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Light-dependent activation of G proteins by two isoforms of chicken melanopsins

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Abstract

In the chicken pineal gland, light stimuli trigger signaling pathways mediated by two different subtypes, G_t and G_{11}. These G proteins may be activated by either of the three major pineal opsins, pinopsin, OPN4-1 and OPN4-2, but biochemical evidence for the coupling has been missing except for functional coupling between pinopsin and G_t. Here we investigated relative expression levels and functional difference among the three pineal opsins. In the chicken pineal gland, pinopsin mRNA level was significantly more abundant than the others, of which *OPN4-2* mRNA level was higher than that of *OPN4-1*. In G protein activation assays, G_t was strongly activated by pinopsin in a light-dependent manner, being consistent with previous studies, and weakly activated by OPN4-2. Unexpectedly, illuminated OPN4-2 more efficiently activated G protein(s) that was endogenously expressed in HEK293T cells in culture. On the other hand, G_q, the closest paralogue of G_{11}, was activated only by OPN4-1 although the activity was relatively weak under the condition. These results suggest that the OPN4-1 and OPN4-2 couple with G_q and G_t, respectively.

Two melanopsins, OPN4-1 and OPN4-2, appear to have acquired mutually different functions through the evolution.

Introduction

The pineal gland is an endocrine organ synthesizing and secreting melatonin in a circadian rhythmic manner at a significantly higher level during the night (or in the dark). The pineal gland of non-mammalian vertebrates is intrinsically light-sensitive, representing extra-retinal photoreceptive organs. In particular, the chicken pineal gland has been widely used as a good experimental model because it produces melatonin in a circadian and light-dependent manner in culture. Even in culture, melatonin production of isolated chicken pineal gland is sensitive to light stimuli with two distinct modes; one is acute suppression of melatonin production by light, and the other effect of light is the phase-shift of the melatonin production rhythm. The pioneering pharmacological study showed that pertussis toxin (PTX) blocks the acute suppression of melatonin but not the phase-shift of its rhythm[1], indicating that the two effects are mediated by at least two distinct signaling pathways. Subsequent studies identified transducin (G_t) as PTX-sensitive heterotrimeric G protein[2], whereas G_q-type G protein, G_{11}, as PTX-insensitive G protein expressed in the pineal gland[3]. A combination of genetic and pharmacological manipulation of G_{11} signaling[3] together with earlier studies[1,2] revealed that the acute suppression of melatonin production by light is mediated by activation of G_t and that the activation of G_{11} leads to the phase-shift of the circadian clock regulating melatonin production[3]. These pineal G proteins are thought to be activated by opsin-type photoreceptor molecules such as pinopsin[4,5], iodopsin[4], and two melanopsin paralogues, *i.e.*, OPN4-1 (OPN4X) and OPN4-2 (OPN4M)[6,7,8]. Iodopsin is a photoreceptor of the red-sensitive cones in the chicken retina and activates G_t[9]. On the other hand, the expression level of iodopsin gene (*OPN1LW*) in the pineal gland is quite low relative to that of pinopsin (*OPNP*). Iodopsin is more similar to the other vertebrate "visual" pigments that scarcely photoactivate G_q[10], and therefore it is unlikely that iodopsin couples with G_{11}. Pinopsin, a non-visual opsin, is specifically expressed in the pineal gland and activates G_t in a light-dependent manner[11,12], while it is not known whether pinopsin activates G_{11}. A sequence similarity of the two members of vertebrate melanopsin (OPN4-1 and OPN4-2) to G_q-coupled invertebrate visual pigments suggests that the two melanopsins couple with the G_{11} in the chicken pineal gland. This idea may be supported by a previous report that melanopsin of a cephalochordate, one of the closest species to vertebrates, activates G_q in a light-dependent manner[13], although activation of G_t by mammalian melanopsin has been also reported[14]. In the present study, we compared the G protein activation abilities of major pineal opsins, and demonstrated diversity in the G protein coupling among the pineal opsins.

