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Characterisation of the RNA interference response against the long-wavelength receptor of the honeybee

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ABSTRACT

Targeted knock-down is the method of choice to advance the study of sensory and brain functions in the honeybee by using molecular techniques. Here we report the results of a first attempt to interfere with the function of a visual receptor, the long-wavelength-sensitive (L-) photoreceptor. RNA interference to inhibit this receptor led to a reduction of the respective mRNA and protein. The interference effect was limited in time and space, and its induction depended on the time of the day most probably because of natural daily variations in opsin levels. The inhibition did not effectively change the physiological properties of the retina. Possible constraints and implications of this method for the study of the bee's visual system are discussed. Overall this study underpins the usefulness and feasibility of RNA interference as manipulation tool in insect brain research.

1. Introduction

The compound eye of the honeybee contains approximately 5000 ommatidia, each comprising 9 photoreceptor cells that form the rhabdom, a fused light-guide structure around its central axis, containing photopigments (Snyder et al., 1973). There are 3 different photoreceptor types, the short (S)-, medium (M)-, and long (L)-wavelength receptors, that show maximal sensitivity respectively in the UV, blue and green regions of the UV–visible light spectrum (Menzel and Blakers, 1976; Peitsch et al., 1992). Each receptor expresses a single photopigment that is composed of a chromophore, 11-*cis*-retinal, and a protein of the opsin family (Chang et al., 1996; Townson et al., 1998; Velarde et al., 2005). The L-receptor is implicated in the coding of chromatic and achromatic visual information (e.g. Kien and Menzel, 1977a,b; Lehrer et al., 1988; Giurfa et al., 1996; Hempel de Ibarra et al., 2000; Niggebrugge and Hempel de Ibarra, 2003). This dual function of the L-receptor signal poses a number of interesting questions, for instance, how L-receptor outputs are processed in parallel by the chromatic and achromatic systems, or whether there could be segregation of L-receptors at the retinal level.

A problem posed by the study of the L-receptor functions in psychophysical and physiological experiments is the strong overlap of receptor spectral sensitivities in the middle and short-wavelength range. The maximum sensitivity of the L-receptor of the worker honeybee is at 540 nm but its sensitivity range spans across the whole bee-visible spectrum. In this regard, molecular biology methods to knock-down the L-receptor would help to better understand its role in the coding of visual information. RNA interference (RNAi) is a valuable tool to inhibit specific gene products and to study their function. In particular, in animal models unsuitable for genetic engineering, it solves the problem of specificity often associated with pharmacological approaches, even if precautions have to be taken to avoid off-target effects (Jarosch and Moritz, 2012).

In the honeybee, RNAi is applied by either injecting (Beye et al., 2002; Amdam et al., 2003; Gempe et al., 2009) or feeding (Aronstein et al., 2006; Maori et al., 2009; Nunes and Simoes, 2009) embryos, larvae or adults with double-stranded RNA (dsRNA).

Abbreviations: RNAi, RNA interference; L-receptor, long-wavelength receptor; DsRNA, double-stranded RNA; LOp1, long-wavelength opsin 1; ERG, electroretinogram.

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It has been used in developmental studies where strong phenotypical effects were generally observed (Kucharski et al., 2008; Hasselmann et al., 2008; Liu et al., 2010; Kamakura, 2011; Jarosch et al., 2011; Wilson and Dearden, 2012). In adults, the technique has been used to successfully targeting several gene candidates, mostly by intra-abdominal injection of dsRNA (Schlüns and Crozier, 2007; Mackert et al., 2008; Wang et al., 2012; Ament et al., 2012). It was proposed that this administration route induces inhibitions at distant locations from the injection site (Gatehouse et al., 2004; Meng et al., 2012). However other studies suggested that the silencing effect is restricted to the fat body after intra-abdominal injections (Wang et al., 2010; Jarosch and Moritz, 2011). Although generally in transgenic approaches it has been demonstrated that RNAi can also be induced in the brain, its induction, especially by acute introduction of dsRNA into the system, has the reputation to be complicated particularly in worms (Kamath et al., 2003). In the honeybee, a few studies reported successful RNAi responses by direct dsRNA injection into the brain (Farooqui et al., 2003, 2004; Müßig et al., 2010; Mustard et al., 2010; Louis et al., 2012; El Hassani et al., 2012). These studies characterised the inhibitory response either at the mRNA or at the protein levels. The inhibitions were of moderate amplitude and were associated with changes in behavioural phenotypes. The spatio-temporal aspects of the inhibition were considered in some studies. They were shown to be first initiated at the injection site and then to migrate to adjacent tissues (Farooqui et al., 2004) or to be localised at the injection site during a limited time window (Müßig et al., 2010; Louis et al., 2012; El Hassani et al., 2012).

In this paper, we report the results of a first attempt to combine experimental methods aiming to advance our understanding of the L-receptor response properties and functions. We describe an RNAi-based protocol to inhibit the long-wavelength opsin 1 (LOp1) in the receptor cells of the honeybee's retina. We show that the injection of dsRNA induces a reduction at the mRNA and the protein levels. We report a set of experiments describing some aspects of the inhibitory response. We lastly evaluated experimentally the changes in the physiological responses at the level of the retina.

2. Material and methods

2.1. Preparation of dsRNA

The sense and anti-sense RNA were transcribed *in vitro* from sense and anti-sense cDNA templates after Müßig et al. (2010). Briefly, for the synthesis of dsLOp1, specific for the LOp1 mRNA, the amplification of the cDNA template for the transcription of the sense and the anti-sense strands were performed with the direct primer 5′-CGGCACCGATTACTTCAACAGA-3′ and the reverse primer 5′-CAAGCCATAAACCATAAAGAGATT-3′ modified or not at their 5′ extremity by the addition of a T7 promoter sequence 5′-TAATACGACTCACTATAGGGCGA-3′. The PCR protocol consisted of an initial denaturation step of 3 min at 95 °C, 25 cycles of 30 s at 95 °C, 1 min at 55 °C, 1 min at 72 °C and a final elongation step of 10 min at 72 °C. The plasmid DNA of selected clones was digested with *Pme*I restriction enzyme (Fermentas, St Leon-Rot, Germany) and the products were used to generate the sense and the anti-sense RNA by *in vitro* transcription using the T7 Ribomax Express Large scale production system (Promega, Mannheim, Germany). The synthesis of dsNEG, the negative control dsRNA specific for the chloramphenicol acetyl transferase (*cat*) gene, was done as previously described (Müßig et al., 2010). The dsRNA solution was resuspended in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at a concentration of 10 μg/μl or 20 μg/μl.

2.2. In situ hybridisation

In situ hybridisation was performed according to the protocol described by Zannat et al. (2006). The sense and anti-sense RNA probes were generated from PCR products amplified from the primers used to generate the templates for the *in vitro* transcription as described above.

2.3. Injection technique

The dsRNAs were directly injected in the retina. Borosilicate glass capillaries (outer diameter 1.0 mm, inner diameter 0.58 mm, WPI, Berlin, Germany) were pulled with a micropipette puller (Mod. P-97, Sutter Instruments, Hofheim, Germany), graduated every mm, filled with a Microfil (MF28G67-5, WPI, Berlin, Germany) mounted on a Hamilton syringe (701 LT, VWR, Darmstadt, Germany) and placed on a micromanipulator linked to a pressure injector (Pneumatic PicoPump PV820, WPI). The bee was placed under a binocular and a hole was pricked with an acupuncture needle in the centre of the right compound eye. The glass capillary was inserted at a 150 μm depth into the retina and 30 s later, ~260 nl of either 10 μg/μl or 20 μg/μl dsRNA was injected. After the injection, the bee was exposed 5 min under a lamp (Highlight 3001, Olympus) to promote the penetration of the dsRNA into the photoreceptors cells (Wunderer et al., 1989).

