Nitric Oxide as an Efferent Modulator of Circadian Pacemaker Neurones in the Eye of the Marine Mollusc *Bulla gouldiana*

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Abstract: An antiserum against the nitric oxide (NO) synthetase labelled varicosities surrounding circadian pacemaker cells ("basal retinal neurons", BRNs) in the eye of the marine mollusc *Bulla gouldiana*. These profiles appear to arise from extrinsic neurons with axons entering the eye *via* the optic nerve and are distinct from FMRFamide containing profiles, which represent the only efferent optic fibres in *Bulla*. In intact, dark-adapted eyes, the NO-donor SNAP (S-nitroso-N-acetylpenicillamine) had no obvious effect on BRN activity recorded from the optic nerve. However, the light response of these circadian pacemaker neurones was reversibly enhanced by 8-bromo-cGMP, a tissue permeable analogue of cyclic guanosine monophosphate, and reduced by L-NAME (N^{ω}-nitro-L-arginine methyl ester), a specific but irreversible inhibitor of NO synthesis. Furthermore, isolated BRNs in primary cell culture responded to the NO-donor SNAP with an elevated intracellular calcium concentration as measured by fluorescent calcium imaging using fura-2. This effect was blocked in the presence of the calcium chelator ethylene glycol tetra acetic acid (EGTA), and mimicked by application of the phosphodiesterase blocker isobutylmethylxanthine (IBMX). We propose that NO released from neurons entering the eyes, modulates the light responsiveness of the circadian pacemaker cells in *Bulla* by activating cGMP-gated calcium channels.

INTRODUCTION

Evolution has witnessed that several types of light sensitive organs emerge, though in all cases they seem to serve two basic functions, visual perception and the mediation of Zeitgeber information to the circadian pacemaker. The marine opisthobranch mollusc *Bulla gouldiana* is now an established animal model in circadian biology (review [1]). The eyes of this gastropod contain a population of approximately 100 circadian pacemaker neurones at the base of the retina, so-called basal retinal neurons (BRNs) [2]. The BRNs in each eye are electrically coupled to each other, and their concerted activity produces a circadian rhythm of autogenous compound action potentials during the subjective day that are propagated through the optic nerve to the central nervous system [2]. Isolated BRNs still produce a circadian rhythm *in vitro* [3] and their activity rhythm *in vivo* is influenced by incident illumination and by efferent fibres from the brain [1, 5, 9].

The input pathway for the Zeitgeber light has been thoroughly investigated (review [4]). BRNs are depolarised by illumination and by experimentally elevated extracellular potassium ions [5], whereby both stimuli produce similar phase-shifts in the circadian rhythm [6]. Light or depolarisation induced phase shifts required for extracellular calcium, and are blocked by calcium channel antagonists [6, 7]. Furthermore, depolarisation of the BRNs causes an influx of calcium ions and a robust elevation of intracellular calcium [8]. Thus, calcium plays a central role in phase shifting the ocular circadian pacemaker in molluscs [4].

The spiking activity of ocular circadian pacemaker cells is modulated by efferent input in *Bulla* and in the related *Aplysia* [9]. Such efferent control is thought to ensure inter-ocular coupling of the pacemakers [10, 11], modify the length and amplitude of circadian oscillations [12], and modulate light induced phase shifts [13-15]. However, knowledge of the involved efferent neurotransmitter systems and the underlying cellular mechanisms is limited. The axons of the BRNs in *Bulla* project *via* the brain to the contralateral eye [16] and immunocytochemistry, suggest that these cells contain glutamate [17], which is released as an excitatory transmitter for synchronizing inter-ocular pacemaker activity [11]. Efferent fibres immunoreactive for the tetraneuropeptide FMRFamide project through the optic nerve to arborize in the eyes' neuropil of *Aplysia*, but not *Bulla* [9, 18], where it suppresses ongoing compound action potentials [9] and modulates light induced phase shifts in both *Bulla* and *Aplysia* [19] by acting on potassium currents [20]. In *Aplysia*, efferent serotonergic fibres that enter and arborize in the eye [21] may act to modulate light's phase shifting effects on the ocular pacemakers [14, 22]. However, this system seems to be absent in *Bulla* [17]. Beyond these findings, and the observation that octopamine and dopamine occur within the connective tissue encapsulating the eye in *Bulla* [17], little is known about the involvement of other transmitters in the efferent control of circadian activity in molluscs.

An important signalling molecule in the vertebrate retina is the gas nitric oxide (NO; [23]). In mammals, NO is also believed to entrain the circadian rhythms generated by the hypothalamic suprachiasmatic nucleus (SCN) clock by acting *via* a cyclic guanosine monophosphate (cGMP) signal transduction pathway [24, 25]. NO is now also an estab-

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lished signalling molecule in the nervous systems of invertebrates (reviews [26-29]). Putative NO-releasing neurones have now been identified in numerous molluscs, including *Bulla* and *Aplysia*, using both NADPH-d histochemistry and specific antibodies to detect the enzyme NO-synthetase (NOS; [30, 31]; review [32]). Most of the effects of NO are exerted *via* the activation of soluble guanylyl cyclase and the production of cGMP (review [33]), and evidence suggests that the same applies to molluscs [34-37].

Despite the accumulated knowledge of NO-signalling in molluscs, there is only sparse information on its role in the molluscan visual system and circadian control. NOS has been detected in the optic nerve of *Aplysia* by NADPH-diaphorase histochemistry [30] and by immunocytochemistry in the retina of the Cephalopod *Sepia* [38]. In *Aplysia*, a membrane permeable analogue of cGMP (8Br-cGMP) is reported to phase-shift the ocular circadian pacemaker in the same way as light [39]. In the related *Bulla*, however, 8Br-cGMP was not found to produce phase shifts [40]. Apart from this we know only of a meeting abstract reporting that NO mediates the circadian rhythm in photoreceptor sensitivity in *Bulla* [41]. Here, we now present an evidence suggesting that nitric oxide (NO) is an efferent neuromodulator in the eye of *Bulla*, that acts *via* cGMP to enhance the light response of the ocular circadian pacemaker cells by elevating intracellular calcium. A preliminary account of some of the findings reported here has been published elsewhere in short form [42].

MATERIALS AND METHODS

Experimental Animals

Adult marine molluscs *Bulla gouldiana* were obtained from a commercial supplier (Marinus Inc. Long Beach, CA) and maintained in artificial seawater tanks (15.1–15.3°C, light/dark cycle: 12/12 hours) at Leipzig University for at least 7 days prior to experiments. All experimental procedures are in accord with animal welfare regulations in Germany.