Experimental

Preparation of mRNA from the chicken pineal gland

Animal experiments were conducted in accordance with guidelines of The University of Tokyo. Newly hatched male chicks (*Gallus gallus*) were purchased from a local supplier and maintained in a 12-hour light/12-hour dark (LD) cycles in the compartments that were kept at 28 ± 0.5°C with a white light provided by fluorescent lamps (≈300 lux at the level of the head of chicks). At the mid-time of the light or the dark period on the fifth day, the chicks were decapitated to isolate the pineal glands. Total RNA was extracted from the pineal glands with TRIzol reagent (Invitrogen) and treated with DNase I. Equal amounts of the total RNA from the pineal glands collected during the light and the dark period were mixed with each other in order to average daily variations of the mRNA levels, and then reverse transcribed with ThermoScript reverse transcriptase (Invitrogen) and with gene specific primers, 5'-AGCAA TAACA GCTGG CACAG-3' (for *OPN4-1*), 5'-TGGCA GAAGC TTTGG

CAATC-3' (for *OPN4-2*), and 5'-TAGCT GTTGT TGTTG CTGCC-3' (for *OPNP*), followed by treatment with RNase H. The cDNA mixtures were stored at −80°C until use.

Quantification of mRNA expression level by quantitative RT-PCR analysis

Quantitative PCR was carried out using the GeneAmp 5700 Sequence Detection System (Applied Biosystems), by which the amount of the PCR product was monitored through progression of PCR cycles by the fluorescence intensity of SYBR Green I intercalated in the double-stranded DNA. Three kinds of plasmid vectors harboring the opsin genes, *OPN4-1*, *OPN4-2* and *OPNP*, were used for the standard templates and their concentrations were estimated by the absorbance at 260 nm. A series of dilutions over three orders of magnitude were prepared for these standard cDNA plasmids, and they were subjected to the real-time PCR using the gene specific primer pairs, 5'-GCGTT TGTGG TCATC ATTGT G-3' and 5'-ATACG GCGTT AGGGT GTTTC-3' for *OPN4-1*; 5'-TGCAC TGATC GTCAT CTTGC-3' and 5'-GAGAA TACCC AGCAA AAGC-3' for *OPN4-2*; and 5'-GGAGA TTTCC AGTTC CAACG-3' and 5'-TCAAC CCTTC AGGCA CGTAG-3' for *OPNP.* The PCR reaction was initiated by heat-activation of AmpliTaq Gold DNA polymerase with 95°C for 9 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 1 min. The reaction mixture (25 µL) was composed of 0.625 units of AmpliTaq Gold (Applied Biosystems), 1× SYBR Green PCR buffer (Applied Biosystems), 3 mM $MgCl_2$, 0.3 mM each of dNTP, 50 nM each of primers. The standard regression line was obtained for each opsin gene from the negative linear correlation between logarithmic values of the initial DNA concentrations and the threshold cycles, each of which was defined as the cycle at which the fluorescence intensity exceeds a threshold. For all of the three genes, values of the coefficient of determination (r^2) were between 0.99 and 1.0.

For accurate comparison of mRNA abundance among different genes, the gene-to-gene variation in the reverse transcription (RT) efficiencies was evaluated according to a literature[15]. The sense-strand RNA was transcribed *in vitro* from the corresponding cDNA cloned in the pBluescript plasmids. The reaction was carried out using 1 µg of linearized plasmid, T7 RNA polymerase (Roche), 1× Transcription buffer (Roche), 1 mM each of rNTP in total volume of 40 µL at 37°C for 90 min. Then, the template plasmid was digested by incubating with DNase I (TaKaRa) at 37°C for 30 min, and the reaction was terminated by adding EDTA to a final concentration of 208 mM and holding the mixture at 80°C for 10 min. The transcribed RNA was purified by the aid of RNeasy MinElute Cleanup Kit (Qiagen). The concentration of the purified RNA was determined from the absorbance at 260 nm and the size of the RNA was confirmed by denaturing agarose gel electrophoresis followed by the staining with SYBR Green II (Cambrex). Then 10 fmol (≈4 ng) each of the RNAs thus synthesized from the opsin plasmids was mixed together and was reverse transcribed using their gene specific primer as described for those of the pineal RNA. The resulting solution was subjected to the real-time PCR in the condition described above. The initial DNA amount in the PCR solution was estimated by using the standard regression line for each opsin gene and was defined as the RT-efficiency for each opsin gene.

