al. (2014), the recording of cellular light responses from PVO–CSF neurons was performed using a white light source that contained a UV component rather than a monochromatic UV light source. It is therefore hard to confirm that these light responses are mediated by either UV light or OPN5, and it is possible that these responses may be mediated by other photopigments expressed within these cells (Wang and Wingfield, 2011; Silver et al., 1988; Wada et al., 1998). Surprisingly the authors did not investigate the loss of PVO–CSF light responses following silencing of OPN5, an experiment that would surely have confirmed the role of OPN5 in the photosensitivity of PVO–CSF contacting cells. Furthermore, OPN5 is widely expressed in the avian brain, and administration of anti-OPN5 siRNA may potentially lead to a reduction of OPN5 expression in several brain regions including, but not limited to the PVO. It is therefore hard to confirm the PVO as the site of UV light driven photoperiodic responses mediating TSHβ release under long days (Menaker, 2014). Furthermore the authors show only a role for OPN5 in TSHβ induction under UV conditions. Similar experiments performed under full spectrum lighting conditions would have enabled the overall impact of OPN5 to be determined under conditions where other photopigments might contribute to these responses.

Reconciling whether OPN5 forms a UV- (chicken) or violet- (quail) sensitive photopigment requires further work, but either way these λ_{max} would not support OPN5 as the primary photopigment driving the photoperiodic response, based upon the 492 nm action spectrum data (Foster et al., 1985), and the observation that relatively little UV light penetrates into the hypothalamus (Foster and Follett, 1985; Hartwig and van Veen, 1979). OPN5 has been shown to be highly expressed in the adrenal glands of birds, leading to the suggestion that it may have a chemosensory, rather than photoreceptor function (Ohuchi et al., 2012). Collectively there is currently too little evidence to support a central role for OPN5 in the avian photoperiodic response. Either critical data are missing or contradictory, and further work will be needed to define precisely what the function of this opsin is within the PVO, and whether it has a role in photoperiodic time measurement.

5. Avian vertebrate ancient (VA) opsin

Vertebrate ancient (VA) opsin was first isolated from ocular cDNA from Atlantic salmon (*Salmo salar*) by Foster and colleagues (Soni et al., 1998; Soni and Foster, 1997) and has been identified subsequently in several other teleost fish including zebrafish (*Danio rerio*) (Kojima et al., 2000), the common carp (*Cyprinus carpio*) (Moutsaki et al., 2000), smelt (*Plecoglossus altivelis*) (Minamoto and Shimizu, 2002), roach (*Rutilus rutilus*) (Jenkins et al., 2003) and the agnatha (Yokoyama and Zhang, 1997). When expressed *in vitro* and reconstituted with 11-*cis*-retinal, VA opsins form functional photopigments with a λ_{max} between 460 and 500 nm (Soni et al., 1998; Kojima et al., 2000). The teleost VA opsins are expressed in a subset of neurons in the horizontal and ganglion cell layers of the retina, the pineal and in two bilateral columns of sub-ependymal cells of the MBH (Kojima et al., 2000; Philp et al., 2000). VA opsin-expressing retinal horizontal cells have been shown to be directly photosensitive and to utilize an opsin/vitamin A photopigment with a λ_{max} near 477 nm (Jenkins et al., 2003). These results in teleost fish stimulated the search for orthologues of the VA opsins in other vertebrate classes, but until recently all attempts had met with failure. This restricted taxonomic distribution of the VA opsins was puzzling as most other opsin classes are found across multiple vertebrate taxa. This anomaly, along with the distribution of VA opsin within the teleost brain and the absence of any other strong candidate for the avian photoperiodic photoreceptor, prompted another search for homologues of this gene within birds.

The chicken genome database was searched for VA opsin-like sequences and a region on chromosome 6 was detected that showed approximately 70% identity to the teleost VA opsins. The full-length sequences of two isoforms of chicken VA opsin (cVA and cVAL) were then subsequently isolated by PCR from both ocular and hypothalamic cDNA. The gene (GenBank accession numbers EF055883 and GQ280390) encodes open reading frames of 972 bp (cVA) and 1080 bp (cVAL) which encode predicted proteins of 323 and 359 amino acids respectively (Halford et al., 2009). Additional bioinformatic analyses of other genome databases has also allowed the identification of VA opsin-like genes in multiple vertebrate classes including the Amphibia (*Xenopus laevis*), Reptilia (*Anolis carolinensis*) and the Elasmobranchii (*Callorhinchus milii*), but interestingly failed to find any VA opsin homologues within the mammalian databases. As a result, mammals have not only lost DBPs (see Section 1) but also the VA opsin gene. A phylogenetic analysis of the deduced VA proteins places all the identified sequences in a distinct phylogenetic clade (Halford et al., 2009). In this regard, VA opsin resembles many of the other recently discovered "orphan opsin families" (Davies et al., 2010). The next question was to see whether it met any of the photobiology criteria predicted for the avian DBP.

5.1. Functional expression of chicken VA (cVA) opsin

To determine whether cVA opsin is capable of forming a functional photopigment and can mediate phototransduction, an *in vitro* heterologous expression system was employed (Melyan et al., 2005). Full-length cVA and cVAL were both cloned into the expression vector pIRES2-AcGFP and transiently transfected into Neuro-2A cells. Whole-cell patch clamp recordings from transfected fluorescent cells, expressing either cVA or cVAL, demonstrated light dependent inward currents in the presence, but not the absence, of chromophore (Fig. 1). These results demonstrate that both cVA and cVAL can elicit a retinal-dependent light response *in vitro*, consistent with the activity of a fully functional opsin photopigment that can couple to a ubiquitous G-protein cascade in Neuro-2A cells. Thus one criterion has been met, namely that the biochemistry of the molecule must be shown to be capable of signaling light.

5.2. The spectral sensitivity of chicken VA (cVA) opsin

The first approach to determine the spectral sensitivity of cVA involved the *in vitro* expression of a full-length cVA sequence in HEK293T cells (at 37 °C), reconstituted with 11-*cis* retinal and subjected to UV–Vis spectrophotometry. Despite repeated attempts,

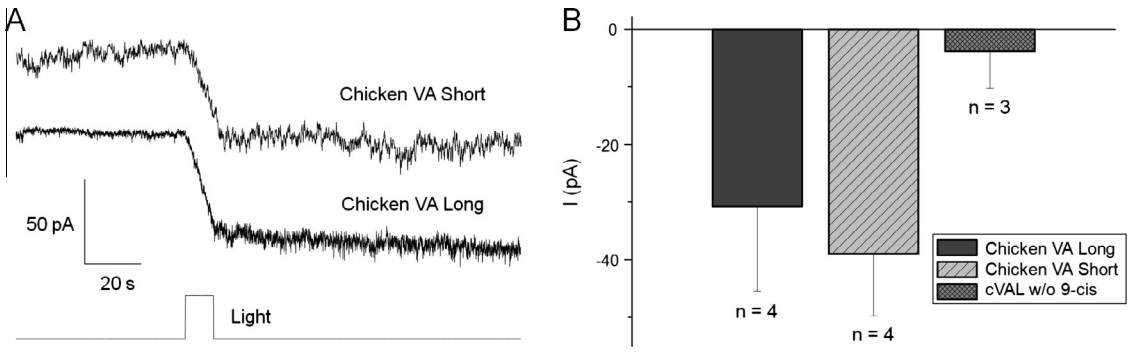

Fig. 1. (**A**) Transient expression of either chicken VA opsin short or VA opsin long (cVA or cVAL) in Neuro-2A cells leads to a light-evoked, 9-*cis*-retinal-dependent inward current in response to a 460 nm light stimulus (8×10^{14} photons cm^{-2} s^{-1}). Note 9-*cis*-retinal can be substituted for the naturally occurring chromophore 11-*cis*-retinal. These light induced currents were not seen in sham-transfected cells, or in the absence of 9-*cis*-retinal. (**B**) Amplitudes of light evoked currents recorded from cVAS and cVAL transfected Neuro-2A cells in the presence or absence of chromaphore (9-*cis*-retinal). The holding potential was −50 mV. Data shown is mean ± SEM. Figure modified from Halford et al. (2009).

no spectra were obtained (Fig. 2A), almost certainly for the reasons articulated below. Two additional approaches were then undertaken. The first involved the direct extraction of *in vivo* VA protein from a lysate made from the hypothalami of chick and adult chicken by affinity immunochromatography, using an antibody specific for cVA (VA85) (Halford et al., 2009). Darkadapted brain tissue ensured the purified native cVA protein remained conjugated to its endogenous 11-*cis* retinal chromophore. As shown in Fig. 2B, UV–Vis spectrophotometry resulted in a difference spectrum that could then be fitted to a Govardovskii vitamin A_1-based photopigment template. The result was an opsin/vitamin A 13 photopigment with a spectral peak at 491 nm ($R^2 = 0.5$). The second method involved repeating the failed *in vitro* regeneration experiments with cVA-transfected HEK293T cells but this time incubating at 30 °C instead of 37 °C. The rationale was that the lower temperature would reduce the rate of translation and endocytic membrane clearance, and provide more time for correct protein folding and trafficking of viable opsin to the cell membranes. Indeed this approach yielded protein, which when reconstituted with excess 11-*cis* retinal and subjected to UV–Vis spectrophotometry, and produced a characteristic opsin/vitamin A difference spectrum with a λ_{max} value of 490 nm ($R^2 = 0.7$) (Fig. 2C).

These *in vivo* and *in vitro* approaches yielded absorbance maxima at ~490 nm, which is 2 nm away from the reported 492 nm peak of the action spectrum for the avian photoperiodic response (Hankins et al., 2008; Young, 1962). Thus another requirement for assigning a role for VA opsin in the avian photoperiodic response was demonstrated.

5.3. The spectral sensitivity of chicken VA opsin and the light environment of the brain

The avian skull and brain are permeable to light across the visible spectrum, but scatter and absorption of photons by the overlying tissues significantly affects the spectral composition of sunlight that is transmitted into the hypothalamus of birds (Foster and Follett, 1985). Specifically, as sunlight passes through the feathers, skin, skull and brain, wavelengths within a range of 400–450 nm (and to a lesser extent 525–550 nm) are greatly attenuated, due to the presence of hemoglobin with absorbance peaks at 430 nm and 550 nm (Fig. 2D). However, there is a small, yet clearly defined "spectral window" in hemoglobin absorption and light penetrates into the brain between 450 and 550 nm, with a peak at 489 nm ($R^2 = 0.9$) (Fig. 2D–1E). This value is significant as it very closely corresponds to the spectral absorbance peak for VA opsin photopigment (Fig. 2B–1C) and the action spectrum of the photoperiodic response (Fig. 2E). Photons at wavelengths beyond 590 nm penetrate into the hypothalamus with greater efficiency than 489 nm (Fig. 2D). But there is a problem in generating a red-shifted photopigment to maximize photon capture in most terrestrial vertebrates. Opsin-based photopigments are either conjugated to vitamin A_1- or A_2-based chromophores. Vitamin A_2 retinal chromophore confers longer wavelength sensitivity compared to A_1 retinal. Birds, however, are unable to manufacture A_2 retinal and so generating a photopigment with a λ_{max} at 590 nm or longer to maximize photon capture would not be possible. But even if this were possible, any increase in photon capture would be outweighed by increased thermal noise and a drop in overall signal-to-noise. A long-wavelength-shifted photopigment in the 40 °C temperature environment of the avian brain would have a greatly diminished capacity to detect light-induced isomerisation from thermally induced isomerisation of the chromophore. As a result, these data fully support the criteria listed in Section 2, that the absorbance characteristics of VA opsin photopigment should be appropriately matched to the spectral qualities of the available light within the hypothalamus.

