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Article

G Protein-Coupled Receptor Kinase 2 Is Required for Rhythmic Olfactory Responses in *Drosophila*

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Summary

Background: The *Drosophila* circadian clock controls rhythms in the amplitude of odor-induced electrophysiological responses that peak during the middle of night. These rhythms are dependent on clocks in olfactory sensory neurons (OSNs), suggesting that odorant receptors (ORs) or OR-dependent processes are under clock control. Because responses to odors are initiated by heteromeric OR complexes that form odor-gated and cyclic-nucleotide-activated cation channels, we tested whether regulators of ORs were under circadian-clock control.
Results: The levels of *G protein-coupled receptor kinase 2* (*Gprk2*) messenger RNA and protein cycle in a circadian-clock-dependent manner with a peak around the middle of the night in antennae. *Gprk2* overexpression in OSNs from wild-type or cyc^{01} flies elicits constant high-amplitude electroantennogram (EAG) responses to ethyl acetate, whereas *Gprk2* mutants produce constant low-amplitude EAG responses. ORs accumulate to high levels in the dendrites of OSNs around the middle of the night, and this dendritic localization of ORs is enhanced by GPRK2 overexpression at times when ORs are primarily localized in the cell body.
Conclusions: These results support a model in which circadian-clock-dependent rhythms in GPRK2 abundance control the rhythmic accumulation of ORs in OSN dendrites, which in turn control rhythms in olfactory responses. The enhancement of OR function by GPRK2 contrasts with the traditional role of GPRKs in desensitizing activated receptors and suggests that GPRK2 functions through a fundamentally different mechanism to modulate OR activity.

Introduction

Many sensory systems are regulated by the circadian clock. Various insects including flies, moths, and cockroaches show circadian rhythms in odor-dependent electrophysiological and behavioral responses [1–5]. In mammals, the firing rate of isolated mouse olfactory bulb neurons is regulated by the circadian clock [6, 7], as are odor-evoked brain activity waves (e.g., event-related potentials [ERPs]) in humans [8]. Daily rhythms in neuronal activity or sensitivity have been reported for other sensory systems, such as the visual and auditory systems [9–12].

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We previously reported that the circadian clock modulates olfactory responses in *Drosophila*: Robust electroantennogram (EAG) responses are seen during the middle of the night, and weak EAG responses are seen during the middle of the day [2]. These rhythms in EAG responses are controlled by the olfactory sensory neurons (OSNs) in *Drosophila*, which act as independent peripheral circadian oscillators [13]. Colocalization of the circadian oscillator and a rhythmic output to the OSNs indicates that the abundance and/or activity of odorant receptors (ORs) and/or OR-dependent processes are under clock control. *Drosophila* ORs are seven-transmembrane-domain proteins that share some structural similarities with G protein-coupled receptors (GPCRs) [14]. However, recent studies demonstrate that *Drosophila* ORs have an inverted membrane topology compared to canonical GPCRs [15, 16] and function as odor-gated and cyclic-nucleotide-activated cation channels [17, 18]. To understand how the clock modulates odor-dependent responses, we determined whether factors that modulate ORs were regulated by the circadian clock.

G protein-coupled receptor kinases (GPRKs) and arrestins act to terminate GPCR signaling in mammals, thereby protecting cells from receptor overstimulation. GPRK-phosophorylated GPCRs are internalized by arrestin and subsequently degraded or recycled [19]. Two *Gprk* genes, *Gprk1* and *Gprk2*, have been reported in *Drosophila* [20]. *Gprk1* messenger RNA (mRNA) is enriched in photoreceptor cells, and expression of a *Gprk1* dominant-negative mutant in photoreceptors increases the amplitude of electroretinogram (ERG) responses [21]. *Gprk2* is required for egg and wing morphogenesis, as well as embryogenesis in *Drosophila* [22, 23]. In mammals, seven *Gprk* genes are divided into three subfamilies on the basis of sequence homology: the rhodopsin kinase or visual *Gprk* subfamily (*Gprk1* and *Gprk7*), the β-adrenergic receptor kinase subfamily (*Gprk2* and *Gprk3*), and the *Gprk4* subfamily (*Gprk4*, *Gprk5*, and *Gprk6*) [24]. *Gprk3* knockout mice are unable to mediate odor-induced desensitization of odorant receptors [25]. In contrast, loss of *Gprk2* function in *C. elegans* olfactory sensory neurons results in reduced chemosensory behavior, suggesting that *Gprk2* is necessary for GPCR signaling [26]. These results suggest that GPRKs play different roles in vertebrate and invertebrate olfaction.