Functional expression of melanopsins and membrane preparation

We previously reported that each of chicken OPN4-1 and OPN4-2 has two splicing variants, S (short) and L (long) isoforms, with diverged C-terminal tails[8]. In this study, OPN4-1S and OPN4-2L isoforms were used for OPN4-1 and OPN4-2, respectively. Each of the coding regions of OPN4-1, OPN4-2, and pinopsin was modified so as to tag the protein with an 8-amino acid epitope sequence (ETSQVAPA) of anti-rhodopsin monoclonal antibody 1D4[16] at the C-terminus, and was subcloned into a mammalian expression vector, pUSRα[17]. These opsins were heterologously expressed in HEK293T cells and reconstituted with 11-*cis*-retinal as described previously[8]. The membrane fraction of the cells was isolated by centrifugation flotation in a stepwise sucrose gradient according to the method described in the literature[18].

Purification of G_t and G_q

G_t was extracted and purified as the trimeric form from the membrane of bovine rod outer segment with a hypotonic buffer plus GTP according to the literature[19,20]. G_q subunits ($G\alpha_q$, $G\beta_1$ and $G\gamma_2$) were produced in the baculovirus-infected *Sf9* cells and purified as previously described[21,22].

G protein activation assay

G protein activation assay (GTPγS binding assay) was carried out by measuring the amount of guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) bound to the α-subunit. Membrane preparations of the recombinant photopigment were prepared as described above. A small portion of the membrane sample was extracted in 1% (w/v) *n*-dodecyl-β-D-maltoside and spectrophotometrically analyzed to estimate the pigment content in the membrane sample according to the literature[8], while the rest was used for the GTPγS binding assay as follows. To adjust the pigment content and the total membrane proteins, the membrane fraction of the cells expressing either of the opsins was mixed with that of the cell transfected with the pUSRα empty vector. The membrane sample thus prepared for OPN4-1, OPN4-2 or pinopsin was mixed with GTPγS and GDP, and then irradiated with blue (450 nm for OPN4-1 and OPN4-2) or yellow light (>480 nm for pinopsin) for 1 min (*Light*) or kept in the dark (*Dark*) at 4°C. The *Light* or *Dark* sample was then immediately mixed (in the dark) with a solution of bovine G_t or mouse G_q. The buffer composition of the opsin–G protein reaction mixture was 10 mM of MOPS-NaOH (pH 7.5 at 4°C), 30 mM of NaCl, 60 mM of KCl, 2 mM $MgCl_2$, 0.195 mM of $CaCl_2$, 0.2 mM of EGTA, 1 mM of DTT, 4 μg/mL of aprotinin, 4 μg/mL of leupeptin, 0.5 μM of [^{35}S]GTPγS, 25 μM of GDP, 0.07% CHAPS, and 0.2 μM of melanopsin or pinopsin, 1.2 μg/μL of membrane protein, 2.2 μg/μL of ovalbumin, and 0.1 μM of G protein. After the reaction mixture was incubated for the selected time in the dark at 4°C, its aliquot (10 μL) was mixed with 100 μL of stop solution (20 mM Tris-HCl, 100 mM NaCl, 25 mM $MgCl_2$, and 5 μM GTPγS, pH 7.4) and was immediately filtered through the nitrocellulose membrane to trap [^{35}S]GTPγS bound to G proteins. The nitrocellulose membrane was then washed four times with 200 μL of washing buffer (20 mM Tris-HCl, 100 mM NaCl, and 25 mM $MgCl_2$, pH 7.4) to eliminate free [^{35}S]GTPγS, put into 0.9 mL of scintillator (ACS II, GE Healthcare) and subjected to quantification by a liquid scintillation counter.

Statistical analysis of the G protein activation assay

To find out light-dependent and G protein-dependent incorporation of GTPγS, we first conducted a multiple comparison test among the four conditions ("*Light, G+*", "*Light, G−*", "*Dark, G+*", "*Dark, G−"*) within each time point of 2 and 4 min because a full set of the data for all the four conditions were available only at these two time points. This test compares all possible pairs of means for the four conditions. Significant differences between "*Light, G+*" and "*Light, G−*" are described in the figures 2 and 3. Then, to further analyze by taking into account the both time points, we conducted a repeated measures ANOVA with the three factors (G protein addition, illumination, and reaction time) for the data sets from the time points of 2 and 4 min. The significant interaction among all the three factors is found only in the combination of pinopsin and G_t, and hence the other combinations were subjected to the Tukey's multiple comparison tests after omitting the time effect. All of the results were summarized in supplementary table1.