Immunohistochemistry

The basic procedures employed for detecting bound antisera in sections of paraffin wax embedded tissue were published elsewhere [17]. Briefly, animals were immobilised by injecting isotonic $MgCl_2$ (10 ml, 374 mM) after which the head ganglia and both eyes were excised within 1 minute. Tissue was passed rapidly through iced artificial seawater, pinned out and immersion fixed for 2 hours at room temperature in paraformaldehyde (4%) in phosphate buffer (pH 7.4) supplemented with sucrose to obtain isotonic conditions (1000 mOsm). Tissues were subsequently dehydrated in an ascending ethyl alcohol series, passed through xylene and embedded in paraffin. Serial sagittal sections (10 μm) were cut with a rotary microtome (Jung Biocut, Leica, Nussloch, Germany) and mounted on poly-D-lysine coated slides (0.4 mg/ml). After removing paraffin in xylene (2 changes, each 5 minutes), sections were rehydrated in a descending alcohol series, washed in buffer (0.1 M Tris-HCl, pH 7.6) containing 0.3% hydrogen peroxide to block endogenous peroxidase activity (10 minutes) and then fresh buffer (10 minutes). Hereafter all sections were washed for 15 minutes in buffer containing 0.1% Triton X-100 (Sigma, Steinheim, Germany, "Tris-HCl-Tx") and then incubated first in normal goat serum (Dako, Hamburg, Germany, 1:10 in Tris-HCl-Tx, 30 minutes) followed by incubation in primary antiserum (in Tris-HCl-Tx with 1% normal goat serum, 15-18 hours, 20°C). Bound antibody was detected by the avidin-biotin technique using a commercially available kit (Vectastain, Vector, Burlingame, CA, USA), with adherence to recommended dilution and incubation times and employing diaminobenzidine (DAB, 0.02% in buffer, Polyscience, St. Goar, Germany) with hydrogen peroxide (0.001%) as chromogen. As an alternative, bound primary antibody was detected using Cy3-conjugated goat-anti-rabbit antibody (Dianova, Hamburg, Germany) diluted 1:200 in Tris-HCl-Tx. Finally, sections were washed in Tris-HCl, dehydrated in an ascending alcohol series, cleared in xylene and mounted under cover slips in either Entellan (Merck, Darmstadt, Germany) for light-, or Fluoromount (Serva) for fluorescent- microscopy.

Antibodies and Controls

An established rabbit polyclonal antiserum ("universal" nitric oxide synthetase, anti-NOS, Affinity Bioreagents) was employed to detect putative nitric oxide producing nerve cells and neuropils in cerebral ganglia and the eye. This antibody was raised in rabbit using the peptide DQKRYHEDIFG and affinity purified using the same peptide. The peptide comprises amino acid residues 1113–1122 of murine NOS, with an added N-terminal aspartyl residue, and is highly conserved in all animal NOS. In molluscs, this antibody is now well established, having been shown to recognise NOS by western blot analysis and in the tissues of several different species (e.g., [43-46]). We used this serum at the recommended dilution of 1:200.

Neurones and neuropils containing the molluscan tetrapeptide FMRFamide family were localised using a polyclonal rabbit antiserum originally obtained from Dr. E. Weber, Portland, USA which detects peptides with the terminal peptide sequence arginine-phenylalanine (RF) amide ("RFamide-like peptide"; cf. [47] for details). We used this serum as recommended diluted 1:4000.

The resemblance in the staining pattern of *Bulla* retina and cerebral ganglia has been reported in an independent study [9]. The tissue staining patterns obtained with the NOS and FMRFamide antisera used here and elsewhere [48] are mutually exclusive, so that cross-reactivity between the two can be excluded. For all antisera tested omission of the primary antiserum and/or secondary antiserum resulted in negative staining in the eye and brain of *Bulla*.

Image Acquisition and Reproduction

The photomicrographs for Figs. (**1A-D**) were taken using a slow-scan 12-bit CCD camera (Sensicam, PCO, Kelheim, Germany) mounted to a compound microscope (DM RBE, Leica, Wetzlar, Germany) equipped with a 20x/0.50 PH2 objective (Leica) using automatic exposure and colour/brightness compensation. The photographs for Figs. (**1E-F**) were obtained with a TCS confocal microscope (Leica) equipped with a 20x/0.50 PH2 objective (Leica). Images were saved on a hard disc without compression in tiff file format. Afterward they were scaled, trimmed, arranged for final figures and converted to 300 dpi 8 bit colour images