5.4. The distribution of VA opsin containing neurons within the chicken and quail hypothalamus

In order to provide a cellular localization of avian VA opsin protein within the avian hypothalamus, immunocytochemistry was undertaken using a fully characterised antibody (VA-85) raised against a peptide corresponding to an amino terminus region predicted to be extracellular and common to both isoforms (Halford et al., 2009). This antibody was then used to localize VA opsin within chicken and quail brain using immunocytochemistry. Birds were adult and sexually mature. In both species strongly labeled perikarya and fibers were identified within the MBH with an extensive projection of fibers into the median eminence (Figs. 3 and 4). In the anterior hypothalamus of the chicken VA-immunoreactive (VA-ir) perikarya extend in a rostro-caudal direction from the preoptic area to the paraventricular nucleus (PVN). The number of VA-ir neurons decrease in the caudal part of the PVN, and finally all immunoreactive perikarya disappear at the level of the tuberal hypothalamus. However, VA-ir fibers run ventrally and medially through the basal hypothalamus to the ME. These VA-ir fibers occupy the inner region of ME and form a compact tract. Notably, VA-ir fibers extend from this tract to pass through the external

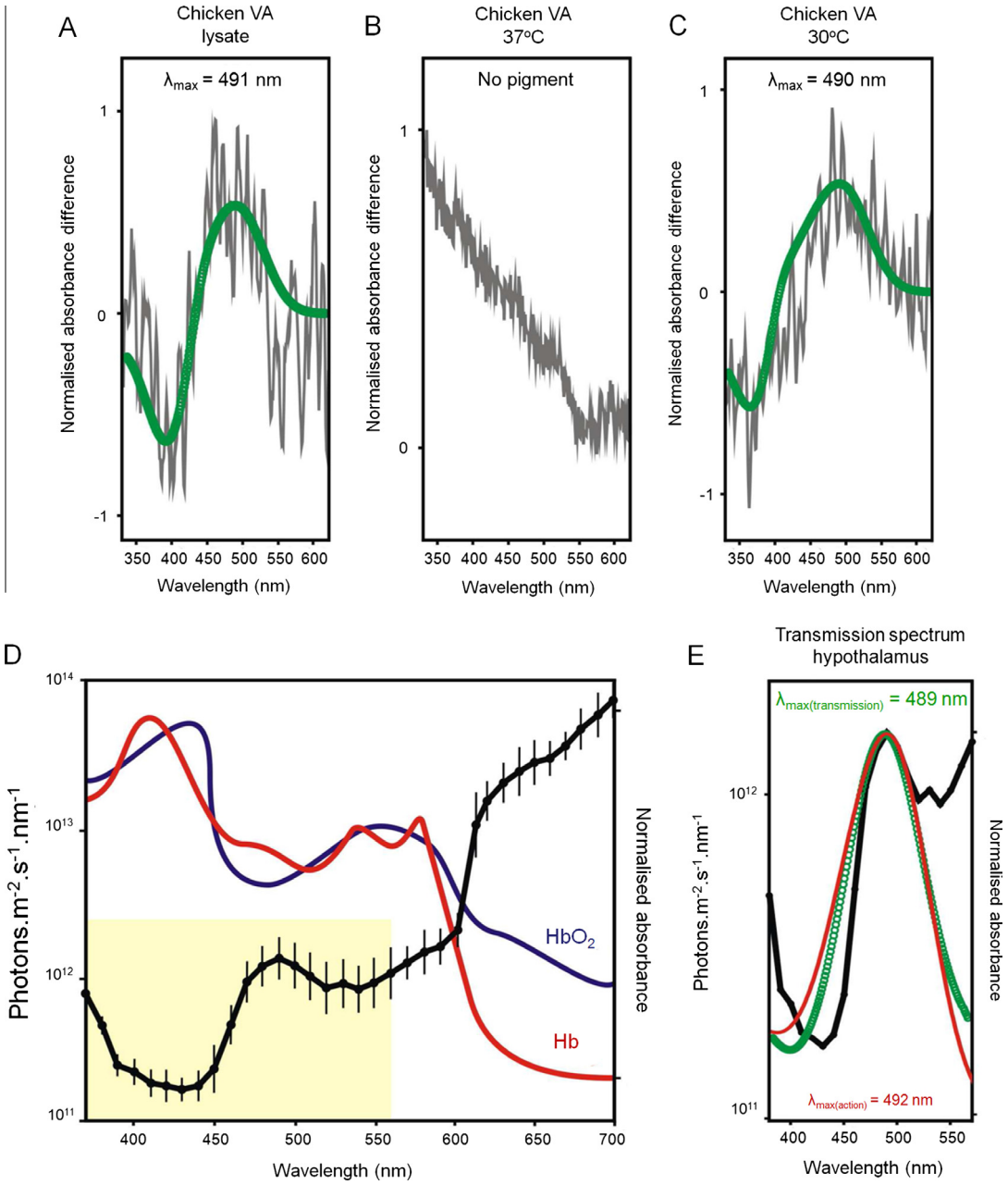

Fig. 2. Absorbance difference spectra (gray line) for chicken VA opsin (cVA) photopigment protein extracted from (**A**) cell lysate generated from the hypothalami of chick and adult chickens, (**B**) HEK293T cells transfected with full-length cVA coding sequence and incubated at 37 °C (failed regeneration), and (**C**) HEK293T cells transfected with full-length cVA coding sequence and incubated at 30 °C (modified from Davies et al. (2012)). (**D**) Spectral composition of sunlight passing through the head (feathers, skin, skull and brain) of birds to reach the basal hypothalamus (modified from Foster and Follett (1985)) (black line) compared to the absorbance profile of oxygenated (HbO_2) (blue line) and deoxygenated (Hb) (red line) hemoglobin. The yellow shading refers to the transmission spectrum highlighted in (E). (**E**) Comparison of the transmission spectrum for light at the avian hypothalamus (black line), within a physiological range for vitamin A1-based photopigments that exhibit spectral peak of absorbance (λ_{max}) values between 360 nm and 560 nm, fitted to a vitamin A_1-based photopigment template (open green circles) with the action spectrum for the avian photoperiodic response (modified from Foster and Follett (1985), Foster et al. (1985)) (red line). In all cases, λ_{max} values for each experiment are shown, as determined by fitting with a vitamin A_1 photopigment template curve (open green circles). For full details see (Davies et al., 2012).

zone of the median eminence to terminate adjacent to the pars tuberalis (PT) (Fig. 3C). For further details see Section 6.3 below.

The anatomical distribution of VA-ir perikarya and fibers within the MBH is consistent with previous findings suggesting that the avian photoperiodic photoreceptors are located primarily within this region of the brain. As a result VA opsin has been shown to fulfill all the four criteria that would link this photopigment to the deep brain photoreceptors mediating seasonal reproduction in birds. However, co-localization studies have added an additional dimension to VA opsin's role and possible function within the avian brain.

6. Co-expression of VA opsin with arginine-vasotocin (AVT) and gonadotropin releasing hormone (GnRH) neurosecretory neurons

The distinctive pattern of VA opsin expressing neurons illustrated in Fig. 4 is remarkably similar to the distribution of two

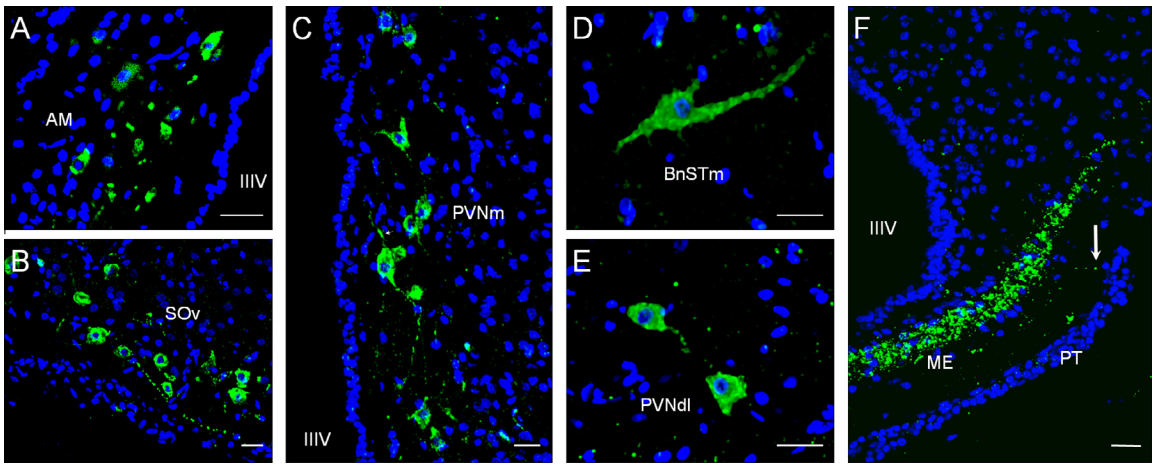

Fig. 3. Location of VA opsin immunoreactive neurons and fibers in the hypothalamus of the chicken. (**A**) VA opsin immunoreactive neurons and fibers in the nucleus anterior medialis hypothalami (AM) at the level of the recessus praeopticus of the third ventricle (IIIV); (**B**) the nucleus supraopticus, pars ventralis (SOv); (**C**) the medial part of the paraventricular nucleus (PVNm) near the columnar ependymocytes of the third ventricle (IIIV); (**D**) the bed nucleus of stria terminalis (BnStm); (**E**); the nucleus paraventricularis magnocellularis, pars dorsolateralis (PVNdl); and (**F**) VA opsin immunoreactive fibers in the median eminence (ME). These positive fibers occupy the inner region of ME and form a compact tract, with some axons clearly innervating the external zone of the median eminence, and proceeding to the pars tuberalis (PT) (arrowhead). Scale bar = 20 μm.