For the validation of the injection technique, 3% Alexa Fluor 488 Dextran resuspended in 1× PBS was injected in the retina as described above. One hour later, the animal was decapitated; a small window was cut in the head capsule and it was incubated 24 h in 4% paraformaldehyde in 1× PBS at 4 °C. The brain was extracted from the head capsule, washed twice in 1× PBS for 10 min, dehydrated in ethanol (50%, 70%, 90%, 99%, 2 × 100%, each 10 min), cleared in methyl salicylate and analysed with a confocal microscope (Leica TCS SP2).

2.4. Animal treatment and RNA and protein extraction for reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot analyses

Forager honeybees, *Apis mellifera carnica*, were caught at the hive entrance outdoors during the summer or in a flight room during the winter. The animals were anaesthetised on ice and fixed in small tubes, kept in an air conditioned room at 20 °C in a constant day/night cycle (12 h/12 h; daylight spectrum lamp, Arcadia Birdlamp, UK, illumination between 6:00 AM and 6:00 PM) and fed with 30% (w/v) pollen-enriched bee fondant (Neopoll N) twice a day (in the morning and in the evening) during the whole experiment. The animals were injected with dsRNA on the next day for the RT-qPCR, and 3 days later for the Western blot analysis. The animals were anaesthetised on ice and decapitated at different post-injection times. The head capsule was opened and the glands and tissues covering the brain were removed. In the RT-qPCR experiment, the complete brain or brain hemispheres were recovered and immediately homogenised in a Teflon-glass homogeniser containing 800 μl of Trizol (Life technologies, Darmstadt, Germany). When the whole brains were analysed, 4–9 bees were pooled for the different conditions and 10–11 if the treated and non-treated sides were analysed separately. Each pooled extract was analysed 3 times, except for the separate analysis of the injected and the non-injected site that were analysed twice. Total RNA extraction was performed according to Trizol user's manual and the material was resuspended in 20 μl of RNAse free water. In Western blot analysis, the retina and the optic lobe still attached to the cornea were removed, mixed with 32 μl of ice cold 1× PBS, 2 mM EDTA, 2 mM EGTA and immediately frozen in liquid nitrogen.

The tissues were homogenised in a Teflon-glass homogeniser, sonicated for 5 min in an ultrasonic bath and finally mixed with 8 μl of 5× Lämmli buffer (0.225 M Tris–Cl pH 6.8, 50% Glycerol, 5% SDS, 0.05% Bromophenol blue, 0.25 M dithiothreitol (DTT)).

In the experiment evaluating the variation of LOp1 levels during a day/night cycle, animals from a single-age cohort hive were used. They were all 28 days old at the beginning of the experiment. The animals were first kept 3 days in cages and harnessed 24 h before the start of the experiment. Extracted proteins from two optic lobes of four animals were combined and analysed four times.

2.5. RT-qPCR

Two micrograms of total RNA were reverse transcribed with the Superscript first strand synthesis kit (Life technologies) by following the manufacturer's protocol for random hexamer primer. The RT-qPCR was done with an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Each reaction contained 5 μl SybrGreen (Applied Biosystems), 0.6 μM of direct and reverse primers, 1.8 μl water and 2 μl cDNA. Following primers were used: LOp1 fwd: 5′-CGGCACCGATTACTTCAACAGA-3′, LOp1 rev: 5′-CAAGCCATAAACCATAAAGAGATT-3′, rp49 fwd: 5′-AAACTGGCGTAAACCTAAAGGTAT-3′, rp49 rev: 5′-CAGTTGGCAACATATGACGAGT-3′, gapdh fwd: 5′-TAATTGTTTAGCACCTCTTGCT-3′, gapdh rev: 5′-AGCAGTAACAGCATGAACAGTA-3′. The amplification protocol consisted of an initial denaturation step of 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 40 s at 56 °C and a final phase of 15 s at 95 °C, 15 s at 56 °C, 15 s at 95 °C.

Different parameters were considered to demonstrate the quality of the RT-qPCR conditions. The melting curves for each pair of primers generated only one peak, indicating that no primer dimers were formed (Fig. S1A). Probes from untreated animals were used to perform dilutions curves (10^0, 10^{-1}, 10^{-2} dilutions) to calculate the efficiency of the amplification using the formula $E = 10^{[-1/\text{slope}]}$: LOp1: 2.0; RP49: 1.65; GAPDH: 1.76. The quantification of the three genes on preparations of the left and the right compound eyes revealed no abnormal variation in expression levels (Fig. S1B). This result validated the choice of reference genes and showed that the dissection of the compound eye was not biasing the measured LOp1 mRNA levels. The differences in expression levels were analysed by using the programme REST 2005 (Corbett Research (Pfaffl et al., 2002)) and by comparing for each tested condition dsLOp1 and dsNEG injected animals.

2.6. Western blot

To examine LOp1 and α-tubulin, protein samples were heated for 5 min to 40 °C before being subjected to sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). The equivalent of 1/8 optic lobes were loaded per well. The proteins were transferred on nitrocellulose membranes. The membranes were blocked in 1× PBS, 0.1% Tween-20, 3% non-fat milk powder (blocking solution), 1 h at room temperature (RT). The membranes were probed with the rabbit polyclonal antibody LWRh1 (1:1000, Quality Controlled Biochemicals, Hopkinton, MA, USA) dissolved in the blocking solution, overnight at 4 °C. The membranes were washed with 1× PBS, 0.1% Tween-20 (PBS-T), 3 times for 10 min at RT and then incubated with the secondary antibody directed against rabbit IgG coupled to horseradish peroxidase (1:10,000 – Sigma, Munich, Germany) dissolved in the blocking solution, 1 h at RT. The membranes were washed with PBS-T, 3 times for 10 min at RT and were developed by enhanced chemiluminescence detection (ECL, Perkin Elmer, Rodgau, Germany) and the signals were acquired with a LAS1000 camera and the software Image Reader LAS1000 2.60 (Fujifilm, Düsseldorf, Germany). Then, the membranes were stripped to allow the detection of α-tubulin. The membrane were washed for 30 min at 60 °C in 30 mM Tris–HCl pH 6.7, 2% SDS, 20 mM DTT and washed 3 times for 10 min at RT in PBS-T. The detection of α-tubulin was done as described above. The anti-α-Tubulin antibody (DM1A, Merck, Nottingham, UK) and the secondary antibody (anti-mouse IgG coupled to horseradish peroxidase, Sigma) were dissolved 1:10,000 in the blocking solution. The quantification of the LOp1 levels was done by relating the intensities of the LOp1 signals to the α-tubulin signals by using the programme MultiGauge v3.0 (Fujifilm, Düsseldorf, Germany). The relative LOp1 levels were normalised to the highest LOp1/α-tubulin ratio of the membrane in the experiment evaluating the variation of LOp1 levels during a day/night cycle, and to the LOp1/α-tubulin ratio of the corresponding control group (dsNEG) in the experiment evaluating the RNAi effect. The difference between dsLOp1 and dsNEG injected honeybees was tested for significance with the Mann–Whitney-*U* test implemented in Statistica '99 Edition (Statsoft, Tulsa, OK).