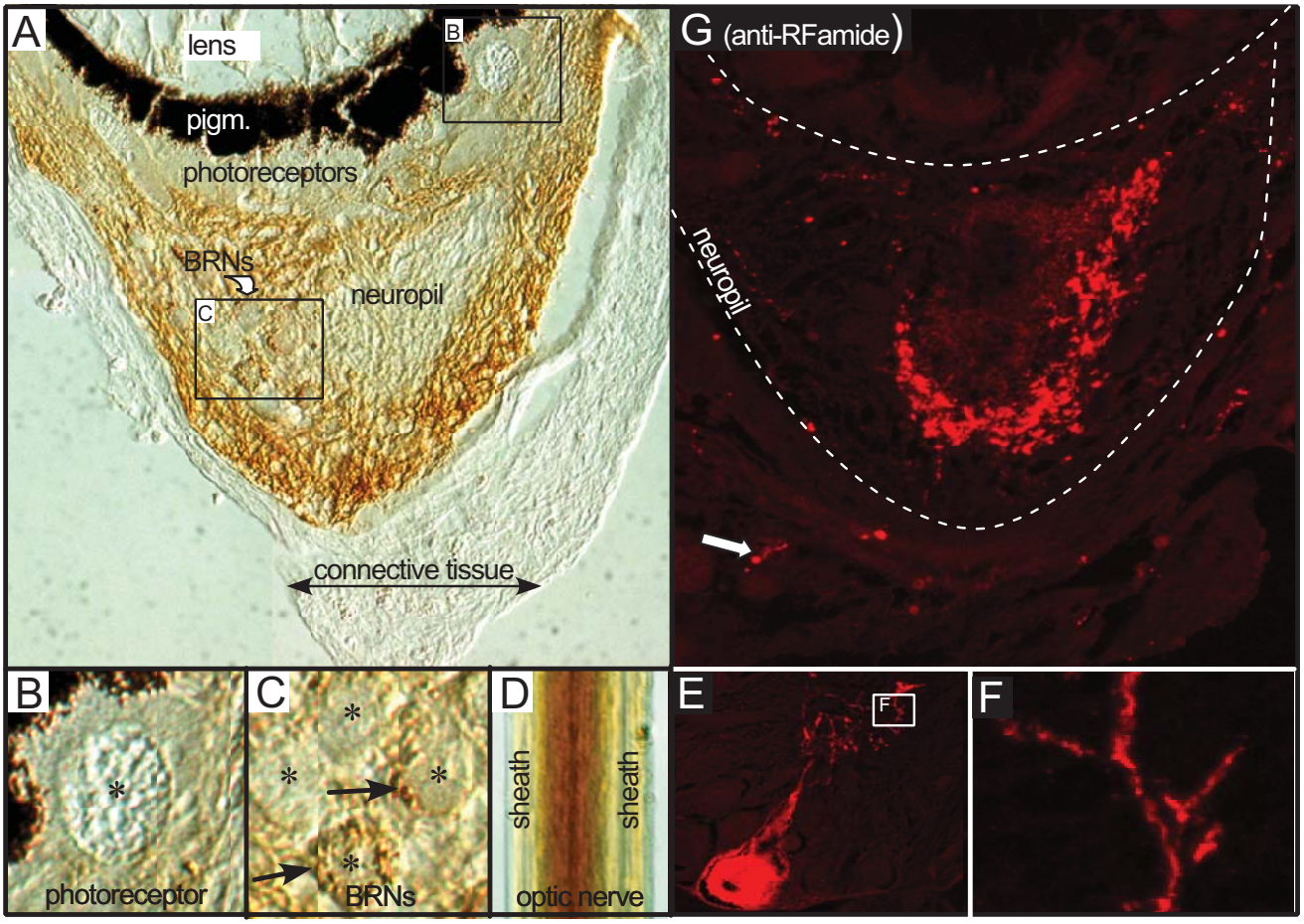

Fig. (1). NOS Photomicrographs of paraffin sections showing NOS-like immunoreactivity in the *Bulla* eye (**A-D**) and brain (**E, F**) compared to RFamide-like immunoreactivity (**G**). **A**. Overview of the eye showing the lens, photo-pigment layer, (pigm.) photoreceptor cell-layer, the eye neuropil with NOS-like immunoreactive label (brown) and its encapsulating connective tissue sheath (double-headed arrow; boxes outline a photoreceptor cell and 4 BRNs shown in **B** respectively **C**). **B**. Detail of an unlabelled photoreceptor cell (* nucleus). **C**. Detail of 4 unlabelled BRNs (*) of which 2 are covered with labelled varicosities (arrows). **D**. Optic nerve. Note only the core, and not the sheath is labelled. **E**. An NOS-like immunoreactive soma in the brain (box outlines detail shown in **F**). **F**. Detail of branches from the neurone shown in **E**. **G**. RFamide-like immunoreactive label (red) in the eye, which is concentrated in regions showing sparse NOS-like immunoreactivity in **A** (dashed line outlines the neuropil, arrow indicates labelling in the sheath). Bound antiserum was visualised in **A-D** using DAB as chromogen and Nomarski optics and in **E-G** using a fluorescent (cy3) secondary antibody and confocal microscopy. Calibration bar: **A, D, G** 100 µm**; B, C, D, F** 40 µm; E 400 µm.

using standard software (Canvas, version X, Daneba Systems, Inc. Miami, USA). Beyond this, no further image processing was undertaken.

Extracellular Recordings

The concerted activity of the electrically coupled circadian pacemaker neurones in *Bulla* (BRNs) was recorded extracellularly from the optic nerve of intact eyes as compound action potential (CAPs). Snails were taken during their subjective day (CT 10-11), immobilised by injection of isotonic magnesium chloride solution and the brain with attached eyes excised and placed in artificial seawater (ASW). The brain was hemi-sectioned by cutting the cerebral and pedal commissures and the two brain halves transferred to a custom built Teflon recording chamber having three small pools. Each eye was lifted into a separate adjacent pool filled with 100 µl ASW, while the attached brain-half remained in the large pool filled with 500 µl ASW. Vaseline served to electrically isolate the eye from its respective brain-half (Fig. **2**). Four silver wire electrodes placed adjacent to each eye and brain-halves were connected to separate differential AC amplifiers (Biopulse unit, ADI Instruments; gain 1000, 1 kHz high pass, 10 Hz low pass), which fed into an analogue - digital converter (PowerLab, AD-Instruments, 10 kHz sampling frequency/channel) operating with an Apple-Macintosh computer (Cupertino, CA, USA) installed with analysis software (Chart 4, AD-Instruments). An output facility of the PowerLab provided an appropriate signal to illuminate a LED connected to an optic fibre ending 5 mm equidistant from both eyes.

Each eye's responses to 1 minute light pulses interposed by 15 minute long dark periods was monitored continuously for several hours. Each eye's bathing solution was exchanged manually with a pipette within less than 15 seconds under ambient lighting just after each light pulse. In most experiments the responses to 3 changes of ASW were recorded before applying a drug dissolved in ASW to one eye (test)