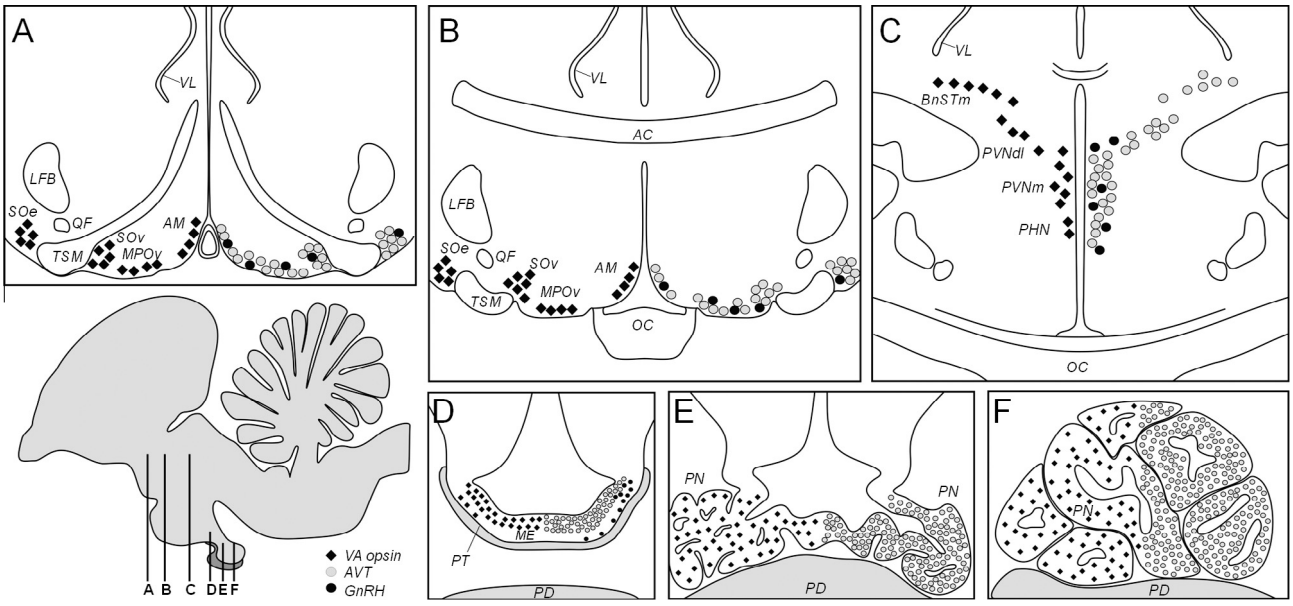

Fig. 4. Summary of the distribution of VA opsin, AVT and GnRH immunolabeling in the quail and chicken brain. The profile of the brain in sagital section (lower left drawing) indicates the approximate location of the cross sections represented in A–F. The key for the symbols used is indicated above. VA opsin immunolabeling is represented on the left half of the drawings, whilst AVT and GnRH immunolabeling are represented on the right. Symbols represent neuronal somata in A–C and axons in D–E. VA opsin or AVT immunolabeled axons with Herring bodies are found in the median eminence (ME) and in the pars nervosa (PN) of the pituitary gland. GnRH axons are restricted to the ME (D). Note that this part of the pituitary is constituted by a number of lobules originated by evagination from the median eminence. Each lobule contains a lumen. *Abbreviations*: AC, anterior commissure; AM, nucleus anterior medialis hypothalami; BnSTm, bed nucleus of stria terminalis, medial part; LFB, lateral forebrain bundle; ME, median eminence; MPOv, nucleus magnocellularis preopticus, pars ventralis; OC, Optic Chiasm; PD, Pars Distalis; PHN, nucleus periventricularis hypothalami; PN, pars nervosa; PT; pars tuberalis; PVNdl, nucleus paraventricularis magnocellularis, pars dorsolateralis; PVNm, nucleus paraventricularis magnocellularis, pars medialis; QF, tractus quintofrontalis; SOe, nucleus supraopticus, pars externus; SOv, nucleus supraopsticus, pars ventralis. VL, ventriculus lateralis.

"classical" neurosecretory systems of the avian hypothalamus: the arginine-vasotocin (AVT)/mesotocin neurosecretory cells (Oksche et al., 1964; Goossens et al., 1977; Bons, 1980a,b; Sanchez et al., 1991; Panzica et al., 2001) and the gonadotropin releasing hormone (GnRH) neurosecretory neurons (Foster et al., 1987, 1988; D'Hondt et al., 2000). To determine whether VA opsin is indeed co-localized within these two neurosecretory networks, a series of detailed immunocytochemical co-localization studies were undertaken and the results are presented here for the first time. As a result we provide below full methodological details for these findings.

6.1. Antibodies

Three polyclonal antisera were utilized; (i) VA opsin antibody (VA-85) raised in rabbit against the amino-terminal region of chicken VA opsin diluted 1:2000 to 1:4000. The specificity of this antibody has been outlined previously (Halford et al., 2009) and

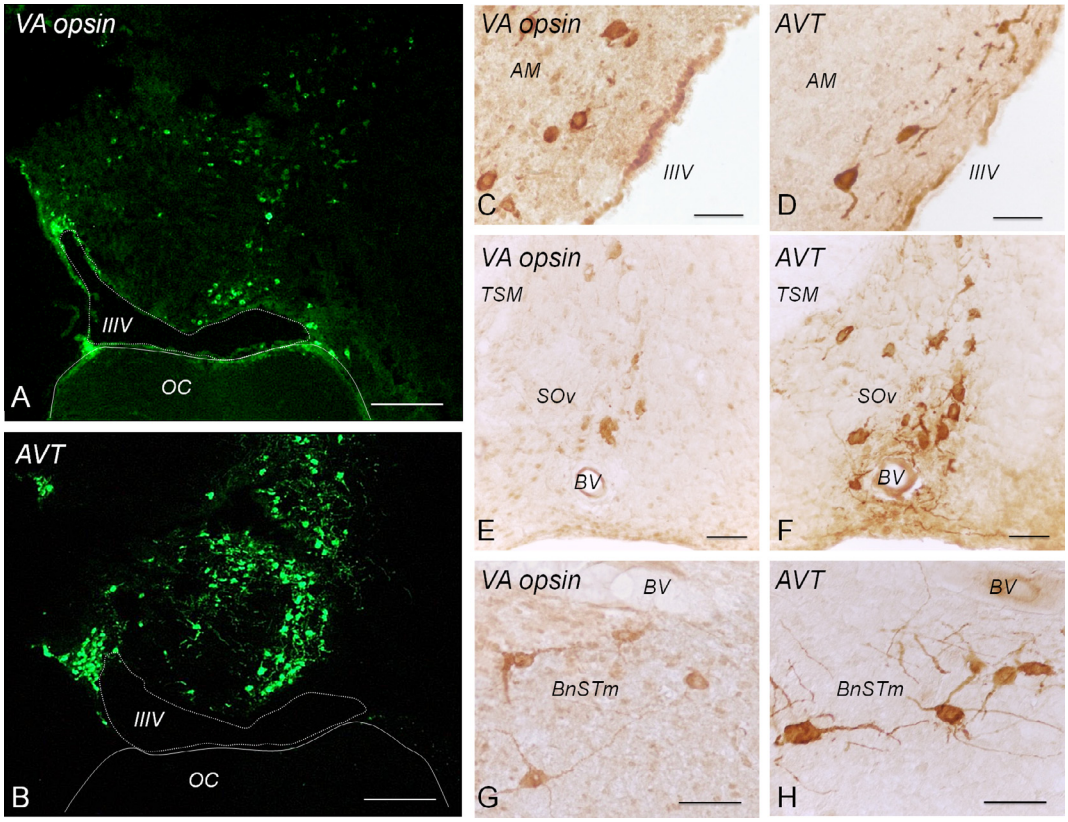

Fig. 5. Adjacent parasagittal 15 μm-thick sections showing VA opsin (**A**) and AVT (**B**) immunolabeling with a tyramide-based amplification method in the anterior and medial hypothalamus dorsal to the preoptic and supraoptic ventricular recess. The number of VA opsin immunolabeled neurons in the paraventricular nucleus (PVN) is smaller than that of AVT immunolabeled neurons. **C–H**: Cross-sections (immunostained with the ABC method) of three different hypothalamic areas showing VA opsin (left) and AVT immunolabeling (right): **C–D**, nucleus anterior medialis hypothalami (AM); **E–F**, nucleus supraopticus, pars ventralis (SOv); **G–H**, bed nucleus of stria terminalis, medial part (BnSTm). (BV, blood vessel; IIIV, third ventricle; OC, optic chiasm; TSM, tractus septomesencephalicus). Scale bars; A–B, 100 μm; C–H, 25 μm.

is confirmed by labeling of mammalian cell lines transiently transfected with plasmids encoding chicken VA opsin (results not shown); (ii) Anti-vasopressin antibody (Chemicon, Tremula, CA, USA) was raised in rabbit against synthetic vasopressin hormone, diluted 1:4000. The specificity of this antibody has been demonstrated previously (Shen et al., 1992; Arai et al., 1993; Alvarez-Viejo et al., 2003); (iii) Anti-GnRH antibody (Chemicon, Tremula, CA, USA) raised in rabbit against synthetic gonadotropin-releasing hormone (GnRH1), diluted 1:12,000. This antiserum has been used widely in many vertebrate species (Alvarez-Viejo et al., 2003; Kozlowski and Coates, 1985). In our hands this antibody also produced very clear and specific labeling of GnRH cells and fibers in the mouse brain (data not shown).

6.2. Immunostaining

Single labeling of primary antibodies was performed according to standard immunohistochemcial protocols, using the ABC "Elite" staining kit (Vectastain; Vector laboratories, Burlingame, CA, USA.) with peroxidase-conjugated avidin-biotin secondary antibody complexes and diaminobenzidine (DAB) as the chromogen. Primary antibodies were diluted in PBS containing 1% serum and 0.2% Triton-X, and incubated for 72 h in a humid chamber at 4 °C. For double labeling of VA opsin and vasopressin antibodies (both raised in rabbit) fluorescent approaches were employed using modified protocols aimed at minimizing cross reactivity of the primary antibodies. The VA opsin antibody was incubated first, diluted 1:10,000, followed by detection with Tyramide Signal Amplification (TSA™) conjugated to Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA). The vasopressin antibody was then incubated at 1:4000, followed by labeling with a Texas red conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). For controls, sections were processed with one or both primary antibodies replaced by non-immune serum. No specific immunoreactions were observed in these control sections. For double labeling of VA opsin and GnRH antibodies (both raised in rabbit), the individual primary antibodies were incubated and labeled sequentially, with additional blocking steps to minimize sequestering and cross reactivity of primary antibodies (Hughes et al., 2013). The VA opsin antibody was incubated first, diluted 1:2000, followed by donkey anti-rabbit Alexa 568 for 2 h at RT. Sections were then incubated with unconjugated rabbit anti-goat IgG (Sigma Aldrich, Dorset, UK) 1:200 for 2 h at RT and then unconjugated donkey anti-rabbit monovalent FAB fragments 1:100 (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 2 h at room temperature. The GnRH antibody was then incubated at 1:10,000 followed by donkey anti-rabbit Alexa 488 (Molecular Probes, Carlsbad, CA, USA). This double rabbit antibody protocol has been used previously for successful specific co-localisation studies. For details see (Hughes et al., 2013).