Results

The opsin genes dominantly expressed in the chicken pineal gland are pinopsin (*OPNP*) and two melanopsins, *OPN4-1* and *OPN4-2*. To investigate the relative abundance of pinopsin and melanopsins in the pineal gland, the mRNA levels were compared by quantitative RT-PCR analysis (Figure 1). The cDNA abundance shown in Figure 1A does not necessarily reflect their accurate mRNA levels, because the efficiency of the reverse transcription (RT-efficiency) may vary among the opsin genes due to differences in the oligonucleotide primers and/or the mRNA sequences in the RT reactions[15]. Indeed, the RT-efficiencies differed as much as 2.5-fold among the three opsin genes (Figure 1B). Hence, the pineal cDNA levels (Figure 1A) were normalized with the RT-efficiencies (Figure 1B) to estimate the relative mRNA levels in the pineal gland (Figure 1C). The mRNA level of *OPNP* was 24.4-fold higher than *OPN4-1* and 9.3-fold higher than *OPN4-2*. Thus, *OPNP* mRNA is the most abundant opsin mRNA in the chicken pineal gland, while *OPN4-2* mRNA level is higher than *OPN4-1*. In this study, we focus our attention to these three opsins, pinopsin, OPN4-1, and OPN4-2.

As described above, light stimuli trigger two signaling pathways mediated by two different G proteins, G_t and G_{11}. To examine which combination of the opsin and the G protein functions in a light-dependent manner, we conducted GTPγS-binding assays by using opsin-containing membranes and the purified G proteins at 4°C. When G_t was mixed with pinopsin-containing membrane (abbreviated as pinopsin membrane), GTPγS incorporation to G_t was considerably accelerated by light illumination (Figure 2A), being consistent with the previous studies[11,12]. Light illumination of OPN4-1 membrane caused no significant effect on GTPγS incorporation to G_t (Figure 2B, $p > 0.05$ by Tukey's multiple comparison test among the four group, "*Light,* G_t+", "*Light,* G_t−", "*Dark,* G_t+", "*Dark,* G_t−", within the each time point of 2 min and 4 min). Consistently, when the 2 min and 4 min time points were both taken into consideration, a statistical significantly difference ($p < 0.05$) was detected only between “*Light,* G_t+” and “*Dark,* G_t+” but not between “*Light,* G_t+” and “*Light,* G_t–” (Supplementary table1). Hence, it is unlikely that the light-dependent GTPγS incorporation to OPN4-1 membrane under the “G_t+” condition is derived from the exogenously supplied G_t.

In OPN4-2 membrane, GTPγS incorporation was accelerated by light even in the absence of G_t (Figure 2C), indicating that OPN4-2 photoactivated the G protein(s) that is endogenously expressed in the HEK293T. The addition of G_t to OPN4-2 membrane further increased the light-dependent GTPγS incorporation, indicating light-dependent G_t activation by OPN4-2. Although the G_t-activation by OPN4-2 (Figure 2C) was much lower than that by pinopsin serving as a positive control (see also Figure 2E, where one tenth concentration of pinopsin was used for the assay), this activation was specific for photoactivated OPN4-2 because no activation was observed in the absence of any opsins (Figure 2D). Taken into consideration of the highest *OPNP* expression level in the pineal gland (Figure 1C), it is likely that G_t activation by light stimulation in the pineal gland is mainly triggered by pinopsin rather than by OPN4-2 (see discussion).

Activation of $G_{q/11}$ subtype was estimated by GTPγS binding assays with G_q. Among the three opsins, only OPN4-1 significantly activated G_q in a light-dependent manner (Figure 3B) although the activation was not so strong. In contrast, OPN4-2 showed no significant activation ability on G_q (Figure 3C), while GTPγS incorporation in OPN4-2 membrane was stimulated by light regardless of the G_q-addition in a manner similar to the G_t assay (Figure 2C). The lack in G_q activation by OPN4-2 is not due to a rapid decay of the light-activated OPN4-2 intermediate nor due to saturation of OPN4-2 activity by the G protein(s) endogenously present in the HEK293T membranes, because the light-dependent G_t activation by OPN4-2 was detected in a similar condition (Figure 2C). Addition of G_q to the photoactivatable pinopsin appeared to slightly reduce the GTPγS incorporation (Figure 3B, *Light,* G_q+, open circles), though it had no statistical significance to any of the others ($p >$ 0.05 by Tukey's multiple comparison test). Taken together, only OPN4-1 activates G_q in a light-dependent manner among the three opsins tested in this study.