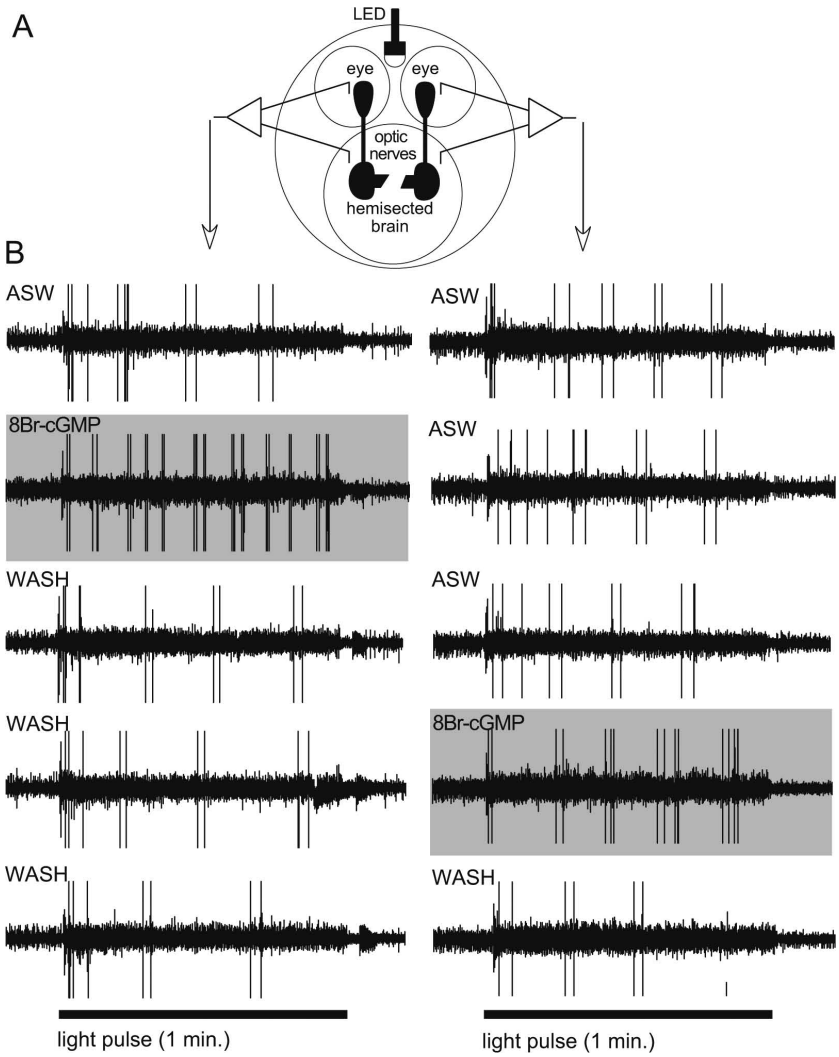

Fig. (2). Enhanced light response of BRNs in an intact, dark adapted, eye preparation following bath application of 8-Br-cGMP. **A**. Schema of the setup for extracellular recordings of the left and right optic nerves following brief illumination of the eyes with a light emitting diode (LED). **B**. Simultaneous records from the left and right eye nerves showing bursts of compound action potentials of the BRNs in response to 1 min. light pulses. Consecutive trials, each 15 min apart, are shown from top to bottom, during which each separate eye is bathed first in artificial seawater (ASW), then in 8-Br-cGMP (100 μM, shaded background) followed by washing in artificial seawater (WASH). Compare with Fig. (**3**).

and ASW to the other (control). These experiments were carried out at room temperature (20-22°C).

Primary Cell Culture

Enzymatic dissociation of excised eyes was performed as previously described [3]. The culture medium was prepared from Leibovitz L15 medium [49] and additional salts [50]: 500 ml Leibovitz L15 medium (Sigma) were supplemented with (in g): 7.680 NaCl, 0.716 $CaCl_2$ • 2 H_20, 0.147 KCl, 1.576 $MgSO_2$ • 7 H20, 2.644 $MgCl_2$ • 6 H20 (p.a., Fluka), 0.084 $NaHCO_3$, 3.575 HEPES, 0.050 L-Glutamine, 3.000 D-Glucose (all cell culture tested, Sigma), pH 7.8 adjusted with 10 N NaOH (Titrisol, Merck). Medium was sterile filtered and stored at 4°C. Poly-D-lysine coated cover slips were prepared with 10 mg Poly-D-Lysine (hydrobromide, mol. wt. over 300,000, Sigma) in 50 ml sterile borate buffer (in g: 0.858 BH_3O_3, 0.682 $B_4Na_2O_7$ • 10 H2O).

Animals were immobilised by injecting isotonic $MgCl_2$ (10 ml, 374 mM), placed on ice, and the eyes dissected out and passed through ASW. The basal retina was carefully dissected from isolated eyes and incubated for 1 hour at 32°C in cell culture medium containing 1 mg/ml Protease VIII (Sigma). Cells were mechanically dispersed in a small drop of cell culture medium on a poly-D-lysine coated cover slip in sterile plastic Petri dish. After 10 minutes the cells became attached to the cover slip and the Petri dish was filled with cell culture medium containing 5-10% haemolymph.

Cell cultures where maintained in a fridge controlled by a custom-built temperature regulator at 14.5–15.5 °C in constant darkness and were used on day *in vitro* (DIV) 2 and 3.

Solutions

Artificial seawater (ASW) [5] contained (in mM): 395 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 28 Na_2SO_4, 30 HEPES at a pH of 7.8. In low calcium artificial seawater (EGTA-ASW) 5 mM calcium was buffered with 10 mM Ethylene-bis(oxyethylenenitrilo)tetraacetic acid tetrasodium salt

(EGTA) to obtain a free calcium concentration of 1,3 µM. Artificial seawater with elevated potassium [5] contained 50 instead of 10 mM KCl and 40 mM less NaCl. All chemicals were obtained from Sigma.

Calcium Imaging

Fura-2 acetoxymethyl ester (Fura-2 AM, Molecular Probes) was used to measure the intracellular concentration of free calcium ions in BRNs as described in detail elsewhere [8]. Briefly, 50 µg Fura-2 AM was diluted in 50 µl DMSO and vortexed, 10 µl of 20% Pluronic (Molecular Probes) in DMSO was then added and the mixture vortexed again. Aliquots of 10 µl were used immediately or stored at -20°C. The loading solution was made by diluting one aliquot in 1.5 ml ASW and had a final concentration of 5.6 µM fura-2 AM. Cells attached to the cover slips were washed with ASW, incubated with loading solution for 30-45 min at 32°C, washed with ASW and allowed to recover for 10 minutes in fresh plain cell culture medium at 14.5–15.5 °C. Cover slips where then mounted on a custom-built recording chamber mounted on an inverted microscope.

Two 20 ms light pulses of 340 nm and 380 nm were delivered every 4-6 s by a monochromator and the emission was recorded with a photomultiplier tube (Till Photonics, Munich, Germany). The data were digitized and recorded using a Macintosh computer and appropriate software (XChart, Heka, Lambrecht, Germany). Fluorescence ratio was calculated after subtraction of background fluorescence.

The fluorescence measurement equipment was calibrated using a standard protocol [8]. Calibration solution was made using a calibration buffer kit (Molecular Probes) in artificial internal solution containing in mM: 360 KCl, 5 $MgCl_2$, 20 MOPS, pH 7.3 (all from Sigma). Fura-2 potassium salt (Sigma) was diluted to a concentration of 50 mM in water, stored at 4°C and was used in a final concentration of 50 µM. Parameters were R_{min}=0.284, R_{max}=2.535 and B=3.837. The mean dissociation constant K_D of Fura-2 was 205 nM (pK_D=9.688 (SEM0.068), N=4). The concentration of free calcium ions was calculated from the background subtracted emission ratio by $[Ca^{2+}]_{free} = K_D \times (R - R_{max}) / (R_{min} - R) \times B$ as described elsewhere [51].

Statistics

P-values were taken from Student's t-test. The probability level for rejecting the null hypothesis was p>0.05. Measurements were described as mean and standard deviations. Graphs were constructed using SigmaPlot (Jandel, San Rafael). Error bars represent standard deviation. Final figures were arranged using standard software (Canvas X, Daneba Systems). Our account is based all together on a critical evaluation of data from 25 eyes taken from 15 individual animals.