Here, we report that *Gprk2* expression is regulated by circadian clocks in antennae and that GPRK2 drives circadian rhythms in olfactory responses by enhancing OR accumulation in the dendrites of basiconic sensilla. *Gprk2* mRNA and protein expression levels were high around the middle of the night, coincident with the peak of olfactory responses. Flies that overexpress *Gprk2* in OSNs show constant high EAG responses to ethyl acetate during 12 hr light:12 hr dark (LD) cycles and accumulate high levels of ORs in OSN dendrites, whereas hypomorphic Gprk2 mutants show constant low EAG responses to ethyl acetate during LD. On the basis of these results, we propose that GPRK2 mediates cycles of OR accumulation in OSN dendrites to generate rhythms in EAG responses.

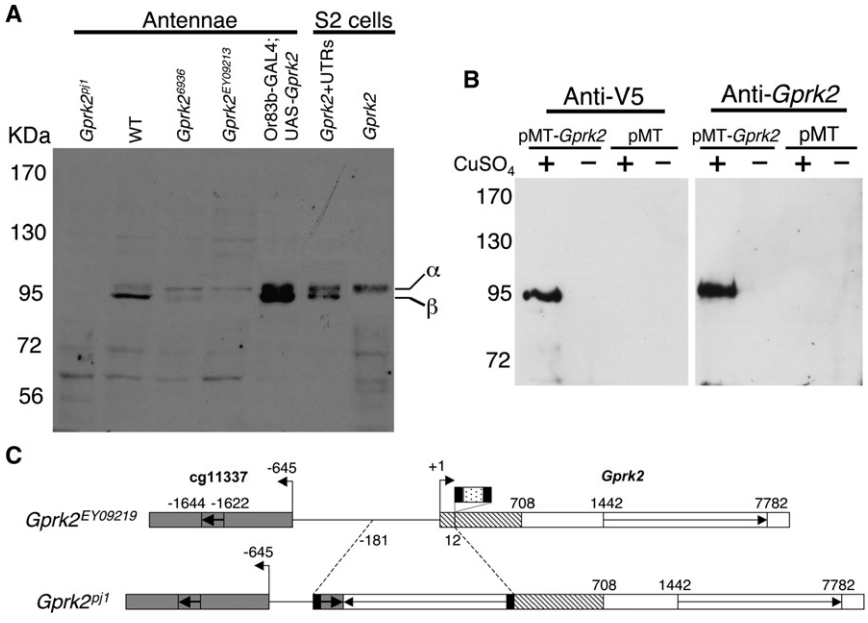

Figure 1. Identity and Expression of *Drosophila* GPRK2

(A) GPRK2 expression in antennae from wild-type (WT), *Gprk2* mutant ($Gprk2^{6936}$, $Gprk2^{EY09213}$, and $Gprk^{pj1}$), and GPRK2 overexpression (*Or83b*-Gal4;UAS-*Gprk2*) flies or in S2 cells transfected with plasmids containing actin promoter-driven *Gprk2* ORF (pAc-*Gprk2*) or actin promoter-driven *Gprk2* ORF + 5′ and 3′ UTRs (pAc-*Gprk2*+UTRs). GPRK2 runs as an α and β isoform in wild-type antennae. Molecular-weight markers, in kilodaltons (KDa), are on the left. GPRK2 isoforms migrate at an apparent molecular weight of 95 KDa.

(B) GPRK2 expression in S2 cells transfected with plasmids containing metallothionine promoter-driven V5-epitope-tagged GPRK2 (pMT-*Gprk2*) or metallothionine promotor alone (pMT) under induced (+) or noninduced (−) conditions, and probed with V5 antibody (Anti-V5) or GPRK2 antibody (Anti-GPRK2).