Color brightfield images were collected on a Nikon Eclipse E400 microscope fitted with a Nikon Digital Sight DS-L2 camera (Nikon, Tokyo, Japan). Fluorescent confocal images were collected using a LSM 710 laser scanning confocal microscope and Zen 2009 image acquisition software (Zeiss). Individual channels were collected sequentially. Laser lines for excitation were 405 nm, 488 nm and 561 nm. Emissions were collected between 440–480, 505–550

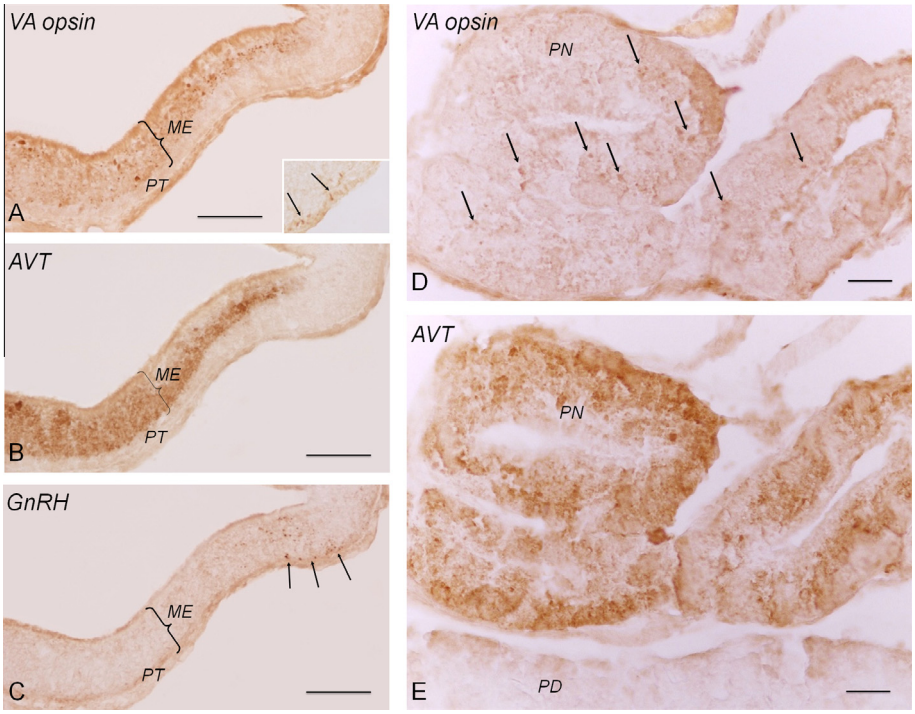

Fig. 6. VA opsin, AVT and GnRH immunelabeling in contiguous sections of the median eminence (ME) (**A–C**) and of the pars nervosa (PN) (**D–E**) of the quail pituitary gland. The distribution of VA opsin immunolabeling (**A**) runs in parallel to that of AVT immunolabeling (**B**). In a contiguous section GnRH fibers are restricted to the lateral areas of the ME and some can also be seen in the outer margins (arrows). The inset in A shows a more rostral area in the outer margins of the ME with some VA opsin immunolabeled fibers (this area also contained GnRH fibers as indicated by arrows in **C**). VA opsin immunolabeling is less intense than AVT at both the ME and the PN; nevertheless, VA opsin immunolabeled Herring bodies, some of which are indicated by arrows, were found throughout the PN. Note that the PN is constituted by lobules. (PD, pars distalis; PT; pars tuberalis). Scale bars; A–C, 50 μm; C–H, 25 μm.

and 580–625 nm for blue, green and red fluorescence respectively. For all images, global enhancement of brightness and contrast was performed using ImageJ software (NIH; rsbweb.nih.gov/ij/).

6.3. Co-localization results

All results are shown in the diagram presented in Fig. 4.

(i) VA opsin immunolabeled cells are located close to the ventricle (subependymal) in the paraventricular (PVNm), periventricular (PHN) and the anterior medial nucleus (AM) and laterally in the supraoptic nucleus. Rostro-caudally, the first VA opsin neurons appear in the preoptic area, lateral to the lamina terminalis and to the medial side of the tractus septo mesencephalicus (TSM). There is also a group of VA opsin neurons lateral to the TSM adjacent to the floor of the brain. These cell groups are the suproaoptic nucleus (pars externus, SOe, and pars ventralis, SOv) and the magnocellular preoptic nucleus, pars ventralis (MPOv) (Fig. 4A and B). More posteriorly and caudally to the anterior commissure (CA) in the paraventricular region there are VA opsin containing neurons along the midline, close to the third ventricle, and in the nucleus paraventricularis magnocellularis (PVN) and the nucleus periventricularis hypothalami (PHN). Some VA opsin neurons are also found scattered laterally and dorsally, in the area of the bed nucleus of stria terminalis in the pretectum, in its medial and lateral parts. The most lateral group of VA opsin immunoreactive cells are at the edge of the fasciculus prosencephalic lateralis (LFB = lateral forebrain bundle) in the lateral hypothalamic region. In more caudal regions the number of VA opsin immunoreactive neurons decreases and labeled cell bodies disappear at the level of the caudal part of the PVN. Immunolabeled VA opsin fibers could be observed in the ventrolateral part of the medial hypothalamus, running toward the basal areas at the level of the decussatio supra-optica dorsalis. At the infundibulum, these immunopositive fibers ran through the inner part of the median eminence (Fig. 4D) toward the neural lobe of the pituitary. VA opsin immunoreactivity was found throughout the pars nervosa (Figs. 4E, F and 6D).

(ii) AVT immunolabeled cell bodies and fiber tracts, show a distribution that has been described previously using both aldehyde-fuchsin staining and antibodies raised against vasopressin (Goossens et al., 1977; Bons, 1980a,b; Oksche et al., 1964; Panzica, 1985). As illustrated in Fig. 5, both the number and the degree of immunostaining of VA opsin cell bodies appeared to be slightly lower than those of AVT, presumably due to different levels of protein expression, and/or antibody binding. VA opsin and AVT fibers are both located in the same inner part of the median eminence and have the same distribution in the neural lobe of the pituitary, as is shown in contiguous sections in Fig. 6. The VA opsin and AVT antibodies label the Herring bodies in both neuroanatomical structures.

(iii) GnRH immunolabeled cell bodies and fiber tracts show a distribution that has been observed previously in the chicken and quail hypothalamus (Foster et al., 1988; D'Hondt et al., 2000). In the anterior hypothalamus GnRH cell bodies are localized adjacent to the floor of the brain in the supraoptic nucleus, medial and lateral to the tractus septomesencephalicus. In the anterior and medial hypothalamic areas, GnRH neurons lie near the third ventricle, as well as in the anterior medialis hypothalamic nucleus and the paraventricular region (shown in Fig. 4).

(iv) Co-localization of VA opsin with AVT. Co-localization studies employed two different techniques; in the first instance 5 μm thick sections were cut that allowed individual cells to be represented on two adjacent sections, and

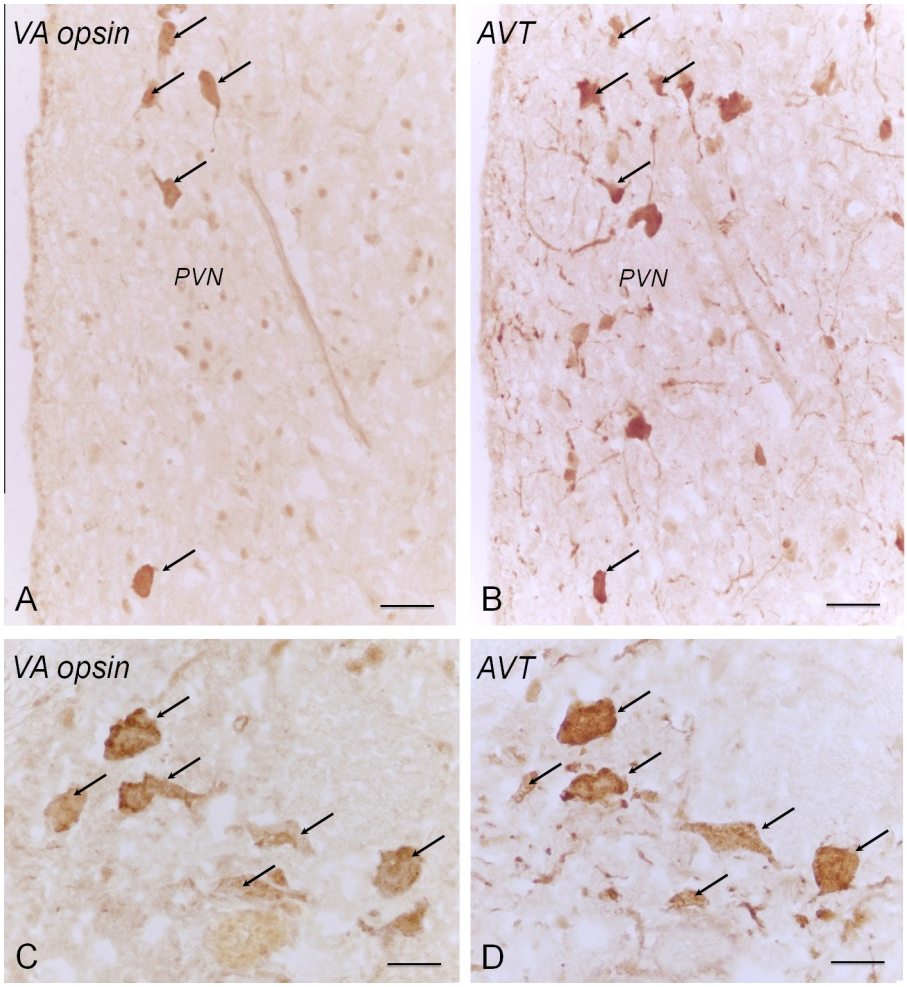

Fig. 7. VA opsin and AVT imnmunolabeling in contiguous sections of the chicken brain. Excerpts of the same neurons appear to be immunolabeled at both sections, demonstrating co-localization. **A–B** show an area of the paraventricular nucleus (PVN) in two 5 μm-thick sections, in which the number of VA opsin immunolabeled neurons (indicated with arrows) is smaller than the number of AVT neurons. Nevertheless, co-localization of both antigens in all the somata can be observed in the supraoptic nucleus (**C–D**) (5 μm-thick contiguous sections). Scale bars; A–B, 25 μm; C–D, 10 μm.

subsequently stained with two different primary antibodies; Secondly, double labeling was performed on the same 15 μm thick sections using modified protocols aimed at minimizing cross reactivity of the primary antibodies and signal contamination (Hughes et al., 2013). Comparison of 5 μm-thick contiguous sections, immunostained separately with AVT and VA opsin antibodies showed clear evidence of co-expression of these two antigens (Fig. 7). In multiple examples, individual neurons can be identified in adjacent sections, and show immunoreactivity for both VA opsin and AVT. Simultaneous double labeling of VA opsin and AVT antibodies on the same 15 μm sections again indicated co-expression of VA opsin within AVT labeled neurons. The majority of the VA opsin positive cells were also AVT positive, although some VA opsin immunoreactive cells can be observed that are not labeled by the AVT antibody (Fig. 8). Specific immunoreactions were absent when one or both primary antibodies were replaced by non-immune serum.

(v) The co-localization of VA opsin with GnRH. To study the possibility of co-expression of GnRH and VA opsin within individual neurons we again adopted two different techniques; single labeling of both antibodies on contiguous 5 μm thick sections, or double labeling of both primary antibodies using modified protocols aimed at preventing cross labeling of both primary antibodies. Single labeling of adjacent 5 μm-thick sections with VA opsin and GnRH antibodies shows that a sub-set of GnRH positive neurons are also labeled with VA opsin antibodies (Fig. 9). Double labeling of VA opsin and GnRH antibodies again shows clear evidence of co-localisation for both antigens, with the majority of GnRH positive neurons also positively stained for VA opsin (Fig. 10). VA opsin staining is detected primarily in the cell body of immunoreactive cells, with VA opsin staining less evident along the processes of these cells. A large number of GnRH positive fibers were identified that lacked detectable levels of VA opsin staining. Both GnRH immunoreactive and VA opsin immunoreactive fibers were detected in the median eminence, close to the pars tuberalis, although the number of GnRH positive fibers is lower than the number of VA opsin positive fibers (Fig. 6).