2.7. ERG (electroretinogram)

Forager honeybees were caught three days before dsRNA injection and kept 2 days in a constant day/night cycle (12 h/12 h). The ERG recording electrode combined a ringer-filled glass capillary and a silver/silver-chloride wire as reference. Recordings were performed in a standard electrophysiological setup. Signals were amplified with a differential amplifier (ISO-DAM, WPI, Berlin, Germany), digitised and registered with an A/D converter (Micro 1401, CED, Cambridge, UK). Data acquisition was performed with Spike 2 (CED, Cambridge, UK). The animals were stimulated with three LEDs that matched the maximum sensitivity of the 3 honeybee photoreceptors (UV: 350 nm – 3/6/5 μW/cm^2, blue: 440 nm – 5/8/15 μW/cm^2, green: 540 nm – 14/20/21 μW/cm^2, stimulus duration 241 ms).

DsRNA was administered between 6:00 AM and 8:00 AM and ERGs were recorded 12 h and 24 h after injection. The recording electrode was inserted in the hole pricked in the right eye for the dsRNA injection and the reference electrode was inserted just under the left eye which had been covered with black paint after the injection procedure. The room was completely dark, and 5 min after dark adaptation, the stimulation started with the green LED, followed by the UV and the blue LEDs (Fig. S2A). The eye was first stimulated with the low-intensity stimulus (3 times with an interstimuli interval of 20 s), then with the middle intensity value, and finally with the high-intensity value. Intensity changes were separated by 1 min of darkness to allow the receptors to re-dark-adapt and by 2 min when switching between LEDs.

A typical honeybee ERG recording is characterised by a short decrease in the recorded potential after the beginning of the stimulation (On-response), then it levels off (plateau response) and it decreases again at the end of the stimulation (Off-response) before returning to the potential in darkness (reference potential) (Fig. S2B). These three components as well as the sustained response, here defined as the potential recorded 1 ms before the end of the stimulation, were analysed.

The absolute changes in potential varied largely between animals. As the response to UV should be the less affected by a possible reduction of the L-receptor sensitivity, each recorded response was normalised to the response to the high-intensity UV stimulation. Further we also analysed the general shape of the ERG, setting the potential at the beginning of the stimulation at 0 and at the end of the stimulation at −1. The values comprised between the beginning of the stimulation and up to 150 ms after the end of the stimulation were treated in this way. These normalised curves were also used to compare the transient components between dsLOp1 and dsNEG

groups. We calculated the integrated values of the first 100 ms after stimulus onset and offset as well as for the time window during which onset peaks occurred (26–76 m after stimulus onset). Due to small sample sizes the data were analysed using non-parametric statistics (Mann–Whitney-*U* test).

3. Results

3.1. Selection of a dsRNA specific for the LW opsin

A 267 bp long dsRNA, dsLOp1, specific for the position 820–1086 of the LOp1 mRNA (Acc. n°: NM_001011639) was designed. In non-mammalian species, dsRNA molecules do not activate defence mechanisms, characterised among others by a general inhibition of protein translation (Holen and Mobbs, 2004). DsRNAs interact with the Dicer enzymatic complex, which reduces them to siRNAs that are 19–23 bp long. Then, siRNAs are incorporated into an enzymatic complex to form the RNA-induced silencing complex (RISC) that induces the degradation of mRNA species matching the sequence of the siRNA (Terenius et al., 2011). The comparison of dsLOp1 against the honeybee genome showed that fully identical stretches of sequences of dsLOp1 longer than 19 bp were only found in the *lop1* gene. Pair-wise comparisons between dsLOp1 and the other opsins mRNA identified in the honeybee (long-wavelength-sensitive opsin 2 (LOp2), acc. n°: NM_001077825), blue-sensitive opsin (AmBLOp, acc. n°: AF004168), UV-sensitive opsin (AmUVOp, acc n°: AF004169) and pteropsin (acc. n°: NM_001039968) confirmed the specificity of the dsRNA molecule (data not shown). Hence, it can be safely assumed that the dsRNA generated from dsLOp1 were only specific for the LOp1 mRNA.

To avoid unspecific effects due to the injection of dsRNAs in the brain, a control dsRNA construct was employed as negative control which is specific for the chloramphenicol acetyl transferase gene (dsNEG) that is expressed only in prokaryotes. The comparison of the dsNEG sequence to the honeybee genome allowed us to exclude the possibility that siRNA specific for any honeybee gene could be generated from this dsRNA and it was already shown that this molecule does not affect olfactory memory (Müßig et al., 2010).

To further confirm the specificity of dsLOp1, the sequence of this molecule was used to generate an *in situ* hybridisation probe to localise the LOp1 mRNA. Signals were detected exclusively in the retina (Fig. 1A–C). No signals were detected in the first micrometres of the outer surface of the retina that comprises the crystalline cones.

Dense signals were detected in photoreceptor cells, characterised by their long elongated morphology and their columnar arrangement. The signals extended to the basal membrane. In some preparations, detection of LOp1 mRNA was more intensive in the central part of the retina (Fig. 1A, B). The specificity of the hybridisation was assessed by showing that a negative control, a sense RNA probe, did not yield any signals (Fig. 1D).

3.2. Validation of the injection into the compound eye

Previous studies suggested that the induction of the RNAi response in the central nervous system requires injecting the dsRNA molecules directly into the brain. Thus, it was necessary to first validate our injection technique by showing that the injection of a fluorescent dye through the cornea effectively reached the nervous tissue. The injection of animals in the right eye resulted in dyeing of the retina, the lamina, the medulla and the lobula of the injected eye (Fig. 2A). The visualisation of the tissue at a higher magnification showed that the dye accumulated within the cell and the nucleus, as expected from this cell permeable dye (Fig. 2B).

3.3. Evaluation of the RNAi effect against LOp1 at the mRNA level

DsLOp1 or the negative control dsRNA (dsNEG) were injected directly into the right compound eye, and different experimental conditions were applied to evaluate the inhibitory effect. Different quantities of dsRNA were injected, once or repeatedly at different times of the day, and the retina and the optic lobe were analysed at different post-injection times. In some conditions, the injected and the non-injected sides were analysed separately. The inhibitory effect was evaluated by RT-qPCR and difference in expression with the REST 2005 software. The LOp1 mRNA levels were normalised to those of two reference genes: ribosomal protein 49 (*rp49*, Acc. n°: GB10903) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*, Acc. n°: GB14798) in dsLOp1 and dsNEG injected groups. Then, these relative mRNA levels were compared to each other for each tested conditions (Table 1, Fig. 3).