(C) Diagram of the *Gprk2* promoter region in $Gprk2^{EY09219}$ and $Gprk2^{pj1}$ mutants. The following are indicated: *Gprk2* exon 1, hatched box; nonduplicated portion of *Gprk2* intron 1, white boxes; duplicated portion of *Gprk2* intron 1, white box with arrow; *P* element inverted repeats, black boxes; cg11337 transcribed sequence, gray box; duplicated sequence in cg11337, gray box with arrow; start of transcription, bent arrows; intergenic region, thin line; insertion site of *P* element, gray line; region deleted in $Gprk2^{pj1}$, dashed lines; internal *P* element sequences, spotted box; and distances relative to the *Gprk2* transcription start, numbers.

Results

Drosophila GPRK2 Is Most Similar to GPRK4 from Mammals

We compared *Drosophila* GPRK2 to members of the three mammalian GPRK subfamilies in humans and found the highest amino acid identity in the kinase catalytic domain, with lower levels of similarity in the flanking N-terminal and C-terminal regions. GPRK2 was most similar to human GPRK4, with 87% identity in the kinase domain and 45% and 47% identity in the N- and C-terminal regions, respectively. GPRK2 displays substantially less sequence identity to mammalian GPRK3, which is necessary to desensitize odorant receptors [25]. The N-terminal region of GPRK2 includes a unique stretch of amino acids (Gly124–Gly261, 138 amino acids) containing asparagine-rich and glycine-rich clusters (Figure S1 available online). This unique amino acid region is not present in other GPRK subfamilies and is not homologous to sequences in other proteins, on the basis of Basic Local Alignment and Search Tool (BLAST) searches. These data suggest that GPRK2 is a member of the GPRK4 family from mammals but may have additional functions compared to other GPRK4 family members.

Two GPRK2 Isoforms Are Expressed in Olfactory Sensory Neurons

We used a newly generated *Drosophila* GPRK2 antibody to demonstrate that GPRK2 protein is expressed in antennae (Figures 1A, 2A, and 2B). GPRK2 mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is 95 KDa in antennae (Figure 1A), though the estimated molecular weight of the 714 aa GPRK2 open reading frame is approximately 80 KDa. So it could be determined whether these approximately 95 KDa bands corresponded to GPRK2, a V5-epitope-tagged GPRK2 was expressed in S2 cells and probed with V5 antibody and GPRK2 antibodies. Both V5 and GPRK2 antibodies detected the same approximately 95 KDa bands (Figure 1B), thereby demonstrating that the 95 KDa bands correspond to GPRK2.

Two GPRK2 isoforms are detected in antennae, a higher molecular weight α isoform and a lower molecular weight β isoform (Figure 1A). The *Gprk2* gene is predicted to generate a single mRNA [27], suggesting that the two GPRK2 isoforms are