Collectively the results from the double labeling studies and labeling 5 μm thick alternate sections show a high level of co-expression of VA opsin with both AVT and GnRH neurosecretory cells across the avian brain. Given that VA opsin has been shown to form a functional photopigment (Halford et al., 2009; Davies et al., 2012), these findings suggest that VA opsin may play multiple photosensory roles in the neuroendocrinology of birds. The broader implications of these results are considered below.

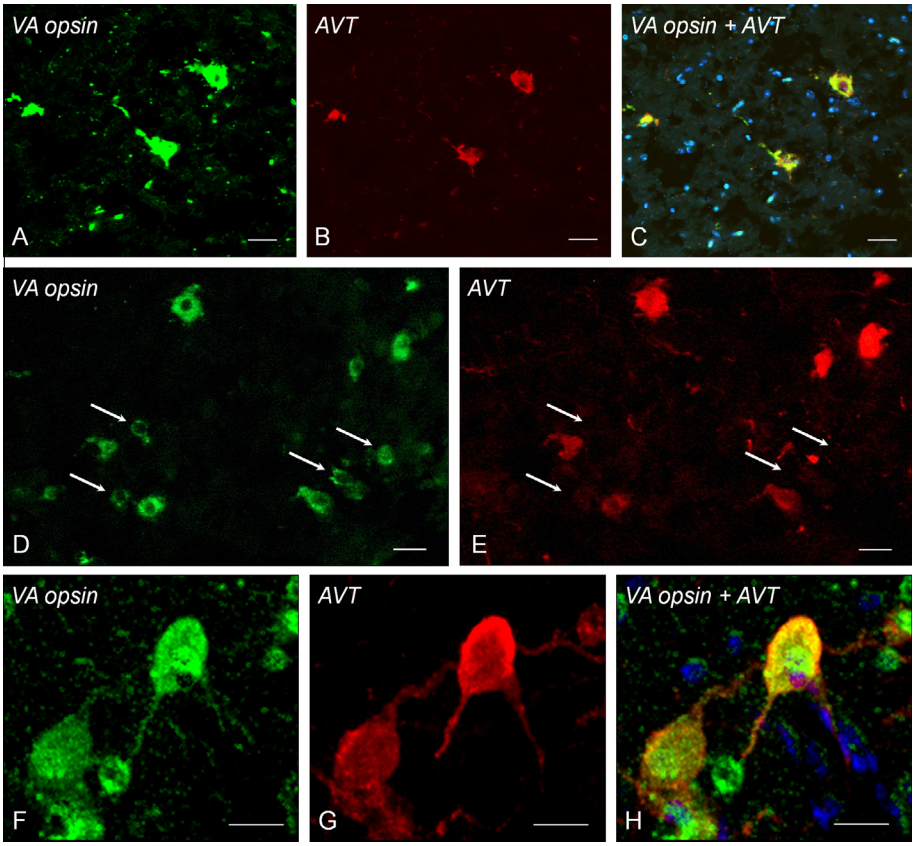

Fig. 8. Confocal images of double immunolabeling demonstrating co-localization of VA opsin (**A**) and AVT (**B**) in the same neurons of the quail hypothalamus (merged image with DAPI nuclear counterstain is shown in **C**). The majority of the VA opsin immunoreactive cells are also AVT positive; however, there are some exceptions (indicated with arrows) that show VA opsin (**D**) but not AVT immunostaining (**E**). Images showing co-localization of VA opsin (**F**) and AVT (**G**) in the same neurons of the chicken hypothalamus (merged image shown in **H**). Scale bars; A–H, 10 μm.

7. Discussion

There are two associated themes that are considered in this paper: The first reflects upon the candidacy of VA opsin as the photopigment of a deep brain photoreceptor regulating one or more avian photoperiodic responses; and the second theme based upon entirely new data considers the unexpected finding that VA opsin may confer endogenous photosensitivity to two populations of neurosecretory cells within the avian brain. Our conclusions are summarized here.

Although we have known since the 1930s that photoreceptors within the MBH regulate photoperiodic responses to daylength (Benoit, 1938) and that an action spectrum for this response published in the 1980s described a photopigment based upon an opsin/vitamin A with a λ_{max} near to 492 nm (Foster and Follett, 1985; Foster et al., 1985), the specific molecular identity of this opsin has remained uncertain. Several opsin classes have been proposed as candidates including rod opsin (Wang and Wingfield, 2011); melanopsin (OPN4) (Kang et al., 2010); neuropsin (OPN5) (Nakane et al., 2010); and vertebrate ancient (VA) opsin (Halford et al., 2009; Davies et al., 2012). The candidacy of these opsins has been evaluated based upon criteria used routinely in photobiology, and although it should be made clear that these opsins have received different levels of attention, based on the currently available data VA opsin and OPN5 both emerge as the strongest candidates. Absorbance spectroscopy has now shown convincingly that cOPN5 forms a photopigment with peak absorbance sensitivities (λ_{max}) in the ultraviolet (UV) part of the spectrum (λ_{max} at 360 nm) (Yamashita et al., 2010). Such a spectral maximum would not support OPN5 as the primary photoperiodic photopigment, based upon the 492 nm action spectrum for the avian photoperiodic response (Foster et al., 1985), and the observation that relatively little UV light can penetrate tissue (Foster and Follett, 1985). Recent data (Nakane et al., 2014) does however support a role for OPN5 and UV light in long day induction of TSHβ, at least under 'UV only' lighting conditions. Until very recently, the absorption spectrum for avian VA opsin photopigment was not known, and so any potential match or mismatch between action and absorption spectra could not be evaluated. The finding that the absorption spectrum for the chicken VA opsin (cVA) photopigment has a λ_{max} at 490 nm (Davies et al., 2012), and differs by only 2 nm from the peak of the action spectrum at 492 nm is striking and strongly implicates VA opsin as the photoperiodic photopigment. Furthermore, because of the absorbance characteristics of hemoglobin, there is a small "spectral window" in light penetration into the hypothalamus that peaks at 489 nm. As a result, the spectral maximum of the cVA photopigment is ideally suited to maximize photon capture within the bird hypothalamus. Of course, other photopigments such as OPN4 or OPN5 might also provide an input to this photoneuroendocrine pathway (Nakane et al., 2014), but neither of these opsins fully meet the criteria of the primary photopigment mediating the photoperiodic response within the MBH.

Although VA opsin has emerged as the strongest candidate, how might VA opsin based photoreceptors regulate seasonal biology? There is good evidence that long daylengths stimulate cells (thyrotrophs) in the pars tuberalis (PT) of the anterior pituitary to release thyrotrophin (TSH) which in turn stimulates ependymal cells (tanycytes) of the third ventricle. The tanycytes express a type II

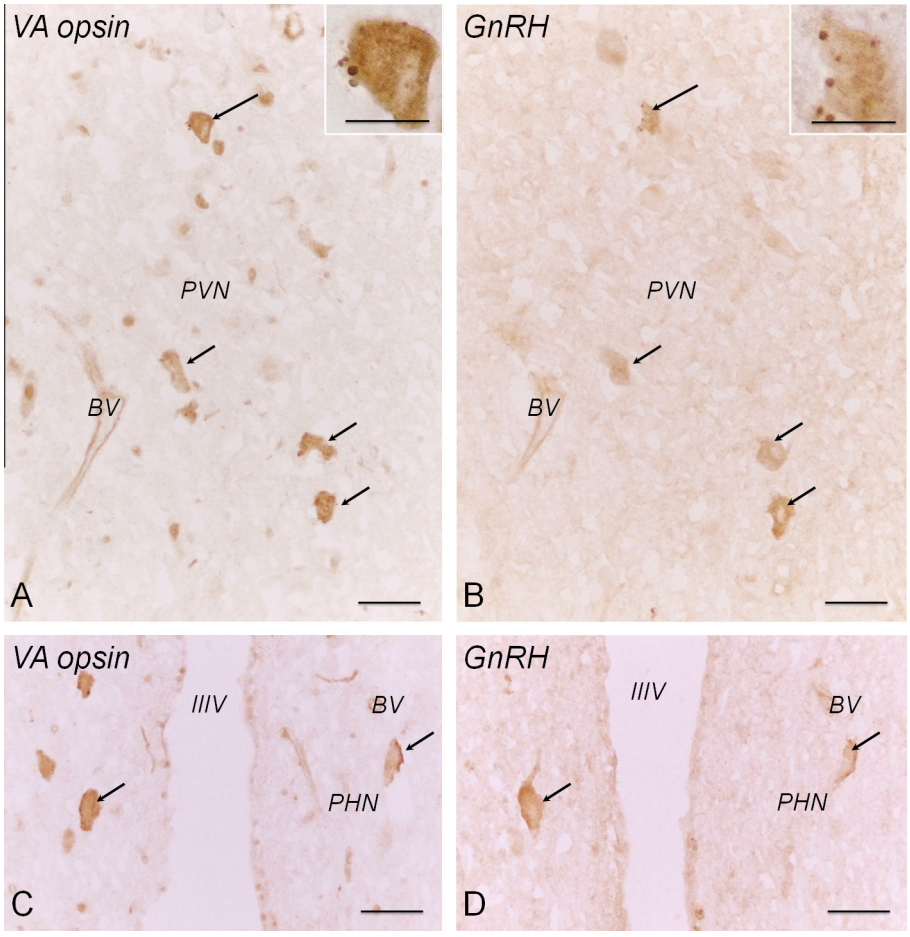

Fig. 9. VA opsin and GnRH immunolabeling in the paraventricular nucleus (PVN) (**A–B**) and in the nucleus periventricularis hypothalami (PHM) (**C–D**) of the chicken hypothalamus in two 5 μm-thick contiguous sections. Cell bodies with fragments immunolabeled in both sections are indicated with arrows. (BV, blood vessel; IIIV, third ventricle). Scale bars; A–D, 25 μm; insets, 10 μm.