Discussion

In the present study, three kinds of opsins expressed abundantly in the chicken pineal gland were compared with each other in terms of their mRNA expression levels and G protein activation abilities (as summarized in Table 1). The quantitative analysis revealed pinopsin as the most abundantly expressed opsin (mRNA level) in the chicken pineal gland (Figure 1, Table 1). It is likely that the PTX treatment-sensitive acute suppression of melatonin production is dominantly mediated by pinopsin–G_t coupling. The weak photoactivation of G_t by the less abundant pineal opsin, OPN4-2, suggests its minimal contribution to the G_t signaling. In contrast to G_t, light-dependent activation of G_q was detected only when mixed with OPN4-1 membrane (Figure 3B). Although the finding of G_q activation by chicken OPN4-1 is consistent with previous reports that mammalian and cephalochordate melanopsins can activate G_q signaling[13,31], the present study first demonstrated that a chicken pineal photopigment can activate a G protein closely related to G_{11}.

In the rhodopsin family[23], melanopsins are most closely related to the invertebrate rhodopsins, whose photoactive intermediates are stable and revert to the original state upon subsequent illumination. Owing to this property, called "bistability", invertebrate rhodopsins keep sensitivity to light without any supply of the chromophore, 11-*cis*-retinal, and hence, are tolerant to bleaching under long-sustained bright conditions. If chicken

OPN4-2 has the bistable property, like melanopsin homolog in amphioxus[24] and mice[25], OPN4-2 may serve as an integrative sensor that mediates long-sustained light signals.

Generally, the processing of light information for entrainment of circadian clock differs from that for visual systems at the following two points[26]. First, the threshold of the light sensitivity for the phase-shifting response of the circadian clock is significantly higher than that of the visual response. Second, the circadian system is insensitive to light stimuli with a short duration but integrates light information over long periods of time (minutes to hours), whereas integration times for visual responses are in the order of subseconds. These signaling properties predict that an opsin(s) with integrative character regulates the circadian clock. If OPN4-1 is a bistable photopigment, this prediction is consistent with the current result that OPN4-1 activates the G_q/G_{11} pathway (Figure 3B), which leads to regulation of the circadian clock.

In the present study, we found that the two chicken melanopsins activate different G proteins, $G_{q/11}$ and G_t, although the activation levels of G_q (by OPN4-1) and G_t (by OPN4-2) appear relatively low (Figure 3B and 2C, respectively). The low levels of G protein activation may be due to, for example, use of bovine G_t and recombinant G_q instead of chicken pineal endogenous G proteins, owing to technical difficulties of preparation of the latter samples. On the other hand, OPN4-1 and OPN4-2 exhibited a striking difference in light-dependent activation of the G protein(s) endogenous to HEK293 cells (*cf.* Figure 2B for OPN4-1 and Figure 3C for OPN4-2). Although this difference in G protein activation may not reflect the one occurring in chicken pineal gland under physiological conditions, it clearly indicates the difference in G protein selectivity between OPN4-1 and OPN4-2 (Table 1). The two chicken melanopsin genes, *OPN4-1* and *OPN4-2*, are paralogous to each other. Phylogenetic analyses of the vertebrate melanopsin genes clearly showed that these paralogues originate from a gene duplication having occurred in the ancestral vertebrate[7,8].

These two lineages of melanopsin genes both encode photopigment with absorption maxima at the blue region[8,25,27]. Although the two melanopsin lineages of zebrafish were reported to be expressed in different cell types of the retina and to have differences in tolerance to bleaching[27], the present study first described a difference in signaling properties of melanopsins. This suggests the two subtypes of melanopsins have acquired diversified G protein-signaling pathways through the evolution of early vertebrates, providing an example for functional differentiation of paralogous opsin genes.

OPN4-2, the chicken orthologue of mammalian *Opn4,* was unable to activate G_q (Figure 3C, Table 1). This result is apparently inconsistent with the accumulating evidence that mammalian melanopsin mediates light information through G_q signaling. The evidence includes abolishment of photoresponse in the isolated melanopsin-positive cells by genetic elimination of PLCβ4[28], a typical effecter of G_q, and by pharmacological inhibition of PLC[29]. We should emphasize, however, that no direct evidence has been presented for the coupling of G_q-type G proteins and mammalian-type melanopsins. In contrast, previous studies showed that G_t is activated by mouse melanopsin[14] and that knock-out of G_q-type G protein genes does not abolish melanopsin phototransduction in mice[30]. OPN4-2 lineage of (or mammalian type of) melanopsins may couple with unidentified G protein(s) other than G_q type.