RESULTS

NOS Immunocytochemistry

Putative NO producing structures in the nervous system of *Bulla* gouldiana were revealed in paraffin sections of the eye and brain, using a universal antibody directed against nitric oxide synthetase (NOS). Our findings are based on an evaluation of 7 favourable eye preparations (from 5 individuals) and 5 brain preparations. Fig. (**1A**) shows a section through the neuropil of the eye, in which NOS-like immunoreactive product is labelled brown. The staining appears to correspond to fine fibres and their processes that are

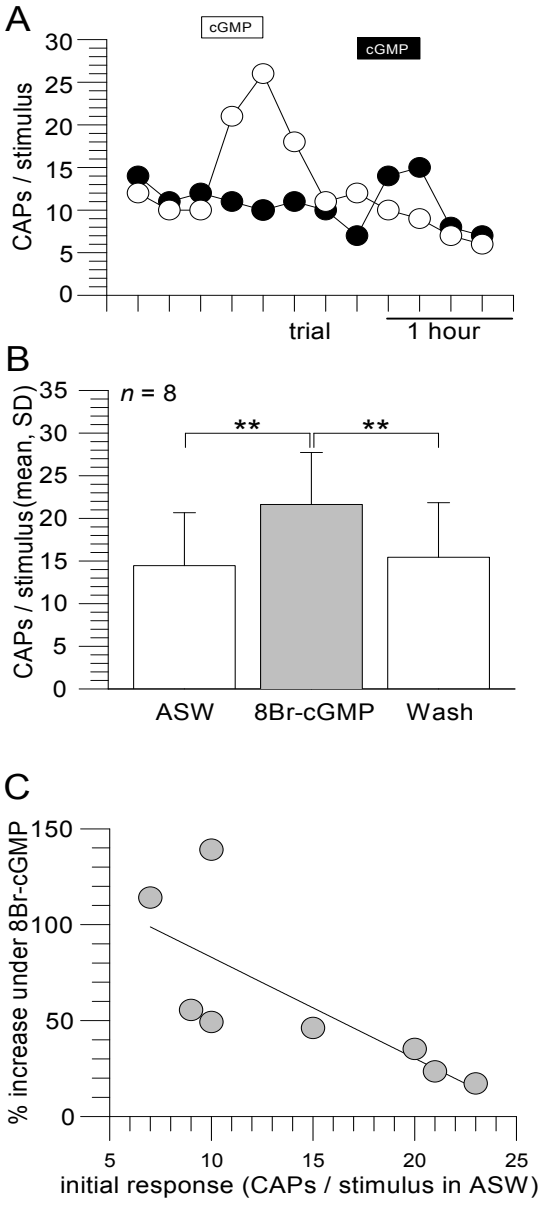

Fig. (3). Evaluation of the effect of 8-Br-cGMP on the light response of BRNs in intact, dark adapted, eye preparations. **A**. Sequential plot giving the number of compound action potentials recorded during each light pulse (CAPs/stimulus) for consecutive trials recorded simultaneously every 15 mins from the left- (white symbols) and right eye (black symbols) of one individual animal (cf. Fig. **2**). Bars indicate periods of incubation in 8-Br-cGMP (100 µM, white: left eye, black: right eye). **B**. Bar chart giving the mean CAPs/stimulus and its standard deviation for 8 individual eyes before (ASW), during (grey bar) and after (WASH) application of 8-Br-cGMP. Significant differences between the means are indicated (**, $p < 0.01$, Student's paired *t*-test). **C**. Linear regression analysis indicating that the percentage increase in CAPs per light stimulus under 8-Br-cGMP (*y*-axis) is inversely correlated to the initial light response in ASW (*x*-axis) for the eight eyes evaluated (correlation coefficient -0,75).

confined within the eye's neuropil. Thus, the connective tissue encapsulating the eye, and the photoreceptor cell bodies (Fig. **1B**) below the pigment layer was void of immunoreactive product. Although the cell bodies of the BRNs did not stain, faintly labelled varicose structures were apparent on the surfaces of some of these circadian pacemaker neurones (Fig. **1C**). NOS-like immunoreactive staining was also evident in the fibre-containing core of the optic nerve, which connects the eye to the cerebral ganglia. Although the staining here was faint, and no individual fibres could be discerned, the labelling was clear in comparison to the negative staining of the connective tissue enclosing the optic nerve (Fig. **1D**). For comparative purposes and as a positive control, Figs. (**1E** and **F**) show the vivid NOS-immunoreactive labelling of a neurone cell body and its dendritic processes in the *Bulla* brain. Fig. (**1G**) shows fluorescent immunocytochemical labelling of the *Bulla* eye using an antiserum that recognises RFamide-related peptides (e.g., FMRFamide). Similar results were obtained for the 4 eyes taken from 2 animals that we critically evaluated in this respect. Since the distribution of RFamide-like immunoreactivity in the eye (Fig. **1G**) clearly differs to that of NOS-like immunoreactivity (Fig. **1A**), it is apparent that the antisera directed against these two neuromodulators label different elements in the neuropil.

In Modulation of BRNs in Intact Eyes

In response to a 1 min. light pulse, a salve of conspicuously large and broad spikes were recorded extracellularly from the optic nerves of dark-adapted eyes during the subjective night (Fig. **2**, n = 8, cf. Fig. **3**). These so-called compound action potentials (CAPs) result from the synchronised spiking of the population of electrically coupled basal retinal neurones BRNs in the eye, which have axons that project to the brain and contralateral eye (cf. [52]). These responses remained stable for several hours. The end of the subjected night was indicated by spontaneous CAP discharges.

Dark adapted, intact eyes, we found no change in the light induced response following bath application of the NO-donor SNAP (100 μM) in the four preparations we tested (data not shown). Since we suspect that NO may not readily traverse the thick connective tissue that encapsulates the eye, and/or that SNAP may be fully degraded before doing so, we continued experiments using the membrane permeable and stable analogue of the second messenger cGMP, 8-bromo-cGMP.

Contrasting the above, 8-bromo-cGMP (100 μM) increased the light induced responses in all 8 eyes (from 4 individuals) tested some 15-30 minutes after bath application (Figs. **2** and **3**). Example of extracellular recordings from two eyes are shown in Fig. (**2B**) before, during, and after 8-bromo-cGMP application, and the number of CAPs evoked by each light stimulus is plotted in Fig. (**3A**) for the whole experiment that lasted 3 hours. Subsequent washing in ASW reversed the effect in all cases (Figs. **2B** and **3A**,**B**). In the mean, the number of light evoked CAPs was significantly greater (approximately 50%) in the presence of 8-bromo-cGMP ($p < 0.01$, students paired t-test,n = 8, Fig. **3B**). The magnitude of the effect varied from eye to eye, and we suspect that this may reflect differences in endogenous cGMP levels. Supporting this idea, the net effect of 8-bromo-cGMP (100 μM) was negatively correlated to the initial light response in ASW (Fig. **3C**, y=136.4-3.4x, coefficient of linear correlation -0.75).