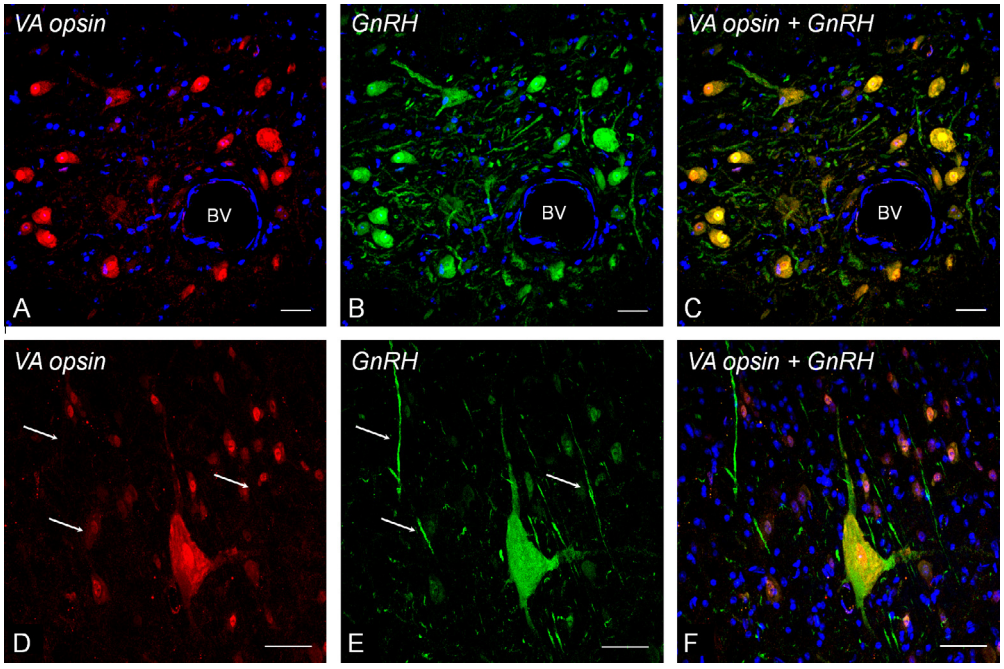

Fig. 10. Confocal images of VA opsin and GnRH double labeling demonstrating co-localization of VA opsin (**A, D**) and GnRH (**B, E**) in the same neurons of the quail supraoptic nucleus (merged images are shown in **C** and **F**). The majority of GnRH immunoreactive cells identified are also VA opsin positive, although a number of GnRH fibers are observed that lack detectable VA opsin expression (**D–F**) (indicated with arrows). (BV, blood vessel) Scale bars; A–C, 20 μm; D–F, 10 μm.

deiodinase (DIO2) enzyme which converts the prohormone thyroxine (T4) to bioactive triiodothyronine (T3). T3 then stimulates GnRH neurosecretory cells to release GnRH from terminals within the ME (Hazlerigg and Loudon, 2008). Our original hypothesis was that VA opsin photoreceptors detect photoperiod and communicate light information to the PT via their projections to the ME (see Figs. 3 and 6). However, the surprising finding that many GnRH neurons co-express VA opsin, and may themselves be endogenously photosensitive, complicates this representation of events enormously. Perhaps the photoperiodic machinery is all contained within GnRH neurons? Interestingly, in the cephalocordate amphioxus, the encephalic light-sensitive Hesse and Joseph cells co-express GnRH (Castro et al., 2006). Alternatively, the GnRH/VA opsin co-expressing neurons may perform another function. For example, birds possess two isoforms of GnRH: a hypophysiotropic GnRH form (GnRH-I) and a second form (GnRH-II) (Sharp et al., 1990). Neurons expressing GnRH-II do not project primarily to the ME but to a number of different brain regions, suggesting that this isoform regulates other physiological events. This led us to consider whether VA opsin is expressed in GnRH II neurosecretory neurons. This possibility seems unlikely, however, as the anti-GnRH antibody used in our study produced a pattern of staining typical of neurosecretory cells expressing the GnRH-I isoform (D'Hondt et al., 2000; Balthazart et al., 1994). Of course, GnRH neurosecretory cells may be regulated by both PT induced release of T3 and an endogenous photoreceptor. Such an overlap of sensory inputs is not unusual in the regulation of non-image forming photosensory tasks such as the regulation of circadian rhythms (van Oosterhout et al., 2012; Lall et al., 2010), and future experiments will address this possibility.

Levels of GnRH expression in birds are known to change in a seasonal manner. The discovery that GnRH neurons may themselves be directly photosensitive raises important questions regarding how the seasonal regulation of GnRH is achieved. Based on our current data it is unclear as to whether the endogenous light responses of GnRH cells may act to regulate levels of GnRH within these same cells under long days and short days. Although this is an intriguing possibility, it is not a necessity. It is equally possible that signals sent from GnRH cells (or AVT cells) that express VA opsin are delivered to a separate population of cells (potentially a circadian clock), which in turn may influence levels of GnRH expression as part of a feedback loop. Further work will be needed to investigate these possibilities.

Along with VA opsin and GnRH co-expression, very many AVT neurons within the chicken and quail brain co-express VA opsin. This suggests that VA opsin performs an as yet unknown photosensory role in vasotocin synthesis and/or release. This striking conclusion is less surprising when placed into a broader context of other non-mammalian vertebrates. The avian hypothalamo-hypophysial system is thought to be homologous to the vasotocinergic T5 hypothalamic region of the lamprey, renamed as the magnocellularis preoptic nucleus in the prosomeric map of Pombal et al. (2009), as well as being homologous to the nucleus preopticus magnocellularis (NPOM) of fishes, amphibians and reptiles (Pombal et al., 2009; Garcia-Fernandez et al., 1997). A series of studies have demonstrated the presence of phototransduction proteins in these homologous regions of the brain across the vertebrates, and specifically within populations of periventricular neurons. For example, opsin expression has been shown in the T5 of the lamprey brain (Garcia-Fernandez et al., 1997; Garcia-Fernandez and Foster, 1994). In fish, VA opsin and other opsins have been demonstrated within periventricular neurons of a subhabenular region (Philp et al., 2000) and NPOM (Alvarez-Viejo et al., 2003) respectively; In the amphibia, OPN4 and other opsins are expressed in the NPOM (Provencio et al., 1998; Alvarez-Viejo et al., 2003; Yoshikawa et al., 1994, 1998; Foster et al., 1994); Several reptiles including *Podarcis sicula* also show opsin labeling of neurons within the NPOM (Pasqualetti et al., 2003). Furthermore, antibodies directed against different opsin photopigments label fibers in the median eminence and neurohypophysis of lampreys, fish and amphibians (Alvarez-Viejo et al., 2003; Garcia-Fernandez et al., 1997; Garcia-Fernandez and Foster, 1994).

Long before these publications, and with remarkable prescience, Ernst Scharrer in 1928 suggested that periventricular neurons of the NPOM are the encephalic photoreceptors regulating skin pigmentation in fish (Scharrer, 1928). Scharrer is more widely recognized for the discovery of neurosecretory cells, but informed by his earlier studies on encephalic photoreceptors, he proposed that many neuroendocrine cells were also likely to be photosensitive (Scharrer, 1964). The results in birds presented here for the first time showing co-expression of VA opsin with AVT and GnRH neurosecretory cells would fully support Scharrer's original concept that the non-mammalian vertebrates possess photoneuroendocrine cells within the paraventricular region of the brain (Scharrer, 1964).

More recently, photoneuroendocrine cells have been clearly identified in the invertebrates. For example, the vasotocinergic neurosecretory cells within the forebrain of an annelid (*Platynereis dumerilii*) also co-express a photopigment opsin (c-opsin) (Tessmar-Raible et al., 2007). Such studies suggest remarkable phylogenetic conservation of photosensitivity within vasotocinergic neurons across both protostome and deuterostome lineages (Tessmar-Raible et al., 2007; Arendt et al., 2004). Addressing why such neurosecretory cells show photosensitivity remains the topic of much speculation. In fish, vasotocinergic release depends on long and short photoperiods (Gozdowska et al., 2006). By contrast, in birds there is little direct evidence that light can modulate AVT secretion, although a single study suggests that AVT release is stimulated by long photoperiods (Singh and Chaturvedi, 2008). Furthermore, there is strong evidence to suggest that the AVT system has little or no involvement in the photoperiodic regulation of reproduction (Sharp and Follett, 1969). As a result we hope that these new findings in the quail and chicken will help stimulate research that addresses the functional significance of photosensory AVT neurosecretory cells in the bird.

8. Conclusions

In conclusion, VA opsin emerges as a prime candidate to be the photopigment of the deep brain photoreceptors regulating the avian photoperiodic response. The results presented here, combined with future gene knockdown approaches e.g. (Nakane et al., 2014; Jagannath et al., 2013) and physiological measures of photosensitivity e.g. (Sekaran et al., 2003) are required to provide definitive answers which link experimentally one or more of the opsin candidates with the photoperiodic reproductive response and/or other photoperiodic responses such as prolactin secretion, or hyperphagia or migration. Furthermore, new data shows that large numbers of AVT and GnRH neurons express VA opsin, and as a result, may be endogenously photosensitive. In the context of AVT neurosecretory cells, this finding is entirely consistent with studies showing co-expression of opsin photopigments within vasotocinergic neurons of fish, amphibians and reptiles. Such findings reinforce Scharrer's proposal first articulated over 80 years ago that the paraventricular region of the vertebrate brain contains photoneuroendocrine cells. Although co-expression of opsins and vasotocin/mesotocin has been shown across varied taxa, the function of this direct photosensitivity remains obscure. Co-expression of VA opsin and GnRH was more surprising, and is even more difficult to place into our current models. The consensus explanation of how photoperiod is detected and transduced to generate seasonal

cycles of reproduction in birds (Hazlerigg and Loudon, 2008; Halford et al., 2009) makes no provision for endogenously photosensitive GnRH neurons. As a result, we hope that the discussion and findings presented in this paper will stimulate new research into understanding how birds encode and respond to daylength.

Acknowledgments

This work was funded by Grants from the Wellcome Trust – United Kingdom (090684/Z/09/Z), Biotechnology and Biological Sciences Research Council and the Universidad de Oviedo (Grant UNOV-12-MA-12). WILD was funded by an Australian Research Council (ARC) Future Fellowship – Australia.

References

Alvarez-Viejo, M., Cernuda-Cernuda, R., DeGrip, W.J., Alvarez-Lopez, C., Garcia-Fernandez, J.M., 2003. Co-localization of mesotocin and opsin immunoreactivity in the hypothalamic preoptic nucleus of Xenopus laevis. Brain Res. 969, 36–43.

Arai, R., Jacobowitz, D.M., Deura, S., 1993. Colocalization of calbindin-D28k with vasopressin in hypothalamic cells of the rat: a double-labeling immunofluorescence study. Brain Res. 632, 342–345.

Arendt, D., Tessmar-Raible, K., Snyman, H., Dorresteijn, A.W., Wittbrodt, J., 2004. Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain. Science 306, 869–871.

Balthazart, J., Dupiereux, V., Aste, N., Viglietti-Panzica, C., Barrese, M., Panzica, G.C., 1994. Afferent and efferent connections of the sexually dimorphic medial preoptic nucleus of the male quail revealed by in vitro transport of DiI. Cell Tissue. Res. 276, 455–475.

Bellingham, J., Whitmore, D., Philp, A.R., Wells, D.J., Foster, R.G., 2002. Zebrafish melanopsin: isolation, tissue localisation and phylogenetic position. Brain Res. Mol. Brain Res. 107, 128–136.

Bellingham, J., Chaurasia, S.S., Melyan, Z., Liu, C., Cameron, M.A., Tarttelin, E.E., Iuvone, P.M., Hankins, M.W., Tosini, G., Lucas, R.J., 2006. Evolution of melanopsin photoreceptors: discovery and characterization of a new melanopsin in nonmammalian vertebrates. PLoS Biol. 4, e254.

Benoit, J., 1935a. Stimulation par la lumiere artificielle du developpement testiculaire chez des canards aveugles par section du nerf optique. C. R. Seances. Soc. Biol. Fil. 120, 133–136.