When the whole brain was analysed after a single injection of dsRNA, which was performed in the morning, a 61% significant reduction of LOp1 mRNA was observed 8 h after the injection of 5 μg of dsLOp1 (Table 1, Fig. 3, c; REST 2005, $p < 0.05$) but not 32 h after the injection (Table 1, Fig. 3, f; REST 2005, n.s.). A single injection of 2.5 μg of dsRNA did not induce a reduction of LOp1 mRNA at any tested time delays: 8 h (Table 1, Fig. 3, b; REST 2005, n.s.), 12 h (Table 1, Fig. 3, d; REST 2005, n.s.) or 54 h (Table 1, Fig. 3, g; REST 2005, n.s.) after the injection. It is notable that the 12 h time delay was the only condition where the injection took place in the evening. In two samples we measured 7 h after injection of 2.5 μg both the injected and the non-injected sides separately. In this case we observed a 86% non-significant reduction of LOp1 mRNA on the injected side (Table 1, Fig. 3, a(tr); REST 2005, $0.05 < p < 0.1$) and the mRNA levels remained unchanged on the non-injected side (Table 1, Fig. 3, a(not); REST 2005, n.s.).

Injecting 2.5 μg of dsLOp1 twice, on two consecutive days instead of a single 5 μg injection, did not drastically improve the RNAi effect, although it led to a significant 42% reduction of LOp1 mRNA (whole-brain preparations, 8 h after second injection; Table 1, Fig. 3, e; REST 2005, $p < 0.05$). Adding a third injection on the third day did not produce an RNAi effect at all (8h after third injection, Table 1, Fig. 3, h; REST 2005, n.s.). Finally we compared in two samples the injected and non-injected sides applying the double injection of 2.5 μg of dsRNA. A non-significant reduction of 60% of LOp1 mRNA was observed on the injected side (29–36 h after second injection, Table 1, Fig. 3, i(tr); REST 2005, $0.05 < p < 0.1$), whilst it remained unchanged on the non-injected side (Table 1, Fig. 3, i(not); REST 2005, n.s.).

3.4. LOp1 protein levels vary on a daily cycle

An antibody specific for the bumble bee (*Bombus terrestris*) long-wavelength sensitive opsin (LWRh1, Acc. n°: AY485301 (Spaethe and Briscoe, 2004)) was newly-generated and used to detect the honeybee LOp1. This antibody is directed against a 19 amino acids antigenic peptide specific for the C-terminal region of LWRh1. It shares a high degree of identity with the honeybee LOp1 (78.9%) and a very low degree of identity with the other opsins (LOp2: 10.5%, AmBLOp: 21.1%, AmUVOp: 31.6% and pteropsin: 15.8%) (Fig. 4).

The antibody detected a double band at ~40 kDa and at ~80 kDa in honeybee optic lobe extracts (Fig. 5). The 40 kDa doublet probably corresponds to LOp1 because its calculated molecular weight is of 42.04 kDa. The low degree of identity of the antigenic peptide for the other opsins strongly suggests that the doublet detected at 40 kDa does not correspond to other opsins. The ~80 kDa doublet probably corresponds to dimers of LOp1.

It has been shown that the LOp1 mRNA levels fluctuate on a daily cycle (Sasagawa et al., 2003; Rodriguez-Zas et al., 2012).

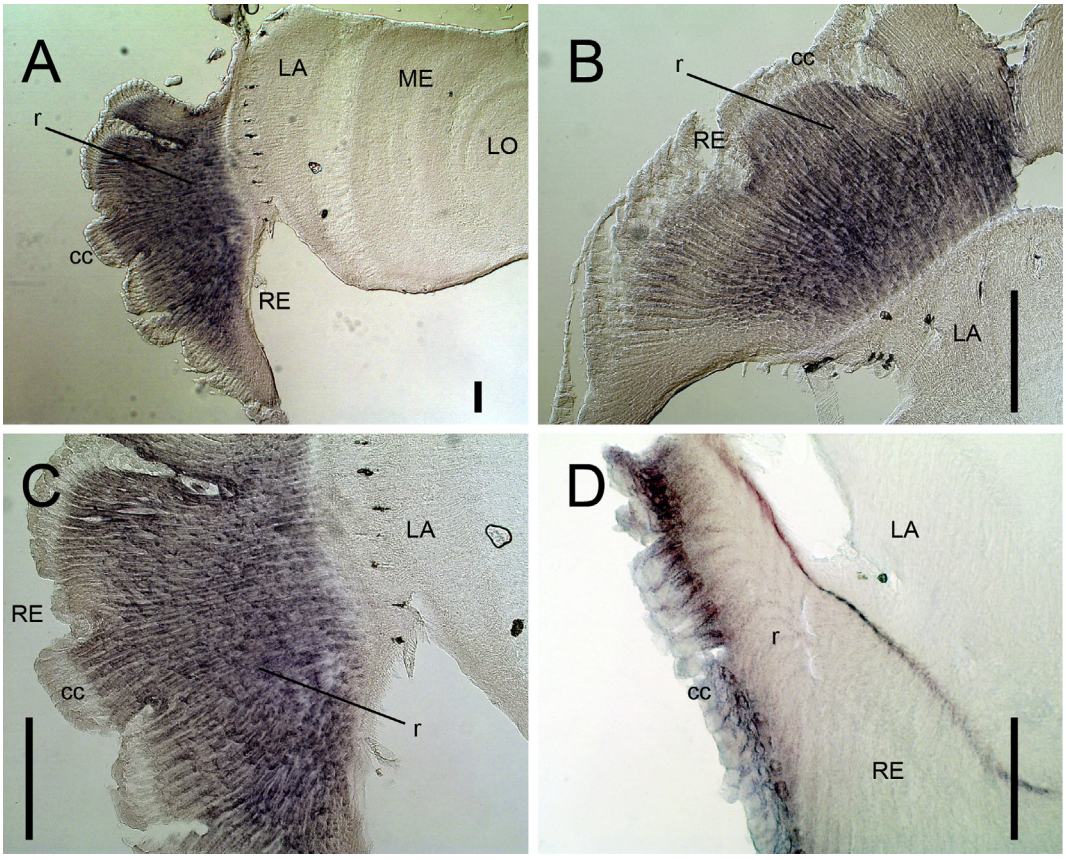

Fig. 1. Detection of *LOp1* in adult eyes by *in situ* hybridisation. Sagittal sections of the optic lobe and the retina of the right eye. The anti-sense probe detected signals in receptor cells (A, B, C). The sense probe did not reveal any signal (D). Retina (RE), lamina (LA), medulla (ME), lobula (LO), receptor cells (r), crystalline cones (cc). Scale bar: 100 μm.

Therefore, we evaluated how these fluctuations affect LOp1 protein levels. We also investigated whether α-tubulin, the reference protein used for the quantification, could be detected in the optic lobes and whether its levels also fluctuated.

Similar to the aforementioned study we kept four-week old bees for 3 days in a 12 h/12 h light cycle and analysed the optic lobes protein extracts that were obtained from dissections every 4 h during a period of 28 h. The protein amount of each probe was evaluated in a Bradford assay (data not shown) prior to Western blotting of equal amounts of each probe (Fig. 5). Whilst the α-tubulin levels remained unchanged, the LOp1 levels fluctuated. They increased from 10:00 AM to reach a maximal value at 2:00 PM. Then they diminished to reach a minimal value at 10:00 PM and they remained low until the next day where an increase was visible at 10:00 AM.