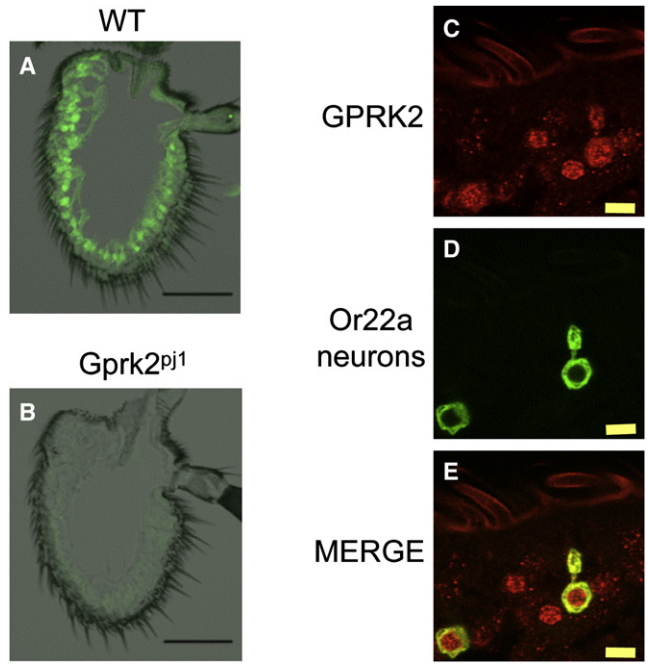

Figure 2. GPRK2 Is Expressed in OSNs

GPRK2 immunostaining in antennae from wild-type (A) and $Gprk2^{pj1}$ (B) flies. Scale bars in (A) and (B) represent 50 μm. GPRK2 immunostaining (C), *Or22a*-driven GFP fluorescence (D), or both GPRK2 immunostaining and *Or22a*-driven GFP (Merge) detection (E) in an antenna from a *Or22a*-Gal4;UAS-GFP fly are shown. Scale bars in (C)–(E) represent 10μm.

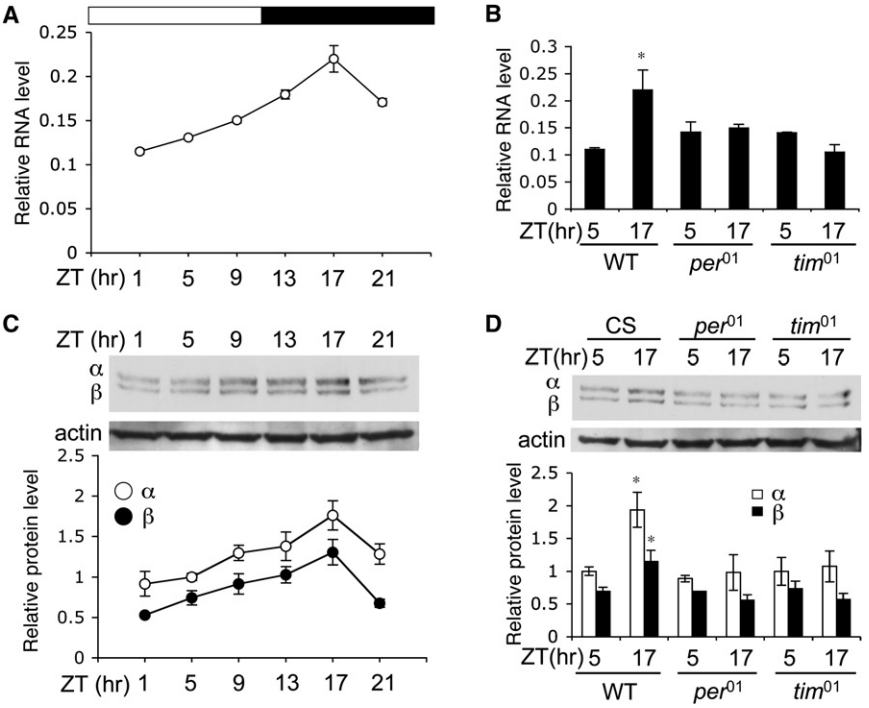

Figure 3. Daily Rhythms in *Gprk2* Expression Are Regulated by the Circadian Clock

(A) Relative levels of *Gprk2* mRNA at the indicated times during an LD cycle. Relative RNA abundance refers to the *Gprk2:rp49* RNA ratio, where *rp49* serves as a control for RNA content in each sample. The white and black bars indicate times when lights were on and off, respectively. The mean ± standard error of the mean (SEM) is shown for each data point from three independent experiments. The overall effect of time of day is significant by one-way ANOVA ($p < 0.0001$).

(B) *Gprk2* RNA levels in per^{01} and tim^{01} flies at ZT5 and ZT17. The mean ± SEM is shown for each data point from three independent experiments. The asterisk denotes a significant ($p < 0.02$) difference between wild-type at ZT17 and wild-type at ZT5, per^{01} at ZT5 and ZT17, and tim^{01} at ZT5 and ZT17.

(C) Western blot showing the levels of GPRK2 α and β isoforms and ACTIN at the indicated ZT times in wild-type flies. The levels of GPRK2 α and β isoforms were quantified at the indicated times during an LD cycle. Relative protein abundance refers to the GPRK2:ACTIN ratio. The GPRK2:ACTIN values at ZT1, ZT9, ZT13, ZT17, and ZT21 are relative to the value at ZT5, which was set to 1.0. The mean ± SEM is shown for each data point from four independent experiments. The overall effect of time of day is significant for both α and β isoforms by one-way ANOVA ($p < 0.005$).