Benoit, J., 1935b. Le role des yeux dans l'action stimulante de la lumiere sur le developpement testiculaire chez le canard. C. R. Seances. Soc. Biol. Fil. 118, 669–671.

Benoit, J., 1938. Action de divers eclairements localises dans la region orbitaire sur la gonadostimulation chez le canard male impubere. Croissance testiculaire provoquee par l'eclairement direct de la region hypophysaire. C. R. Seances. Soc. Biol. Fil. 127, 909–914.

Berson, D.M., Dunn, F.A., Takao, M., 2002. Phototransduction by retinal ganglion cells that set the circadian clock. Science 295, 1070–1073.

Bons, N., 1980a. Immunocytochemical characterization of vasotocin and mesotocin secretory systems in duck brain. Comptes rendus des seances de l'Academie des sciences. Serie D, Sciences naturelles 290, 113–116.

Bons, N., 1980b. The topography of mesotocin and vasotocin systems in the brain of the domestic mallard and Japanese quail: immunocytochemical identification. Cell Tissue Res. 213, 37–51.

Bowmaker, J.K., 1977. The visual pigments, oil droplets, and spectral sensitivity of the pigeon. Vision Res. 17, 1129–1138.

Bowmaker, J.K., Knowles, A., 1977. The visual pigments and oil droplets of the chicken retina. Vision Res. 17, 755–764.

Cassone, V.M., Menaker, M., 1984. Is the avian circadian system a neuro-endocrine loop. J. Exp. Zool. 232, 539–549.

Castro, A., Becerra, M., Manso, M.J., Sherwood, N.M., Anadon, R., 2006. Anatomy of the Hesse photoreceptor cell axonal system in the central nervous system of amphioxus. J. Comp. Neurol. 494, 54–62.

Chaurasia, S.S., Rollag, M.D., Jiang, G., Hayes, W.P., Haque, R., Natesan, A., Zatz, M., Tosini, G., Liu, C., Korf, H.W., Iuvone, P.M., Provencio, I., 2005. Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. J. Neurochem. 92, 158–170.

Dacey, D.M., Liao, H.W., Peterson, B.B., Robinson, F.R., Smith, V.C., Pokorny, J., Yau, K.W., Gamlin, P.D., 2005. Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. Nature 433, 749–754.

Davies, D.T., Follett, B.K., 1975a. The neuroendocrine control of gonadotrophin release in the Japanese quail. II. The role of the anterior hypothalamus. Proc. R. Soc. Lond. B Biol. Sci. 191, 303–315.

Davies, D.T., Follett, B.K., 1975b. The neuroendocrine control of gonadotrophin release in the Japanese quail. I. The role of the tuberal hypothalamus. Proc. R. Soc. Lond. B Biol. Sci. 191, 285–301.

Davies, W.L., Hankins, M.W., Foster, R.G., 2010. Vertebrate ancient opsin and melanopsin: divergent irradiance detectors. Photochem. Photobiol. Sci. 9, 1444–1457.

Davies, W.I., Turton, M., Peirson, S.N., Follett, B.K., Halford, S., Garcia-Fernandez, J.M., Sharp, P.J., Hankins, M.W., Foster, R.G., 2012. Vertebrate ancient opsin photopigment spectra and the avian photoperiodic response. Biol. Lett. 8, 291–294.

Dawson, A., Sharp, P.J., 2007. Photorefractoriness in birds–photoperiodic and non-photoperiodic control. Gen. Comp. Endocrinol. 153, 378–384.

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythms 16, 365–380.

D'Hondt, E., Eelen, M., Berghman, L., Vandesande, F., 2000. Colocalization of arginine-vasotocin and chicken luteinizing hormone-releasing hormone-I (cLHRH-I) in the preoptic-hypothalamic region of the chicken. Brain Res. 856, 55–67.

Follett, B.K., Nicholls, T.J., 1984. Photorefractoriness in Japanese quail: possible involvement of the thyroid gland. J. Exp. Zool. 232, 573–580.

Follett, B.K., Nicholls, T.J., 1985. Influences of thyroidectomy and thyroxine replacement on photoperiodically controlled reproduction in quail. J. Endocrinol. 107, 211–221.

Foster, R.G., Follett, B.K., 1985. The involvement of a rhodopsin-like photopigment in the photoperiodic response of the Japanese quail. J. Comp. Physiol. A 157, 519–528.

Foster, R.G., Hankins, M.W., 2007. Circadian vision. Curr. Biol. 17, R746-51.

Foster, R.G., Helfrich-Forster, C., 2001. The regulation of circadian clocks by light in fruitflies and mice. Philos. Trans. R. Soc. Lond. B Biol. Sci. 356, 1779–1789.

Foster, R.G., Follett, B.K., Lythgoe, J.N., 1985. Rhodopsin-like sensitivity of extra-retinal photoreceptors mediating the photoperiodic response in quail. Nature 313, 50–52.

Foster, R.G., Plowman, G., Goldsmith, A.R., Follett, B.K., 1987. Immunohistochemical demonstration of marked changes in the LHRH system of photosensitive and photorefractory European starlings (Sturnus vulgaris). J. Endocrinol. 115, 211–220.

Foster, R.G., Korf, H.W., Schalken, J.J., 1987. Immunocytochemical markers revealing retinal and pineal but not hypothalamic photoreceptor systems in the Japanese quail. Cell Tissue Res. 248, 161–167.

Foster, R.G., Panzica, G.C., Viglietti-Panzica, C., 1988. Immunocytochemical studies on the LHRH system of the Japanese quail: influence by photoperiod and aspects of sexual differentiation. Cell Tissue Res. 253, 327–335.

Foster, R.G., Grace, M.S., Provencio, I., Degrip, W.J., Garcia-Fernandez, J.M., 1994. Identification of vertebrate deep brain photoreceptors. Neurosci. Biobehav. Rev. 18, 541–546.

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.

Garcia-Fernandez, J.M., Foster, R.G., 1994. Immunocytochemical identification of photoreceptor proteins in hypothalamic cerebrospinal fluid-contacting neurons of the larval lamprey (*Petromyzon marinus*). Cell Tissue Res. 275, 319–326.

Garcia-Fernandez, J.M., Jimenez, A.J., Gonzalez, B., Pombal, M.A., Foster, R.G., 1997. An immunocytochemical study of encephalic photoreceptors in three species of lamprey. Cell Tissue. Res. 288, 267–278.

Gerkema, M.P., Davies, W.I., Foster, R.G., Menaker, M., Hut, R.A., 2013. The nocturnal bottleneck and the evolution of activity patterns in mammals. Proc. Biol. Sci. 280, 20130508.

Goossens, N., Blahser, S., Oksche, A., Vandesande, F., Dierickx, K., 1977. Immunocytochemical investigation of the hypothalamo-neurohypophysial system in birds. Cell Tissue Res. 184, 1–13.

Gozdowska, M., Kleszczynska, A., Sokolowska, E., Kulczykowska, E., 2006. Arginine vasotocin (AVT) and isotocin (IT) in fish brain: diurnal and seasonal variations. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 143, 330–334.

Halford, S., Pires, S.S., Turton, M., Zheng, L., Gonzalez-Menendez, I., Davies, W.L., Peirson, S.N., Garcia-Fernandez, J.M., Hankins, M.W., Foster, R.G., 2009. VA opsin-based photoreceptors in the hypothalamus of birds. Curr. Biol. 19, 1396–1402.

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

Hankins, M.W., Davies, W.L., Foster, R.G., 2014. The evolution of non-visual photopigments in the central nervous system of vertebrates. In: Hunt, D.M., Hankins, M.W., Collin, S.P., Marshall, N.J. (Eds.), Evolution of Visual and Non-visual Pigments. Springer.

Hanon, E.A., Lincoln, G.A., Fustin, J.M., Dardente, H., Masson-Pevet, M., Morgan, P.J., Hazlerigg, D.G., 2008. Ancestral TSH mechanism signals summer in a photoperiodic mammal. Curr. Biol. 18, 1147–1152.

Hartwig, H.-G., van Veen, T., 1979. Spectral characteristics of visible radiations penetrating into the brain and stimulating extra-retinal photoreceptors. J. Comp. Physiol. A 120, 277–282.

Hazlerigg, D., Loudon, A., 2008. New insights into ancient seasonal life timers. Curr. Biol. 18, R795–R804.

Homma, K., Ohta, M., Sakakibara, Y., 1979. Photoinducible phase of the Japanese quail detected by direct stimulation of the brain. In: Suda, M., Hayaishi, O., Nakagawa, H. (Eds.), Biological Rhythms and their Central Mechanism. Elsevier, North-Holland Biomedical Press, Amsterdam, pp. 85–94.

Hughes, S., Watson, T.S., Foster, R.G., Peirson, S.N., Hankins, M.W., 2013. Nonuniform distribution and spectral tuning of photosensitive retinal ganglion cells of the mouse retina. Curr. Biol. 23, 1696–1701.

Jagannath, A., Butler, R., Godinho, S.I., Couch, Y., Brown, L.A., Vasudevan, S.R., Flanagan, K.C., Anthony, D., Churchill, G.C., Wood, M.J., Steiner, G., Ebeling, M., Hossbach, M., Wettstein, J.G., Duffield, G.E., Gatti, S., Hankins, M.W., Foster, R.G.,