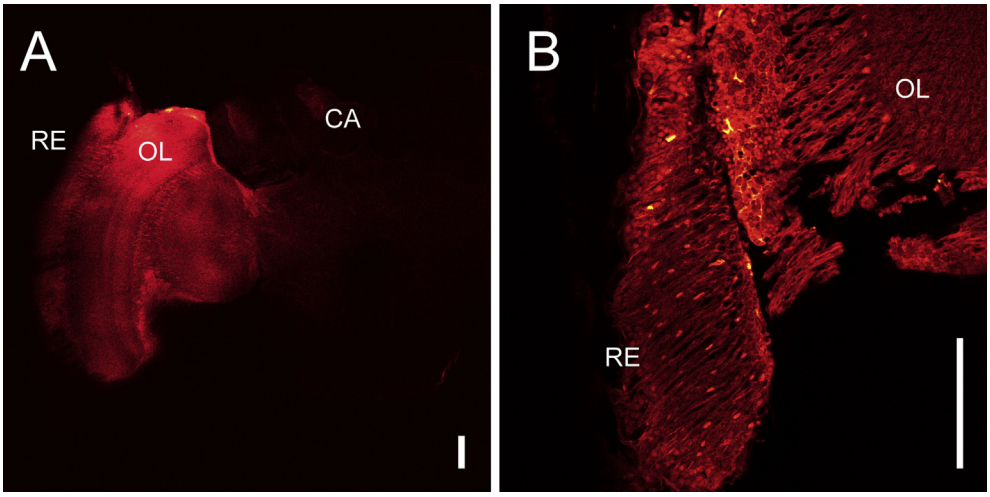

Fig. 2. Validation of the injection technique. Visualisation under the confocal microscope of the brain injected with 3% Alexa Fluor 488 – Dextran (A, B). The brain was dissected 1 h after 260 nl dye injection in the right compound eye. The retina is partially covered by the pigmented layer. Retina (RE), optic lobe (OL), mushroom body calyx (CA). Scale bar: 100 μm.

Table 1
Overview of the different experiments.

Experiment	Nb. of inj.[a]	Time (h) between 1st inj. and diss.[b]	Time (h) between last inj. and diss.[c]	dsRNA qty/inj.[d]	1st Inj. time[e]	2nd Inj. time[e]	3rd Inj. time[e]	Diss. time[f]	Relative *lop1* expr. levels (sample size)[g]
a(tr)	1	7		2.5	8 h30–10 h			14 h30–16 h	0.14 ($n = 2$)
a(not)									1.41 ($n = 2$)
b	1	8		2.5	9–11 h			17–18 h	1.04 ($n = 3$)
c	**1**	**8**		**5**	**8–13 h**			**17–19 h**	**0.39 ($n = 3$)**
d	1	12		2.5	19 h30–20 h30			7 h30–9 h30	0.98 ($n = 3$)
e	**2**	**32**	**8**	**2.5**	**8 h–10 h30**	**8 h–10 h30**		**16 h30–18 h**	**0.58 ($n = 3$)**
f	1	32		5	8–13 h			17 h–20 h30	0.85 ($n = 3$)
g	1	54		2.5	8 h30–12 h			13 h30–15 h30	0.74 ($n = 3$)
h	3	56	8	2.5	8 h–10 h30	8 h–10 h30	8 h30–10 h	16–18 h	0.92 ($n = 3$)
i(tr)	2	56	29–36	2.5	8 h30–14 h	8 h30–12 h		17 h30–20 h	0.4 ($n = 2$)
i(not)									0.75 ($n = 2$)

The table lists the injection schedules and quantities in the different experiments that were conducted in the present study. In experiments a and I (grey shading) the treated (tr) and non-treated (not) sides were analysed separately. Conditions highlighted in bold indicate a significant reduction of *lop1* expression.
[a] Number of dsRNA injections that were performed in each animal.
[b] Time (hours) between the first dsRNA injection and the dissection.
[c] When multiple injections were performed, time (hours) elapsed between the last injection and the dissection.
[d] Quantity of dsRNA inoculated at each injection.
[e] Time of the day of the first injection and during multiple injections of the second and the third injection on consecutive days.
[f] Time of the day of the dissection.
[g] Relative *lop1* expression levels (sample size). The *lop1* expression levels of the dsLOp1 group are shown relatively to the dsNEG injected group that was treated in the same conditions.

3.5. Evaluation of the RNAi effect against LOp1 at the protein level

The RNAi effect at the protein level was evaluated using 5 μg of dsLOp1 or dsNEG. Both the 40 kDa and the 80 kDa doublets were analysed in quantitative Western blots of optic lobe extracts.

To get an overview of the dynamics of the RNAi effect, we first injected a few animals in several different experimental conditions (Fig. 6A, B). The group size ($n = 3$) was too small to attempt a statistical analysis, as we mainly intended to get an overview of the dynamics of the RNAi effect. The injection and the dissection of animals took place at the day/night phase change. Injections were performed either at 6:00 AM or at 5:00 PM and the animals were dissected at several time points post-injection. Morning and evening injections were applied because we hypothesised that the efficiency of the RNAi response might depend on LOp1 mRNA levels at the moment of the dsRNA injection. As expected, we saw a reduction of the 40 kDa and the 80 kDa doublets 12 h after the injection of dsLOp1 in animals that were injected in the morning.

We repeated the experiment concentrating on the RNAi response induced 12 h after the injection of dsRNA, comparing the effectiveness of the morning (6:00 AM) and evening (6:00 PM) injection times (Fig. 6C). A significant 25% reduction of the 40 kDa and the 80 kDa doublets was observed only when bees were injected in the morning at 6:00 AM (Mann–Whitney-*U* test, $p < 0.05$), but no reduction occurred after injecting in the evening at 6:00 PM (Mann–Whitney-*U* test, n.s).

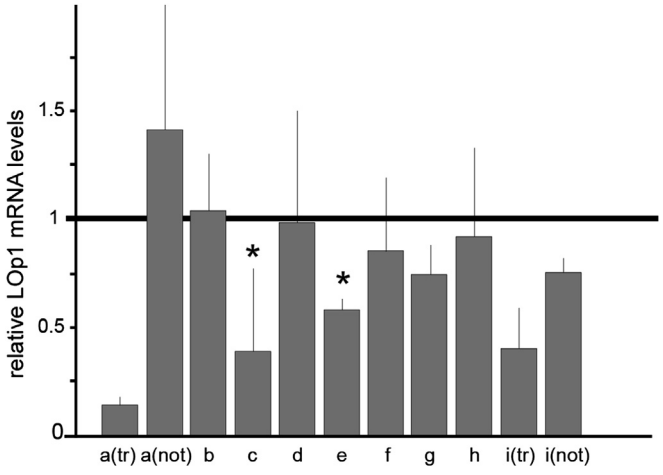

Fig. 3. Evaluation of the RNAi effect against LOp1 at the mRNA level by RT-qPCR. Bars depict the ratios of *LOp1* mRNA levels +/− SE in honeybees injected with dsLOp1 normalised against controls dsNEG injected bees for each experimental conditions, numbered a–i (see Table 1 for detailed experimental conditions). mRNA levels after single 2.5 μg injections 7–8 h (a, b), 12 h (d) and 54 h (g) post-injection. mRNA levels after single 5 μg injections: 8 h (c) and 32 h (f) post-injection. mRNA levels after multiple 2.5 μg injections: 8 h (e) and 29–36 h (i) after the last injection out of two and, 8 h after the last injection out of three (h). The values for both the non-injected side (not) and the injected side (tr) are shown for experiments a and i. $n = 2$ (a, i) and $n = 3$ (b–h). Asterisks indicate a significant reduction in mRNA levels in dsLOp1 treated animals compared to dsNEG treated animals (*$p < 0.05$, REST 2005).