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Figure Legends

Figure 1: Relative mRNA expression levels of *OPNP*, *OPN4-1* and *OPN4-2* in the pineal gland. (**A**) The cDNA amounts were quantified by real-time PCR. The cDNA was reverse transcribed rom the total RNA sample extracted from the chick pineal gland. (**B**) The RT-efficiency was evaluated by using the *in vitro* synthesized RNA pool, which is a mixture of equal mole of each opsin RNA. (**C**) The mRNA level in the pineal gland for each opsin gene was estimated by dividing the cDNA amount (**A**) with the RT-efficiency (**B**) and by subsequent normalization by the level of *OPNP* as 1. *OPNP*, 1.00±0.32; *OPN4-1*, 0.041±0.0073; *OPN4-2*, 0.11±0.017. *n=4*, mean ± sem. * $p<0.05$ by Tukey's post hoc test. Although the multiple comparison test showed no significant difference between *OPN4-1* and *OPN4-2*, there is a significant difference when focused on the two group, *OPN4-1* and *OPN4-2* ($p=0.011$ by Student's *t*-test).

Figure 2: G_t activation by pinopsin (**A**, **E**), OPN4-1 (**B**, **F**), or OPN4-2 (**C**, **G**) was examined by using the opsin-containing membrane fraction. (**A–H**) After irradiation with yellow light (> 480 nm for pinopsin, shown as orange in the graphs) or blue light (450 nm for OPN4-1 and OPN4-2, shown as blue in the graphs), the opsin-containing membrane was mixed with a purified G_t solution at time 0 (min). Open and closed symbols indicate the data from the irradiated (open) and non-irradiated (closed) samples. Circles with solid curves indicate the data with G_t, whereas squares with dotted curves indicate those without G_t. Each series of the data was fit to a single exponential curve. (**A–C**) The reaction was conducted in the presence of 200 nM of photopigment and 1.2 μg/μL of membrane proteins. (**E–G**) A similar assay was conducted with the 10-fold diluted membrane, which contains 20 nM of photopigment and 0.12 μg/μL of membrane proteins. (**D**, **H**) Negative control experiments with the membrane from the cells transfected with the empty vector for panel A–C and E–G, respectively. *n*=3, mean ± sem. Asterisks show statistical significance, $p < 0.05$ (*) and $p <$

0.01 (**), between “*Light,* G_t+” and “*Light,* G_t−” by a Tukey’s multiple comparison test among the four experimental conditions (“*Light,* G_t+”, “*Light,* G_t−”, “*Dark,* G_t+”, “*Dark,* G_t−”) within each time point of 2 and 4 min. All the results of the test were shown in the supplementary table1. Note that the data of all the four conditions were completely obtained only at the time points of 2 and 4 min.

Figure 3: G_q activation by pinopsin (**A**), OPN4-1 (**B**), or OPN4-2 (**C**) was examined by using the opsin-containing membrane and the purified G_q solution with the procedures similar to the figure 2A–D. Open and closed symbols show the data from the irradiated (open) or non-irradiated (closed) samples. Circles with solid curves indicate the data with G_q, whereas squares with dotted curves indicate those without G_q. Each series of the data was fit to a single exponential curve. The reaction was conducted in the presence of 200 nM of photopigment and 1.2 µg/µL of membrane proteins. *n=3*, mean ± sem. Asterisks shows statistical significance ($p < 0.05$) between “*Light,* G_t+” and “*Light,* G_t−” by a Tukey’s multiple comparison test among the four experimental conditions within each time point of 2 and 4 min. All the results of the test were shown in the supplementary table 1. Note that the data of all the four conditions were completely obtained only at the time points of 2 and 4 min.

Table 1: Relative expression levels and G protein activation abilities of the chicken pineal opsins.