Peirson, 2013. The CRTC1-SIK1 pathway regulates entrainment of the circadian clock. Cell 154, 1100–1111.
Jenkins, A., Munoz, M., Tarttelin, E.E., Bellingham, J., Foster, R.G., Hankins, M.W., 2003. VA opsin, melanopsin, and an inherent light response within retinal interneurons. Curr. Biol. 13, 1269–1278.
Kang, S.W., Leclerc, B., Kosonsiriluk, S., Mauro, L.J., Iwasawa, A., El Halawani, M.E., 2010. Melanopsin expression in dopamine-melatonin neurons of the premammillary nucleus of the hypothalamus and seasonal reproduction in birds. Neuroscience 170, 200–213.
Kojima, D., Mano, H., Fukada, Y., 2000. Vertebrate ancient-long opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J. Neurosci. 20, 2845–2851.
Kojima, D., Mori, S., Torii, M., Wada, A., Morishita, R., Fukada, Y., 2011. UV-sensitive photoreceptor protein OPN5 in humans and mice. PLoS ONE 6, e26388.
Konishi, H., Foster, R.G., Follett, B.K., 1986. Diurnal rhythmicity of a rapid luteinizing hormone release induced by electrical stimulation of the hypothalamus in Japanese quail. In: Sapporo Symposium on Biological Rhythms, Sapporo, Japan Abstract 12.
Kosonsiriluk, S., Mauro, L.J., Chaiworakul, V., Chaiseha, Y., El Halawani, M.E., 2013. Photoreceptive oscillators within neurons of the premammillary nucleus (PMM) and seasonal reproduction in temperate zone birds. Gen. Comp. Endocrinol. 190, 149–155.
Kozlowski, G.P., Coates, P.W., 1985. Ependymoneuronal specializations between LHRH fibers and cells of the cerebroventricular system. Cell Tissue Res. 242, 301–311.
Lall, G.S., Revell, V.L., Momiji, H., Al Enezi, J., Altimus, C.M., Guler, A.D., Aguilar, C., Cameron, M.A., Allender, S., Hankins, M.W., Lucas, R.J., 2010. Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. Neuron 66, 417–428.
Meddle, S.L., Follett, B.K., 1997. Photoperiodically driven changes in Fos expression within the basal tuberal hypothalamus and median eminence of Japanese quail. J. Neurosci. 17, 8909–8918.
Melyan, Z., Tarttelin, E.E., Bellingham, J., Lucas, R.J., Hankins, M.W., 2005. Addition of human melanopsin renders mammalian cells photoresponsive. Nature 433, 741–745.
Menaker, M., 2014. Neurobiology: the eye within the brain. Curr. Biol. 24, R613-5.
Minamoto, T., Shimizu, I., 2002. A novel isoform of vertebrate ancient opsin in a smelt fish, *Plecoglossus altivelis*. Biochem. Biophys. Res. Commun. 290, 280–286.
Moutsaki, P., Bellingham, J., Soni, B.G., David-Gray, Z.K., Foster, R.G., 2000. Sequence, genomic structure and tissue expression of carp (*Cyprinus carpio* L.) vertebrate ancient (VA) opsin. FEBS Lett. 473, 316–322.
Nakane, Y., Yoshimura, T., 2010. Deep brain photoreceptors and a seasonal signal transduction cascade in birds. Cell Tissue Res. 342, 341–344.
Nakane, Y., Ikegami, K., Ono, H., Yamamoto, N., Yoshida, S., Hirunagi, K., Ebihara, S., Kubo, Y., Yoshimura, T., 2010. A mammalian neural tissue opsin (Opsin 5) is a deep brain photoreceptor in birds. Proc. Natl. Acad. Sci. U S A 107, 15264–15268.
Nakane, Y., Shimmura, T., Abe, H., Yoshimura, T., 2014. Intrinsic photosensitivity of a deep brain photoreceptor. Curr. Biol. 24, R596-7.
Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y., Kageyama, S., Uno, Y., Kasukawa, T., Iigo, M., Sharp, P.J., Iwasawa, A., Suzuki, Y., Sugano, S., Niimi, T., Mizutani, M., Namikawa, T., Ebihara, S., Ueda, H.R., Yoshimura, T., 2008. Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 452, 317–322.
Ohuchi, H., Yamashita, T., Tomonari, S., Fujita-Yanagibayashi, S., Sakai, K., Noji, S., Shichida, Y., 2012. A non-mammalian type opsin 5 functions dually in the photoreceptive and non-photoreceptive organs of birds. PLoS ONE 7, e31534.
Oksche, A., Mautner, W., Farner, D.S., 1964. The spatial picture of the neurosecretory system in birds under normal and experimental conditions. Zeitschrift fur Zellforschung und mikroskopische Anatomie 64, 83–100.
Oksche, A., Wilson, W.O., Farner, D.S., 1964. The Hypothalamic Neurosecretory System of Coturnix Coturnix Japonica. Zeitschrift fur Zellforschung und mikroskopische Anatomie 61, 688–709.
Oliver, J., Herbute, S., Bayle, J.D., 1977. Testicular response to photostimulation by radioluminous implants in the deafferented hypothalamus of quail. J. Physiol. (Paris) 73, 685–691.
Panzica, G.C., 1985. Vasotocin-immunoreactive elements and neuronal typology in the suprachiasmatic nucleus of the chicken and Japanese quail. Cell Tissue Res. 242, 371–376.
Panzica, G.C., Aste, N., Castagna, C., Viglietti-Panzica, C., Balthazart, J., 2001. Steroid-induced plasticity in the sexually dimorphic vasotocinergic innervation of the avian brain: behavioral implications. Brain Res. Brain Res. Rev. 37, 178–200.
Pasqualetti, M., Bertolucci, C., Ori, M., Innocenti, A., Magnone, M.C., De Grip, W.J., Nardi, I., Foa, A., 2003. Identification of circadian brain photoreceptors mediating photic entrainment of behavioural rhythms in lizards. Eur. J. Neurosci. 18, 364–372.
Peirson, S., Foster, R.G., 2006. Melanopsin: another way of signaling light. Neuron 49, 331–339.
Peirson, S.N., Halford, S., Foster, R.G., 2009. The evolution of irradiance detection: melanopsin and the non-visual opsins. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2849–2865.
Perera, A.D., Follett, B.K., 1992. Photoperiodic induction in vitro: the dynamics of gonadotropin-releasing hormone release from hypothalamic explants of the Japanese quail. Endocrinology 131, 2898–2908.
Philp, A.R., Garcia-Fernandez, J.M., Soni, B.G., Lucas, R.J., Bellingham, J., Foster, R.G., 2000. Vertebrate ancient (VA) opsin and extraretinal photoreception in the Atlantic salmon (Salmo salar). J. Exp. Biol. 203 (Pt 12), 1925–1936.
Pombal, M.A., Megias, M., Bardet, S.M., Puelles, L., 2009. New and old thoughts on the segmental organization of the forebrain in lampreys. Brain Behav. Evol. 74, 7–19.
Provencio, I., Jiang, G., De Grip, W.J., Hayes, W.P., Rollag, M.D., 1998. Melanopsin: an opsin in melanophores, brain, and eye. Proc. Natl. Acad. Sci. U S A 95, 340–345.
Provencio, I., Rodriguez, I.R., Jiang, G., Hayes, W.P., Moreira, E.F., Rollag, M.D., 2000. A novel human opsin in the inner retina. J. Neurosci. 20, 600–605.
Provencio, I., Rollag, M.D., Castrucci, A.M., 2002. Photoreceptive net in the mammalian retina. Nature 415, 493.
Sanchez, F., Panzica, G.C., Viglietti-Panzica, C., Aste, N., Carretero, J., Vazquez, R., 1991. A comparative analysis of the vasotocin and vasopressin systems in the chicken and rat hypothalamus. An immunocytochemical study. J. Hirnforsch. 32, 27–37.
Scharrer, E., 1928. Die lichtempfindlichkeit blinder Elritzen I. Untersuchungen uber das Zwischenhirn der Fische. Z. Vgl. Physiol. 7, 1–38.
Scharrer, E., 1964. Photoneuroendocrine systems: General concepts. Ann. NY. Acad. Sci. 117, 13–22.
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.
Sharp, P.J., 1993. Photoperiodic control of reproduction in the domestic hen. Poult. Sci. 72, 897–905.
Sharp, P.J., Blache, D., 2003. A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long- and short-day photoperiods. Can. J. Physiol. Pharmacol. 81, 350–358.
Sharp, P.J., Follett, B.K., 1969. The effect of hypothalamic lesions on gonadotrophin release in Japanese quail (Coturnix coturnix japonica). Neuroendocrinology 5, 205–218.
Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M., Huskisson, N.S., 1990. Physiological roles of chicken LHRH-I and -II in the control of gonadotrophin release in the domestic chicken. J. Endocrinol. 124, 291–299.
Shen, E., Dun, S.L., Ren, C., Bennett-Clarke, C., Dun, N.J., 1992. Hypotension preferentially induces c-fos immunoreactivity in supraoptic vasopressin neurons. Brain Res. 593, 136–139.
Silver, R., Witkovsky, P., Horvath, P., Alones, V., Barnstable, C.J., Lehman, M.N., 1988. Coexpression of opsin- and VIP-like-immunoreactivity in CSF-contacting neurons of the avian brain. Cell Tissue. Res. 253, 189–198.
Singh, S., Chaturvedi, C.M., 2008. Changes in vasotocin immunoreactivity of paraventricular nuclei and adrenal function of Japanese quail in relation to different phases of sexual development. Domest. Anim. Endocrinol. 34, 293–300.
Soni, B.G., Foster, R.G., 1997. A novel and ancient vertebrate opsin. FEBS Lett. 406, 279–283.
Soni, B., Philp, A., Knox, B., Foster, R., 1998. Novel retinal photoreceptors. Nature 394, 27–28.
Tarttelin, E.E., Bellingham, J., Hankins, M.W., Foster, R.G., Lucas, R.J., 2003. Neuropsin (Opn5): a novel opsin identified in mammalian neural tissue. FEBS Lett. 554, 410–416.
Tessmar-Raible, K., Raible, F., Christodoulou, F., Guy, K., Rembold, M., Hausen, H., Arendt, D., 2007. Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution. Cell 129, 1389–1400.
Torii, M., Kojima, D., Okano, T., Nakamura, A., Terakita, A., Shichida, Y., Wada, A., Fukada, Y., 2007. Two isoforms of chicken melanopsins show blue light sensitivity. FEBS Lett. 581, 5327–5331.
van Oosterhout, F., Fisher, S.P., van Diepen, H.C., Watson, T.S., Houben, T., VanderLeest, H.T., Thompson, S., Peirson, S.N., Foster, R.G., Meijer, J.H., 2012. Ultraviolet light provides a major input to non-image-forming light detection in mice. Curr. Biol. 22, 1397–1402.
Vigh, B., Vigh-Teichmann, I., 1988. Comparative neurohistology and immunocytochemistry of the pineal complex with special reference to CSF-contacting neuronal structures. Pineal Res. Rev. 6, 1–65.
Wada, Y., Okano, T., Adachi, A., Ebihara, S., Fukada, Y., 1998. Identification of rhodopsin in the pigeon deep brain. FEBS Lett. 424, 53–56.
Wang, G., Wingfield, J.C., 2011. Immunocytochemical study of rhodopsin-containing putative encephalic photoreceptors in house sparrow, Passer domesticus. General Comp. Endocrinol. 170, 589–596.
Yamashita, T., Ohuchi, H., Tomonari, S., Ikeda, K., Sakai, K., Shichida, Y., 2010. Opn5 is a UV-sensitive bistable pigment that couples with Gi subtype of G protein. Proc. Natl. Acad. Sci. U.S.A. 107, 22084–22089.
Yasuo, S., Watanabe, M., Okabayashi, N., Ebihara, S., Yoshimura, T., 2003. Circadian clock genes and photoperiodism: comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of Japanese Quail under various light schedules. Endocrinology 144, 3742–3748.
Yokoyama, S., Zhang, H., 1997. Cloning and characterization of the pineal gland-specific opsin gene of marine lamprey (*Petromyzon marinus*). Gene 202, 89–93.
Yokoyama, K., Oksche, A., Darden, T.R., Farner, D.S., 1978. The sites of encephalic photoreception in the photoperiodic induction of growth of the testes in the white crowned sparrow, *Zonotrichia leucophrys gambelii*. Cell. Tiss. Res. 189, 441–467.

Yoshikawa, T., Yashiro, Y., Oishi, T., Kokame, K., Fukada, Y., 1994. Immunoreactivities to rhodopsin and rod/cone transducin antisera in the retina, pineal complex and deep brain of the Bullfrog, *Rana catesbeiana*. Zool. Sci. 11, 675–680.

Yoshikawa, T., Okano, T., Oishi, T., Fukada, Y., 1998. A deep brain photoreceptive molecule in the toad hypothalamus. FEBS Lett. 424, 69–72.

Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., Hirunagi, K., Ebihara, S., 2003. Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. Nature 426, 178–181.

Young, J.Z., 1962. The Life of the Vertebrates. The Clarendon Press, Oxford.