(D) Western blot showing the levels of GPRK2 α and β isoforms and ACTIN in wild-type, per^{01}, and tim^{01} flies at ZT5 and ZT17. The mean ± SEM is shown for each data point from three independent experiments. Asterisks denote a significant ($p < 0.03$) difference between wild-type at ZT17 and wild-type at ZT5, per^{01} at ZT5 and ZT17, and tim^{01} at ZT5 and ZT17.

due to posttranslational modifications or use of alternate translation starts. The *Gprk2* open reading frame, with and without the 5′ and 3′ untranslated sequences, was expressed in S2 cells to determine whether either or both produced the α and β isoforms. The open reading frame produced only the α isoform, whereas the open reading frame plus the 5′ and 3′ untranslated sequences produced both the α and β isoforms (Figure 1A). This result suggests that these isoforms are not due to posttranslational modifications, but how the *Gprk2* untranslated regions (UTRs) generate the smaller β isoform is not known.

Two *P* element insertions in the 5′ UTR of *Gprk2*, $Gprk2^{6936}$, and $Gprk2^{EY09213}$ were identified previously [23, 28]. Reduced levels of the GPRK2 β isoform were observed in both *Gprk2* mutants (Figure 1A), showing that these mutants are hypomorphs and suggesting that the 5′ UTR is important for the generation of the β isoform. To obtain a more severe *Gprk2* mutant, we mobilized the *P* element of $Gprk2^{EY09213}$. One of the resulting mutants, designated $Gprk2^{pj1}$, is a complex rearrangement that deletes 193 bp of *Gprk2* genomic DNA (from −181 to +12 nt), and at the site of this 193 bp deletion adds an inverted portion of *Gprk2* intron 1 (from +1442 to +7782 nt) and a 22 bp sequence upstream of *Gprk2* (from −1644 nt to −1622 nt) that is flanked by 32 bp inverted repeats from the *P* element (Figure 1B). Compared to what is found in wild-type flies, little GPRK2 protein was detected in antennae of $Gprk2^{pj1}$ mutants either on western blots or by immunostaining (Figures 1A, 2A, and 2B), suggesting that $Gprk2^{pj1}$ is a strongly hypomorphic allele.

Gprk2 Expression Is under Clock Control

To determine whether *Gprk2* expression is under circadian-clock control, we measured mRNA and protein levels in antennae from flies collected every 4 hr during LD conditions. *Gprk2* RNA and protein levels oscillate in a diurnal manner with a peak at ZT17 and a trough at ZT1 (Figures 3A and 3C). The levels of GPRK2 α and β isoforms cycled with a similar phase and amplitude (Figure 3C). Cyclic *Gprk2* expression was abolished in per^{01} and tim^{01} mutants, where *Gprk2* mRNA and protein remained at wild-type trough levels (Figures 3B and 3D). Likewise, constant low levels of GPRK2 were present in cyc^{01} mutant flies (Figure S2). These results demonstrate that *Gprk2* mRNA and protein levels are under clock control in antennae.

Cycling of *Gprk2* Expression Is Necessary for EAG Response Rhythms

We previously showed that the circadian clock modulates olfactory responses to ethyl acetate in basiconic sensilla, where robust responses are seen during the middle of the night and weak responses are seen during the middle of the day [2]. GPRK2 is expressed in the OR22a-positive sensory neuron in large ab3 basiconic sensillae, which respond to ethyl acetate (Figures 2C–2E). Given that *Gprk2* expression cycles in antennae, we examined EAG responses in $Gprk2^{6936}$ mutants and flies that overexpress *Gprk2* in OSNs (*Or83b*-GAL4-driven UAS-*Gprk2*). Flies that overexpress *Gprk2* in OSNs displayed constant high-amplitude EAG responses that were near the wild-type peak value in basiconic sensilla, whereas $Gprk2^{6936}$ mutant flies showed constant low-amplitude EAG responses (Figure 4A). Overexpression of *Gprk2* is sufficient to induce robust EAG responses in cyc^{01} flies, indicating that GPRK2 levels determine the magnitude of EAG responses in the absence of a functional clock (Figure 4B). These results demonstrate that the levels of GPRK2 determine the amplitude