	Pinopsin	Melanopsin	
	OPNP	*OPN4-1*	*OPN4-2*
Relative mRNA levels			
	100 ± 32	4.1 ± 0.73	11 ± 1.7
G protein activation abilities			
G_q	−	+	−
G_t	++	−	+
Endo. G*	−	−	+

* Endogenous G protein of HEK293T cells (subtype unidentified)

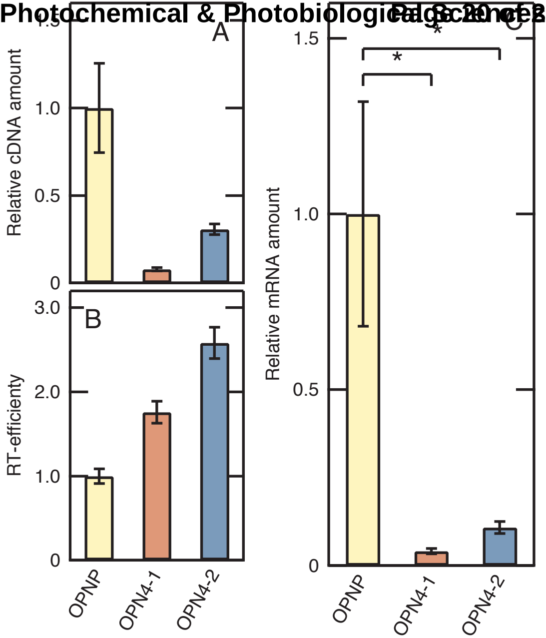
A
Relative cDNA amount
1.5
1.0
0.5
0
B
RT-efficienty
3.0
2.0
1.0
0
OPNP
OPN4-1
OPN4-2
C
*
*
Relative mRNA amount
1.5
1.0
0.5
0
OPNP
OPN4-1
OPN4-2

Figure 1

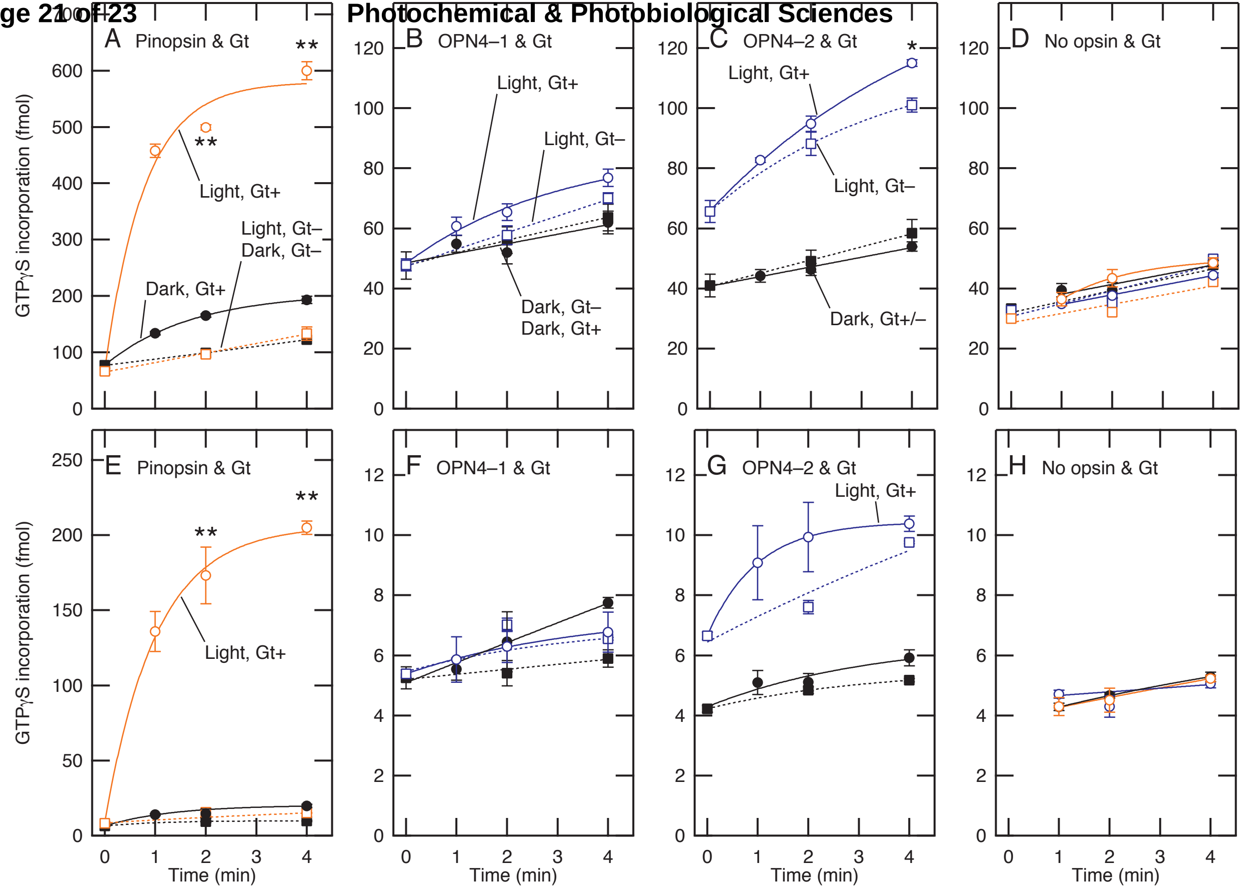
A Pinopsin & Gt
B OPN4–1 & Gt
C OPN4–2 & Gt
D No opsin & Gt
E Pinopsin & Gt
F OPN4–1 & Gt
G OPN4–2 & Gt
H No opsin & Gt
GTPγS incorporation (fmol)
Time (min)
0
1
2
3
4
0
100
200
300
400
500
600
700
0
20
40
60
80
100
120
0
50
100
150
200
250
0
2
4
6
8
10
12
**
*
Light, Gt+
Light, Gt–
Dark, Gt–
Dark, Gt+
Dark, Gt+/–