Further support for endogenous control of the light sensitivity of BRNs by a NO/cGMP pathway was gained using L-NAME, a highly specific irreversible inhibitor of NO synthesis. Due to the dependency of the cGMP effect on the eyes initial light response (Fig. **3C**), we opted to test the effects of L-NAME on eye preparations that produced an average or above average number of CAPs/stimulus (Fig. **4**; hence the larger mean response in ASW, n=5 eyes from 3

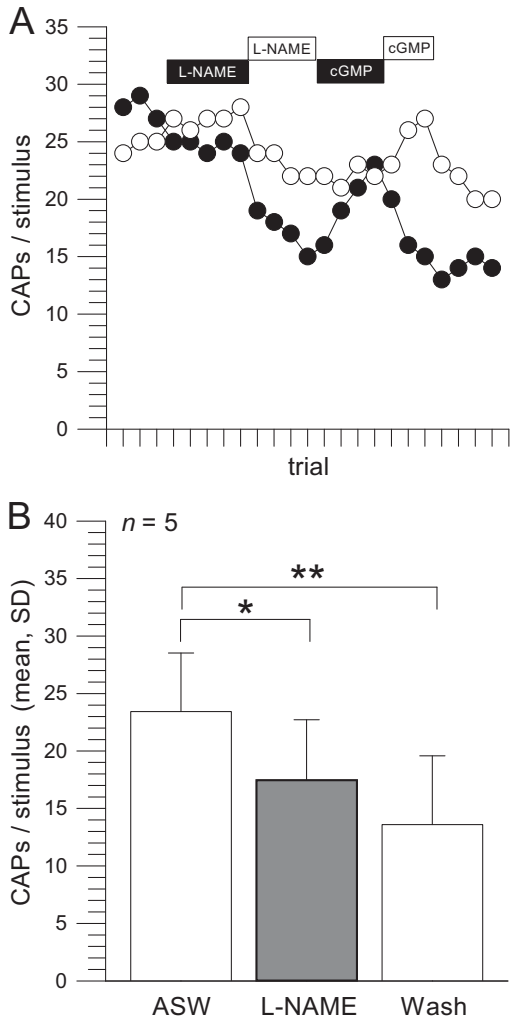

Fig. (4). Depressed light response of BRNs in intact, dark adapted eye preparations following bath application of the irreversible inhibitor or neuronal NOS inhibitor L-NAME (100 μM). **A**. Sequential plot giving the number of CAPs during each light stimulus for consecutive trials recorded simultaneously every 15 mins from the left- (white symbols) and right eye (black symbols) of one individual animal. Bars indicate incubation periods in L-NAME and 8-Br-cGMP (white: left eye, black: right eye). Although the effect of L-NAME cannot be washed out, the enhanced light responses recorded during application of 8-Br-cGMP (cGMP) testifies the viability of the preparation. **B**. Bar chart giving the mean CAP/stimulus and its standard deviation for 5 individual eyes before (ASW), during (grey bar) and after (WASH) application of L-NAME. Significant differences between the means are indicated (*,** $p < 0.05$, <0.01 respectively, Student's paired *t*-test).

individuals). One experiment is depicted in Fig. (**4A**), which plots the number of CAPs for each light stimulus for two eyes over a period of approximately 6 hours. For both eyes, the light response is diminished in the presence of L-NAME (100 µM). As expected, this effect was not washed out in ASW, since the action of L-NAME is irreversible. However, subsequent application of 8-bromo-cGMP (100 µM) increased the light response in both eyes, which verified that the responsiveness of the BRNs had not deteriorated during the course of the experiment. Altogether, we found that the light-responsiveness of the BRNs was reduced by some 26 % after bath application of L-NAME in the 5 individual eyes tested (Fig. **4B**, differences between mean CAPs/stimulus significant, Student's paired t-test, $p<0.05$).

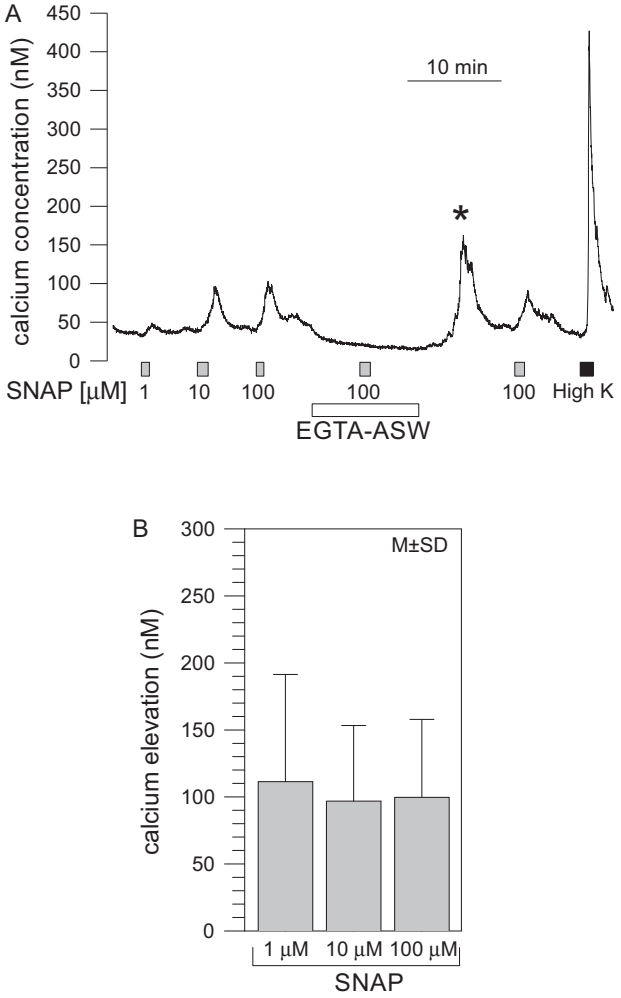

Fig. (5). *In vitro* optical recording of intracellular Ca^{2+} concentration in isolated somata of BRNs in primary cell culture. **A**. Original recording from a single BRN showing the increased Ca^{2+} signal following application of the NO-donor SNAP in ASW (grey bars: 1, 10 and 100 µM), which is prohibited in the presence of the calcium chelator EGTA (white bar; * note that the elevated calcium signal after washing is due to rebound calcium flux). This cell's calcium-response to depolarisation by high extracellular K^+ concentration is shown to the far right (high K). **B**. Bar chart giving the mean change in intracellular Ca^{2+} concentration, and its standard deviation, resulting from application of SNAP (grey bars, *n*= 5). Note we found no clear dependency of the mean response on the concentration of SNAP in the micro-molar range for the 5 cells tested.

Modulation of Isolated BRNs in Primary Cell Culture

Isolated BRNs in primary cell culture still express a circadian activity rhythm [3]. In the intact eye this rhythm is phase-shifted by light *via* a calcium dependant mechanism [53]. We therefore investigated the influence of the NO-donor molecules on the intracellular calcium concentration of isolated BRNs using fura-2.