3.6. Effect of the RNAi response against LOp1 on the physiology of the retina

Knowing the injection parameters and time course of the RNAi manipulation, we next aimed to evaluate whether the reduction of LOp1 opsin modifies the physiological properties of the retina. To measure the ERG response of the manipulated eye, it was stimulated with 3 different LEDs at 3 different intensities (Fig. S2A). The emission wavelengths of the LEDs were selected to excite the photoreceptors within their maximal sensitivity range. ERGs were performed 12 h and 24 h after the morning injection of dsRNA. These time intervals were chosen because a reduction of protein levels was observed 12 h post-injection and to perform evening and morning recordings. For each stimulation, the On- and the

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  1  --CT--------STVSGTTTVADNEKSNA        BtLWRh1 AP
351  --CAAEPSSDAVSTTSGTTTVTDNEKSNA        AmLOp1
348  --LQEKPISD--STSTTTETVNTPPASS         AmUVOp
356  --IH-EP--E--TTSDATSAQTEKIKTDE        AmBLOp
306  SRTA--------VPDSQHTALTALNRQEQRK      AmpterOp
367  --CG--------STEDQ-TAATAGDKASEN       AmLOp2
```

Fig. 4. Analysis of LWRh1 antibody specificity. Comparison of the amino acid sequences of the *Bombus terrestris* LWRh1 antigenic peptide (Bt LWRh1 AP) with the C-terminal sequence of LOp1 (AmLOp1), AmUVOp, AmBLOp, pteropsin (AmpterOp) and LOp2 (AmLOp2). The amino acids identical to the sequence of the antigenic peptide are highlighted in black. The numbers on the left indicate the position of the first amino acid of the alignment sequence within the complete opsin sequence.

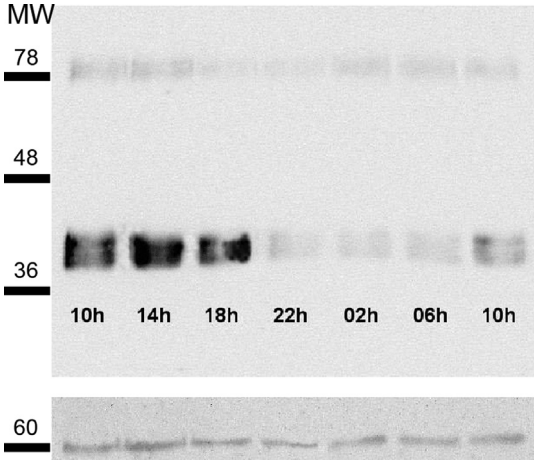

Fig. 5. Detection of LOp1 and α-tubulin during a complete day/night cycle. Honeybees were kept in a 12 h/12 h day/night rhythm (day time from 6:00 AM to 6:00 PM) and optic lobes were analysed at 4 h intervals. The anti-LWRh1 antibody detects 2 doublets at 40 kDa and 80 kDa. The anti-α-tubulin antibody detects a single protein at 60 kDa. Molecular ladder in kDa (MW).

Off-responses, the plateau and the sustained response were measured (Fig. S2B). Receptor and lamina cells are probably implicated in the generation of the transient On and Off components of the ERG responses, while the other components are thought to reflect the activation of the receptor cells only (Goldsmith, 1960; Heisenberg, 1971).

We predicted that a significant reduction of the sensitivity of L-receptors should lead to a lower ERG response in the dsLOp1 injected group as compared to the dsNEG group. For such comparison, it was necessary to normalise the ERGs of each animal to its strongest UV response. ERG responses to UV light have the lowest input from L-receptors and would be least affected by the expected suppression of L-receptor responses after injecting dsLOp1. In some recordings the transient components were lacking or a clear plateau value could not be identified (Fig. S2), therefore we analysed the "sustained response", measured 1 ms before the end of the stimulation. We found no differences in sustained responses to light stimuli between dsLOp1 and dsNEG treated animals, neither 12 h (evening recording) nor 24 h (morning recording) after dsRNA injection (Fig. 7A, Mann–Whitney-*U* test, n.s.). There was also no difference within each of the two groups when comparing responses 12 h and 24 h post-injection (Fig. 7B, Mann–Whitney-*U* test, n.s.). Comparisons of the shapes of the recorded ERG traces in response to blue, green and UV stimulations between dsLOp1 and dsNEG treated animals did not reveal any differences either (Fig. 8).

4. Discussion

In this work, we inhibited adult *lop1* expression by RNAi. The inhibitory effect was shown at the mRNA and the protein levels. The strength of the inhibitions appears to depend on the quantity of injected dsRNA and on the circadian state of the visual system. Already 8 h after the injection the inhibition was observed at the mRNA level and 12 h post-injection at the protein level. The inhibition was probably localised around the injection site and has been found to be stronger at the mRNA level than at the protein level. Moreover, the RNAi response was effective at the protein level only when the animals were injected in the morning but not in the evening, presumably because LOp1 mRNA and protein levels vary in a daily cycle. So far, it was not possible to conclusively demonstrate a physiological effect of the inhibition at the level of the retina. Nevertheless, we gained important insights and will discuss here the potentials and challenges for the further development of the RNAi method as a tool in the study of the honeybee visual system.

Based on computational analysis, we selected a dsRNA molecule, dsLOp1, to induce a specific RNAi response against the LOp1 mRNA. To control for unspecific effects due to the injection of dsRNAs in the brain, molecules specific for the *cat* gene (dsNEG) were used as negative control (Müßig et al., 2010). DsRNA were preferred over siRNA because we have previously found that the later are less potent for inducing RNAi in the bee brain (Müßig et al., 2010). The specificity of the probe for LOp1 mRNA was further demonstrated by showing that it detected mRNA restricted to receptor cells by *in situ* hybridisation. An antibody specific for the *Bombus terrestris* LWRh1 detected 2 doublets at an apparent molecular weight of ~40 kDa and ~80 kDa. The high level of identity between the antigenic peptide used to develop the antibody and the corresponding sequence of LOp1 and the successful induction of RNAi demonstrates the specificity of the antibody. The ~40 kDa doublet is detected at the expected size of LOp1. It was already shown in other insect species that N-glycosylations are necessary for the correct folding and the transport of opsins (Ozaki et al., 1993; Colley et al., 1995; Katanosaka et al., 1998) and 2 potential glycosylation sites are found in LOp1 (Gronenberg et al., 1996). Thus, the doublet probably corresponds to different glycosylated forms of LOp1. It is usual that the detection of insect's opsins by Western blot reveals