Figure 2

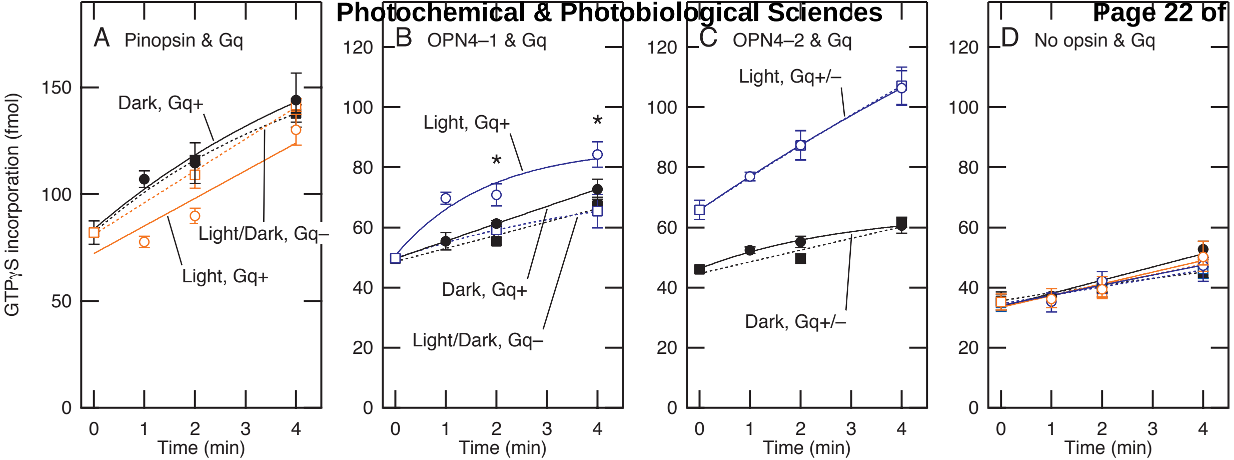
A Pinopsin & Gq
Dark, Gq+
Light/Dark, Gq–
Light, Gq+
B OPN4–1 & Gq
Light, Gq+
*
*
Dark, Gq+
Light/Dark, Gq–
C OPN4–2 & Gq
Light, Gq+/–
Dark, Gq+/–
D No opsin & Gq
GTPγS incorporation (fmol)
0
50
100
150
0
20
40
60
80
100
120
0
1
2
3
4
Time (min)

Figure 3

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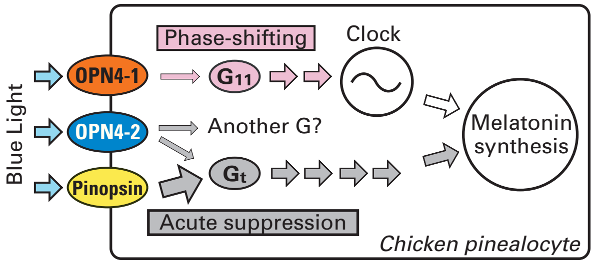

A comparative study of non-visual opsins expressed in the chicken pineal glands showed melanopsins and pinopsin photoactivate mutually different G protein pathways, which regulate melatonin production.