Bath application of the NOS-donor SNAP caused a fast transient elevation of the intracellular concentration of free calcium in the cell bodies of single isolated BRNs. An example recording is depicted in Fig. (**5A**). In this cell the least effective SNAP concentration was 1 µM, which produced a just discernable increase in intracellular calcium concentration of approximately 15 nM above baseline (35 nM). Subsequent application of 10 µM SNAP produced a greater, 60 nM, increase, which was in the same order as the maximum increase in intracellular calcium which we recorded in this cell with 100 µM SNAP (64 nM). This compares to a 390 nM increase in calcium observed in this cell following bath application of ASW with high potassium. All cells recorded showed qualitatively the same response to SNAP, although the net effect varied considerably from cell to cell. For the 5 cells critically evaluated (taken from one eye), we found no clear dependency of the mean calcium elevation on the concentration of SNAP applied (Fig. **5B**, grey bars). The less specific NO-donor sodium nitroprusside was found to have similar, but inconsistent effects depending on the former decomposition of this compound by red light (data not shown).

The response to 100 µM SNAP was abolished in the presence of the calcium chelator EGTA in all 3 cells critically tested (Fig. **5A**; note the elevated calcium signal after washing is due to rebound calcium flux). This indicates that the effect of SNAP is due to an influx of extracellular calcium, possibly *via* cGMP-activated channels. Supporting this, preliminary experiments (n = 2 BRNs) revealed that application of the broad band phosphodiesterase inhibitor IBMX, which prohibits breakdown of endogenous cGMP, resulted in a pronounced elevation of intracellular calcium concentration (> 200 nM).

DISCUSSION

Our data from immunocytochemistry of eye-sections of *Bulla*, together with in situ extracellular electrical recordings, and *in vitro* optical measurements of intracellular calcium of ocular pacemaker neurones, provide the first evidence that the gas nitric oxide (NO) functions as an endogenous neuromodulator of the circadian clock in a mollusc.

Using immunocytochemical techniques, we detected the enzyme NOS, which is used exclusively for NO production, in the eye neuropil and optic nerve. Whereas mammals possess 3 different NOS isomers, only one has been unequivocally identified in invertebrates, though molluscs are suspected as having two [28]. In the absence of specific anti-mollusc NOS, antisera directed against mammalian neuronal-NOS have been used to localise putative NO-producing cells in several gastropods other than *Bulla*, including *Helix aspersa* [54], *Lymnaea stagnalis* [45], *Aplysia californica*, *Pleurobranchaea californica*, *Tritonia diomedea* [46], *Melibe leonina* [55], *Ilyanassa obsoleta* [56]. In most

studies of marine invertebrates, the same structures revealed by neural-NOS immunocytochemistry are also detected by NAPDH-diaphorase histochemistry (review [29]), though there are notable exceptions in molluscs [53, 55]. It has thus been suggested that specific mammalian antisera may not recognise some invertebrate NOS types (cf. [31]). In our study, we employed a "universal" NOS antibody raised against an epitope (DQKRYHEDIFG) conserved in all three mammalian isomers. This serum has been used for localizing NO-producing structures in the nervous tissues of various invertebrates including crustaceans (e.g., [57]) and insects: (*Manduca* [58]; *Drosophila* [59]; *Locusta* [60]), where it detects the same neurones as NADPH-diaphorase histochemistry, but has proved to be superior for revealing efferent fibres [49], and less prone to fixation artefacts (cf. [61]). Pilot experiments with NADPH-diaphorase histochemistry in *Bulla* produced basically the same staining pattern as NOS-immunocytochemistry (Stevenson, unpublished). However, the blue-black staining product shows poor contrast to the frequently dislodged, black photo pigment, so we favoured immunocytochemistry.

NOS has previously been localised by immunocytochemistry in the retina of the cephalopods *Loligo* [62] and *Sepia* [38], but the type and function of the located cells are not known. In our study, NOS-immunocytochemistry revealed no nerve cell bodies within the eye tissues of *Bulla*. Therefore, fine immunoreactive processes in the optic nerve and eye neuropil surrounding the circadian pacemaker cells (BRNs) must be efferent fibres derived from neurones in the CNS.

Wherever critically investigated, NOS-immunoreactivity or NADPH-diaphorase co-localizes in nervous tissue with a more conventional neurotransmitter, in *Aplysia*, for example, with myomodulin [30] and histamine [63]. The only conventional neurotransmitters known to date in the eye of *Bulla* are the peptide FMRFamide [9, 18] and the amino acid glutamate [17]. FMRFamide-related peptide occurs in nearly all efferent nitridergic neurones in an insect [49], and in gastropods this peptide has been identified as a substrate source of NOS [64]. However, we rule out the possibility that NOS is co-localised with FMRFamide in the *Bulla* eye, since the immunocytochemical staining patterns for these two signalling molecules do not coincide. Similarly, NOS does not co-localise with FMRFamide in the CNS of Helix [65]. Glutamate, on the other hand, appears to be the transmitter used in *Bulla* by the BRNs [17] to mediate bilateral coupling between the two circadian pacemakers [11]. Although the cell bodies of these neurones are frequently surrounded by NOS-like immunoreactive varicosities, they themselves remain unlabelled. Thus, putative NO-releasing terminals are ideally located for modulating the ocular circadian pacemaker cells in *Bulla*, although the neurons providing this efferent control remain to be identified.

NO can act as a volume signal, spreading tens of micrometers to distance action sites, as in the mammalian cerebellum [66] and mushroom bodies of the insect brain [67]. In a few systems though, close proximity of NOS to its receptor, soluble guanylyl cyclase (sGC) ensures signal specificity, as in the hippocampus, where NO acts as a synapse specific transmitter [68]. Our experiments in *Bulla*, suggest that the juxtaposition of NOS-immunoreactive varicosities to BRNs could be of similar functional significance. We propose that local release of NO stimulates sGC in BRNs to produce cGMP, which opens cyclic nucleotide gated channels and enables an influx of calcium ions (Ca^{2+}), resulting in an enhanced light response (cf. Fig. **6**). Supporting this, the NOS-donor SNAP (S-nitroso-N-acetylpenicillamine) caused a fast transient elevation of the intracellular concentration of free calcium in the cell bodies of single isolated BRNs.