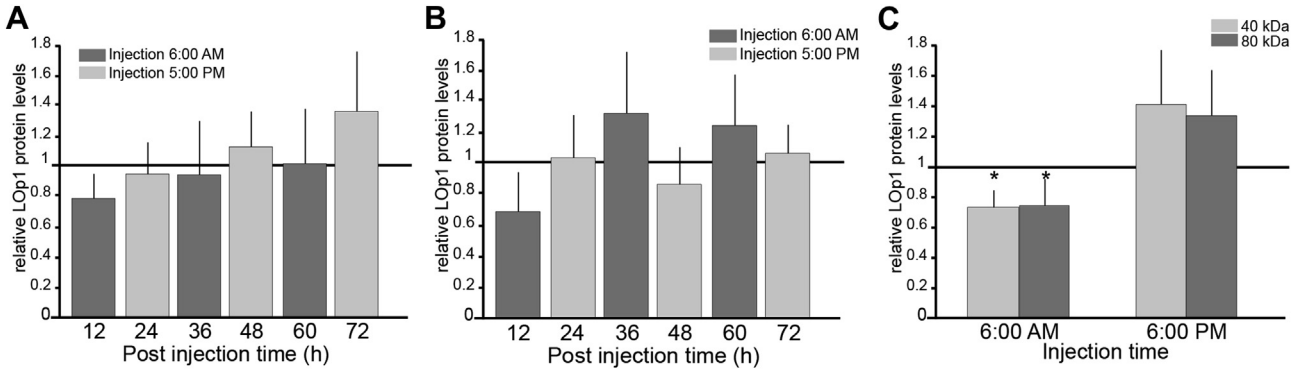

Fig. 6. Evaluation of the RNAi effect at the protein level. Animals were injected with dsLOp1 or dsNEG at 6:00 AM (grey) or 5:00 PM (light grey) and the 40 kDa (A) and 80 kDa (B) doublets were quantified at different post-injection times (3 animals/group were considered at each post-injection time). (C) Animals were injected at 6:00 AM or 6:00 PM with dsLOp1 or dsNEG and the 40 kDa (light grey) and 80 kDa (grey) doublets were quantified 12 h later (10–12 animals/group were injected at 6:00 AM and 6:00 PM, respectively). Each bar represents the mean value of the relative protein levels +/− SE in dsLOp1 injected animals normalised to the levels of dsNEG injected animals. Asterisks indicate a significant reduction ($^*p < 0.05$, Mann–Whitney-*U* test).

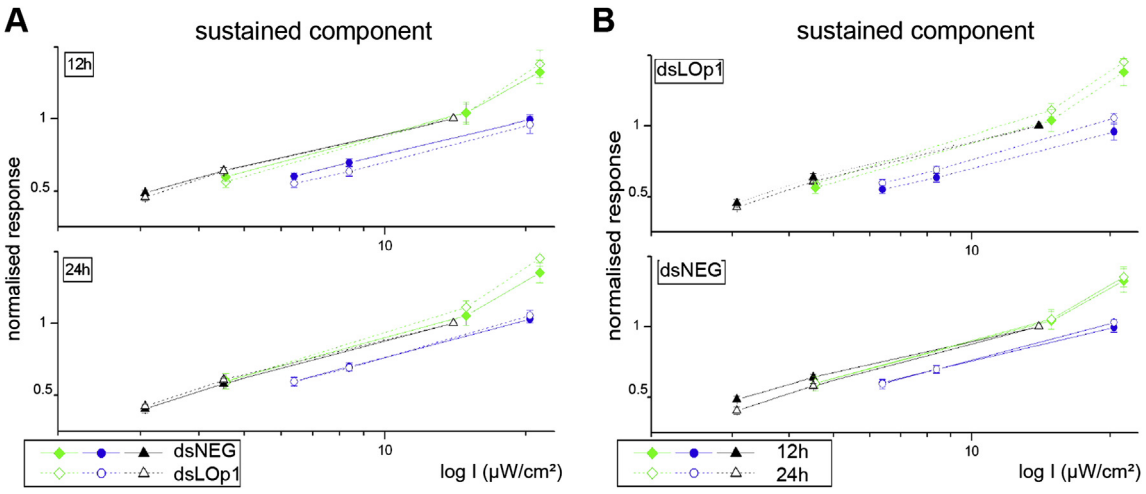

Fig. 7. Evaluation of the RNAi effect on the physiology of the retina by ERG. Normalised response (sustained component) to UV (black lines), blue (blue) and green (green) light of different intensities. (A) Comparison of the responses between dsLOp1 (broken lines, open symbols) and dsNEG (plain lines, filled symbols) injected animals 12 h (dsLOp1, $n = 12$; dsNEG, $n = 12$) and 24 h (dsLOp1, $n = 10$; dsNEG, $n = 9$) after injection. (B) Comparison of the responses 12 h (filled symbols) and 24 h (open symbols) after injection of dsLOp1 (12 h, $n = 12$; 24 h, $n = 10$) and dsNEG (12 h, $n = 12$; 24 h, $n = 9$). Each data point represents the mean value of normalised ERG potentials +/− SE in dsLOp1 and dsNEG injected animals stimulated 12 h or 24 h post-injection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

additional proteins having twice the molecular size of the opsin, in this case ~80 kDa. These are dimers of opsin that are formed directly after the translation (*Drosophila* (Kurada et al., 1998; Kiefer et al., 2002); mice (Wolf et al., 1998)), which can also be glycosylated (Colley et al., 1995). The reduction of this doublet after the injection of dsLOp1 shows that LOp1 also forms doublets in the honeybee.

In many species, the sensitivity of the visual system is regulated by circadian rhythms (insects (Giebultowicz, 2000), crustacean (Katti et al., 2010), vertebrates (Cahill and Besharse, 1995)). Structural changes of the visual system (e.g., size change of rhabdoms, pigment migration, number of synapses, neurotransmitters expression) underlie these changes in sensitivity. In the honeybee, the amplitude of ERGs greatly varies on a daily cycle (Milde, 1985). Daily variations in opsin mRNA regulated by circadian rhythms and triggered by light onset have been described in several species (toad, fish (Korenbrot and Fernald, 1989); mice (von Schantz et al., 1999); *Drosophila* (Claridge-Chang et al., 2001)). Nevertheless much variation exists and the rhythms for the different opsins might differ even within the same organism. For instance, in *Drosophila*, Rh4 and Rh5 opsins are under circadian control but their peaks in mRNA levels occur at different time points that are 4 h apart, whilst Rh1

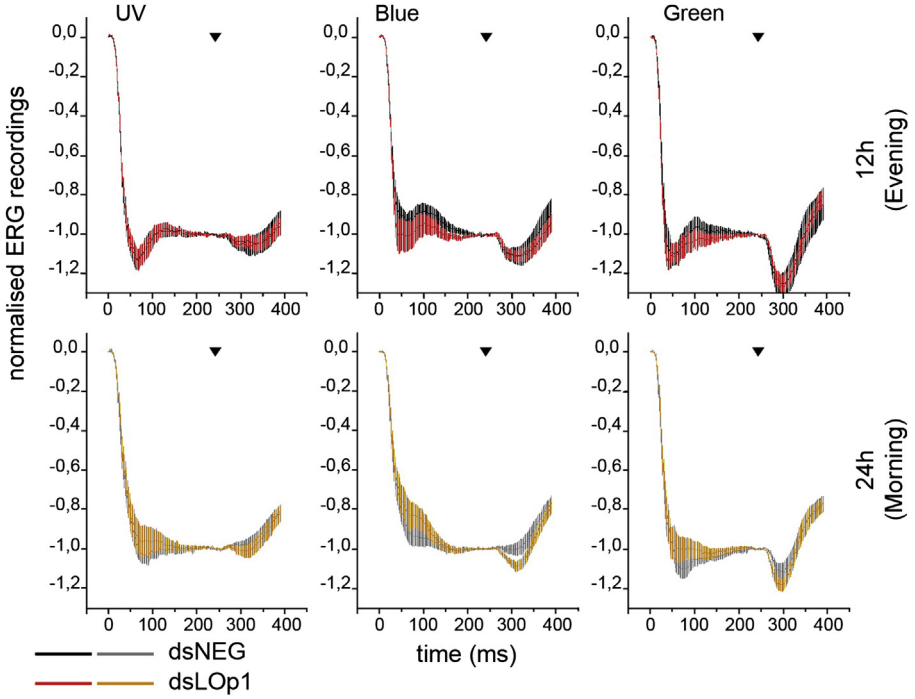

Fig. 8. Comparison of normalised ERG recordings between dsLOp1 and dsNEG treated animals. The normalised ERGs are shown for one stimulation intensity (UV and blue: high, green: middle) 12 h (dsLOp1 ($n = 12$): red, dsNEG ($n = 12$), black) and 24 h (dsLOp1 ($n = 10$): orange, dsNEG ($n = 9$): grey) after the injection. The arrow head indicates the end of the stimulation. Each data point shows the mean value of normalised ERG potentials +/− SE in dsLOp1 and dsNEG injected animals stimulated 12 h or 24 h post-injection.

expression is unchanged over the day (Claridge-Chang et al., 2001). In bees Sasagawa et al. (2003) showed that LOp1 mRNA levels are regulated by a circadian rhythm with a maximum reached 2 h after the beginning of the photoperiod. Here we applied similar photoperiods as in that study and detected high LOp1 protein levels during the day and low levels during the night. The LOp1 protein levels reached a maximum 8 h after the beginning of the photoperiod thus lagging 6 h the mRNA rhythm (Sasagawa et al., 2003). This difference is most likely related to the turn-over of *lop1*. The results strongly suggest that LOp1 translation is also regulated by a circadian rhythm, however further work is required to provide additional evidence for rhythmic daily variations of opsin levels in the bee photoreceptor.

We showed that the inhibitory effect depends on the quantity of injected dsRNA. When the quantification was performed with whole-brain extracts, a single injection of 2.5 μg of dsRNA was not sufficient to reliably induce a reduction whereas a significant reduction of 61% was observed after the injection of 5 μg. The inhibitory effect is transient, a significant reduction of the mRNA was observed 8 h but not 32 h after the injection of 5 μg of dsRNA. The injection in the morning, at the beginning of the photoperiod, led 12 h later to a significant 25% reduction of LOp1 protein levels, which was not present anymore 36 h after injection or later. Such temporal limitation of RNAi effects in the bee brain was also observed in other studies, but with different dynamics (Müßig et al., 2010; Louis et al., 2012; El Hassani et al., 2012). High concentrations of dsRNA and siRNA were also required in studies on the NR1 subunit of the NMDA glutamate receptor (Müßig et al., 2010) and