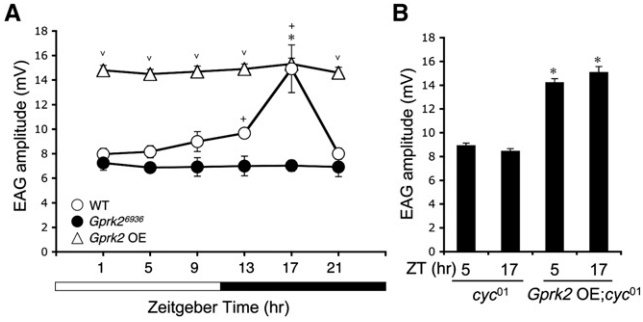

Figure 4. *Gprk2* Expression Levels Control Rhythms in EAG Responses

(A) Diurnal changes in mean EAG responses are plotted for ethyl acetate (10^{-4} dilution) on day 4 of LD. The following are indicated: wild-type (WT), open circles; $Gprk2^{6936}$, closed circles; and *Gprk2* overexpression (*Gprk2* OE), open triangle. Each point represents the mean ± SEM of at least eight females. The white and black bars indicate times when lights were on and off, respectively. The overall effects of time of day, genotype, and their interaction is statistically significant ($p < 0.001$) by two-way ANOVA. The asterisk denotes significant ($p < 0.01$) increases in EAG responses at ZT17 in wild-type flies compared to at all other times of day. The cross indicates significant ($p < 0.01$) differences in EAG responses between wild-type flies and the $Gprk2^{6936}$ mutant at the same time of day. The caret indicates significant ($p < 0.01$) differences in EAG responses between wild-type flies and *Gprk2* OE flies at the same time of day. Post hoc analysis shows no significant differences ($p > 0.9$) as a function of time in either *Gprk2* OE flies or in the $Gprk2^{6936}$ mutant.

(B) EAG responses in cyc^{01} flies and cyc^{01} flies overexpressing *Gprk2* (*Gprk2* OE;cyc^{01}) at ZT5 and ZT17. Each point represents the mean ± SEM of at least eight females. Asterisks denote significant ($p < 0.0001$) differences between cyc^{01} at ZT5 or ZT17 and *Gprk2* OE;cyc^{01} flies at ZT5 and ZT17.

of EAG responses and suggest that clock-dependent rhythms in GPRK2 confer rhythms in EAG responses.

The levels of GPRK2 in $Gprk2^{pj1}$ antennae are lower than those in $Gprk2^{6936}$ antennae (Figure 1A). Given the correspondence between GPRK2 levels and EAG responses, EAG responses should be further reduced in $Gprk2^{pj1}$ flies. Indeed, EAG responses in $Gprk2^{pj1}$ flies at ZT5 were less than half of the amplitude of $Gprk2^{6936}$ or $Gprk2^{EY09213}$ flies and were more than 3-fold lower in amplitude than wild-type flies (Table 1). The low responses in $Gprk2^{pj1}$ flies are consistent with this allele retaining a small amount of *Gprk2* activity.

Cycling Odorant Receptor Accumulation in Dendrites Is Driven by GPRK2

Although many GPRKs function to reduce GPCR-dependent signal transduction by inducing endocytosis of GPCRs [19], *Drosophila Gprk2* enhances olfactory responses. Such an enhancement of olfactory responses is also seen in a *C. elegans Gprk2* mutant, suggesting that GPRK may function through a different mechanism in invertebrate olfactory systems. To assess whether GPRK2 affects the number of ORs in the dendrites, we measured levels of Myc-tagged ORs driven by *Or83b* GAL4 in the dendrites of OSNs. Two Myc-tagged ORs, OR7a and OR22a, were expressed in olfactory sensory neurons (Figure 5). In large basiconic sensilla, high levels of both ORs were detected in dendrites at ZT17 but not ZT5. When these Myc-tagged ORs were coexpressed with GPRK2, they accumulated in the dendrites of large basiconic sensilla at ZT5, indicating that GPRK2 mediates their dendrite localization. No staining with Myc antibody was detected in the antennae of flies that contain the *Or83b*-Gal4 driver or the UAS-*Or7a*