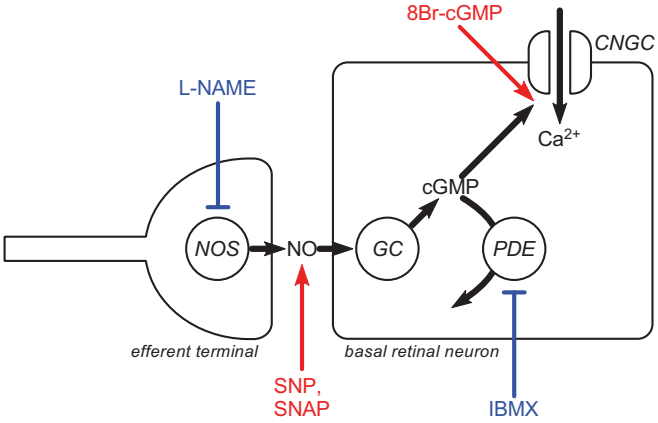

Fig. (6). Schema of the proposed NO-cGMP signalling pathway in the *Bulla* eye. Efferent neurone fibres containing the enzyme nitric oxide synthetase (NOS) terminate on basal retinal neurones (cf. Fig. **1**). Nitric oxide (NO) released by these efferent terminals, or delivered by an NO-donor (SNAP), reaches and enters a basal retinal neuron (BRN) and stimulates guanylate cyclase (GC). This leads to the production of the intracellular second messenger guanine monophosphate (cGMP), which activates cyclic nucleotide gated channels (CNGC) resulting in an influx of calcium ions (Ca^{2+}). Elevated calcium could in turn augment the response of the BRN to illumination *in vivo*. The action of cGMP is terminated by phosphodiesterases (PDE), which are blocked by IBMX.

This must be due to influx of extracellular calcium, since the effect was blocked in the presence of the calcium chelator EGTA. As in vertebrates (review [33]), NO exerts most of its effects in Molluscs *via* activation of soluble guanylyl cyclase, leading to cGMP formation [34, 35, 36, 37], and cyclic nucleotide-gated ion channels represent one of the three known receptor classes for cGMP [69]. However, cyclic nucleotide independent potentiation of cyclic nucleotide gated cation channels by NO has recently been reported in neurons of the gastropod *Pleurobranchia californica* [70]. Nonetheless, our data suggest the presence of a NO-dependent cGMP-signalling cascade in *Bulla* BRNs. Firstly, in isolated BRNs, a fast transient increase in intracellular calcium was also caused by the phosphodiesterase blocker IBMX, which can elevate endogenous cGMP by preventing its degradation. This implies a basal activity of guanylyl cyclase in *Bulla* BRNs, as observed for mammalian retinal guanylyl cyclase [71]. Supporting this, application of the tissue permeable analogue of cyclic guanosine monophosphate, 8-bromo-cGMP to the intact eye reversibly enhanced the light response of the BRNs, recorded as a burst of compound action potentials (CAPs) from the optic nerve. Our observation that the magnitude of this effect was inversely proportional to the initial light responsiveness, also suggests basal activity of the NO/cGMP signalling pathway in BRNs. Finally, although we saw no effect of SNAP on intact eyes,

L-NAME (N^{ω}-nitro-L-arginine methyl ester) a specific but irreversible inhibitor of NO synthesis reduced the response to light, indicating that endogenous NO production may continually regulate light sensitivity of the circadian pacemaker in *Bulla*.

While our data fit the idea that an NO/cGMP signal cascade increases the light sensitivity of BRNs by activating cyclic nucleotide gated channels, we cannot exclude additional mechanisms. NO may also influence light-evoked CAPs by modulating calcium channels, as shown for lizard cone terminals [72] and salamander rod terminals [73]. In snail feeding neurones, NO modulates voltage dependent L-type calcium channels, which have also been identified in *Bulla* BRNs [74], *via* cGMP induction of channel phosphorylation by protein kinases [37]. NO may modulate the conductance of GAP junctions, as shown in fish retinal neurones, by activating a cGMP-dependent protein kinase [75]. A similar action on the electrically coupled BRNs in *Bulla* seems unlikely, however, as we observed no obvious effect on the form of their CAPs. Since phototransduction in most invertebrates involves cGMP (review [76]), nitridergic modulation of photoreceptor sensitivity in the *Bulla* eye, as claimed by Roberts and Willis [41], also seems feasible and has been described in various other animals (molluscs [77]; insects [78, 79]; vertebrates [80]; review [23]). Effects on light adaptation are also possible (vertebrates [72], review: [81]; insects [82]).

We speculate that efferent nitridergic input to the *Bulla* ocular circadian clock may be involved in phase entrainment. Light pulses synchronize circadian rhythms by inducing phase delays during the early night and phase advances during the late night [6]. In the mammalian suprachiasmatic circadian clock, photic resetting depends on activation of voltage gated calcium channels [83], and a similar mechanism is postulated in *Bulla* [4, 53]. The BRNs are intrinsically photosensitive and transduce light signals that shift the phase of their pacemaker [84], possibly *via* an opsin-like photopigment [54]. Furthermore, light induced depolarization leading to a transmembrane calcium influx is essential for light entrainment in both *Bulla* [5, 6, 83, 85, 86] and *Aplysia* [7]. In mammals, it is hypothesised that the NO/cGMP signalling pathway mediates the phase advancing effects of light on the circadian clock (cf. [87]; review [24]), although there is still no complete consistency in the literature (review [25]). In *Aplysia*, light increases the levels of cGMP, and 8-bromo-cGMP phase shifts the circadian rhythm in the same direction and magnitude as light [39]. However, the site at which cGMP acts in *Aplysia* remains to be determined. In *Bulla* unpublished findings (S. B. Khalsa, cited by [1]) suggest that 8Br-cGMP does not lead to phase shifts, possibly because of limited tissue penetration. The later seems unlikely, since we found that 8Br-cGMP enhances the light response of BRNs in the intact eye. One alternative is that cGMP modulates, rather than mediates, light induced phase shifts, as shown for FMRFamide [19].

NO induced cGMP production may also antagonize the action of cAMP in BRNs. In *Bulla*, 8-bromo-cAMP applied during the subjective day decreased CAP frequency and produced phase shifts in the circadian rhythm of magnitude and direction depending on the circadian phase [40]. In *Aplysia*, cAMP is suspected to mediate serotonergic modulation of photic phase shifting [14, 88], by activating a hyperpolarizing potassium current, resulting in decreased calcium flux [7]. Serotonin does not occur in the nervous tissues of the *Bulla* eye [17], but FMRFamide may fulfil a similar role. This tetrapeptide suppresses the light response of basal retinal neurons [9] and blocks light-induced phase changes [19], possibly by attenuating a hyperpolarising, potassium current in the BRNs [20].

Whether FMRFamide influences cAMP in BRNs is not known, although it does not appear to influence calcium flux [20]. Nonetheless, the attractive hypothesis emergent from our study is that the neuromodulator nitric oxide acts as a functional antagonist to FMRFamide in the efferent regulation of light sensitivity of the circadian clock in the eye of *Bulla gouldiana*. This might contribute to robust entrainment to the natural light dark cycle of day and night even under changeable lighting conditions.

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