on the glutamate chloride channel (El Hassani et al., 2012). This is in contrast with other studies where 500 pg of dsRNA were sufficient to induce the inhibition, including one case in which the induction of a long-lasting effect was reported (Farooqui et al., 2003, 2004; Mustard et al., 2010; Louis et al., 2012). It might be that the dsRNAs used in these studies are very potent or that local injections in some neuropiles induce higher inhibitory responses. Overall varied outcomes of RNAi studies in insects have been documented (Terenius et al., 2011) and more studies using RNAi in the bee brain are needed to evaluate the differences between studies and injection methods. Also the role that circadian regulation of gene expression plays for the effectiveness of RNAi manipulations needs to be understood further. In the present study the RNAi responses at the protein level were dependent on the injection time (or the dissection time). The injection in the evening, at the beginning of the dark period, had no significant effect on LOp1 levels. It is likely that the circadian rhythm regulating the expression of *lop1* is responsible for this effect. The LOp1 mRNA levels are high in the morning and low in the evening (Sasagawa et al., 2003). Thus it seems that the RNAi response can be triggered efficiently only when the mRNA levels are high or when the levels begin to decline. We further explored whether multiple injections of LOp1 dsRNA would strengthen the inhibitory effect. Two injections of 2.5 μg induced in one case a significant reduction of LOp1 mRNA 8 h after the last injection, but three injections of 2.5 μg did not. These multiple injections were done at the same site. It could well be that the dsRNA did not penetrate efficiently and/or damaged the tissue too much during repeated injections. The comparison of LOp1 mRNA levels between the injected and the non-injected eyes of the same animals suggests that the RNAi effect is restricted to the injected eye; however, the reduction was not significant probably because the sample size was too small. This conclusion is further supported by dye injection that only penetrated to areas close to the injection site and by other brain-RNAi studies. For instance, in the study by Müßig et al. (2010) the RNAi effect was localised and this was also suggested in the study by El Hassani et al. (2012). So far, only the study inhibiting the bee octopamine receptor AmOA1 (Farooqui et al., 2004) reported an inhibitory effect that spread through the whole brain.

A reduction of LOp1 should influence the properties of the retina because it is expressed in 4 or 6 photoreceptors out of 9 that compose the rhabdom of the ommatidium (Menzel and Blakers, 1976; Wakakuwa et al., 2005). But 12 h and 24 h after the injection of dsRNA there was no reduction of the ERG response to the stimulation with the green LED relative to the UV response. No effect was found when the shape of the ERGs recordings were compared between dsLOp1 and dsNEG injected animals for every LED stimulations, and there were no indications for a change of the relative spectral sensitivity as measured for the three wavelengths. Possibly a much stronger inhibition of LOp1 would have modified physiological responses of the retina. Whilst L-receptors have a clear peak in their sensitivity spectrum around 544 nm, they also have a broad tail of low sensitivity that extends across short and middle wavelength ranges in which the S and M-receptors are maximally sensitive (Menzel and Blakers, 1976; Peitsch et al., 1992). Thus L-receptor responses cannot be fully excluded when measuring summed retinal responses to UV and blue light, and in the present ERG recordings this could have masked some small-scaled RNAi effects on the physiology. In addition, the use of age-matched bees would have probably reduced the variation of the different measurements and helped to unmask small RNAi effects. It is also possible that physiological mechanisms in the retina might have compensated for the reduction of LOp1 opsin. For instance, pigment cells that are found in the ommatidia of many insects have a function similar to the pupil in different light conditions. When photoreceptors are illuminated, the pigment cells move in the direction of the rhabdom and regulate the light flow (Kirschfeld and Franceschini, 1969; Menzel and Lange, 1971). A reduction of LOp1 results in a photoreceptor that is less sensitive to light which could have reduced the movement of pigment cells to the rhabdom thus leading to weaker adaptation responses. This hypothesis could be explored further using hand-reared white-eyed mutants in honeybees lacking screening pigments in their eyes (Gribakin and Chesnokova, 1982). Nevertheless, the effectiveness of retinal adaptation mechanisms has been well documented in other visual systems. For instance, it was shown that *Drosophila* and mice mutants with low rhodopsin concentrations did not always have reduced ERG amplitudes (Bentrop et al., 1997; Gorbatyuk et al., 2005, 2007). Future studies will have to address the link between opsin expression and physiological adaptations again. The present method also allows studying whether environmental factors modulating the sensory periphery influence opsin expression. To do this, one could interfere by mean of RNAi with the signalling of the periphery onto sensory neurons in age- and environmental-matched bees and analyse how it influences opsin expression. In conclusion, this study and our current results provide a further stepping stone towards a wide use of RNAi as tool to study neural functions in genetically non-tractable insect model systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.07.006.

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