Table 1. *Gprk2* Levels Control the Amplitude of EAG Responses

Strains	EAG Responses (mV)	Number Tested
WT	10.40 ± 0.74[a]	n = 8
$Gprk2^{pj1}$	2.68 ± 0.19[b]	n = 15
$Gprk2^{6936}$	6.87 ± 0.32	n = 8
$Gprk2^{EY09213}$	6.77 ± 0.51	n = 8

EAG responses to a 10^{-4} dilution of ethyl acetate were recorded at ZT5 in wild-type (WT), $Gprk2^{pj1}$, $Gprk2^{6936}$, and $Gprk2^{EY09213}$ flies that were entrained for at least 3 days in LD cycles. The mean EAG response ± SEM is shown.

[a] Denotes a significant difference ($p < 0.0006$) between WT and $Gprk2^{EY09213}$ or $Gprk2^{6936}$ EAG values.

[b] Denotes significant difference ($p < 0.0001$) between $Gprk2^{pj1}$ and WT, $Gprk2^{EY09213}$, or $Gprk2^{6936}$ EAG values.

and UAS-*Or22a* responders alone (data not shown). The correspondence between GPRK2 levels and OR localization in basiconic sensillae suggests that rhythms in GPRK2 levels control the rhythmic accumulation of ORs in OSN dendrites in basoconic sensillae.

Discussion

Cycling of *Gprk2* Expression

We demonstrate that *Gprk2* is a circadian output gene whose mRNA and protein peak during the middle of the night in antennae. This phase of mRNA expression is similar to that of *per*, *tim*, and other genes driven directly by CLK-CYC binding to E-box regulatory sequences [29–31]. However, CLK-CYC-dependent genes are expressed at constitutively high levels in per^{01} and tim^{01} mutants and constitutively low levels in Clk^{Jrk} and cyc^{01} mutants [30, 32, 33], whereas *Gprk2* is expressed at low levels in per^{01}, tim^{01}, and cyc^{01} mutants (Figure 3D; Figure S2). Several rhythmically expressed transcripts identified by microarray analysis of heads have low levels of expression in per^{01} and Clk^{Jrk} mutants [34, 35], but the mechanism governing their rhythmic expression has not been explored.

Analysis of arrhythmic clock mutants indicates that cycling levels of *Gprk2* mRNA give rise to rhythms in GPRK2 protein. The levels of GPRK2 protein cycle in phase with *Gprk2* mRNA and correspond to rhythms in EAG responses. Other kinases such as DOUBLE-TIME (DBT), Shaggy (SGG)/GSK3, and Casein kinase 2 (CK2) in *Drosophila* are constitutively expressed proteins [36–40], whereas GPRK2 is the first example of a rhythmically expressed kinase. However, other kinases such as Erk-MAP kinase and Calcium/calmodulin-dependent protein kinase II in the chicken retina are rhythmically activated due to phosphorylation and control cGMP-gated ion channels in cone photoreceptors [41].

GPRK2-Dependent Rhythms in EAG Responses

Cycling levels of GPRK2 are coincident with rhythms in EAG responses; GPRK2 levels and EAG responses peak around the middle of the night and are at their lowest levels during the middle of the day. When GPRK2 levels are constitutively low, as in per^{01}, tim^{01}, and cyc^{01} mutants, EAG responses are also low. In addition, levels of GPRK2 are at or below the normal wild-type trough in $Gprk2^{6936}$ and $Gprk2^{EY09213}$ mutants (Figure 1A) and generate EAG responses that are at or below those at the wild-type trough (Figure 4; Table 1). GPRK2 levels are barely detectable in $Gprk2^{pj1}$ antennae (Figures 1A and 2A) and produce weak EAG responses well below

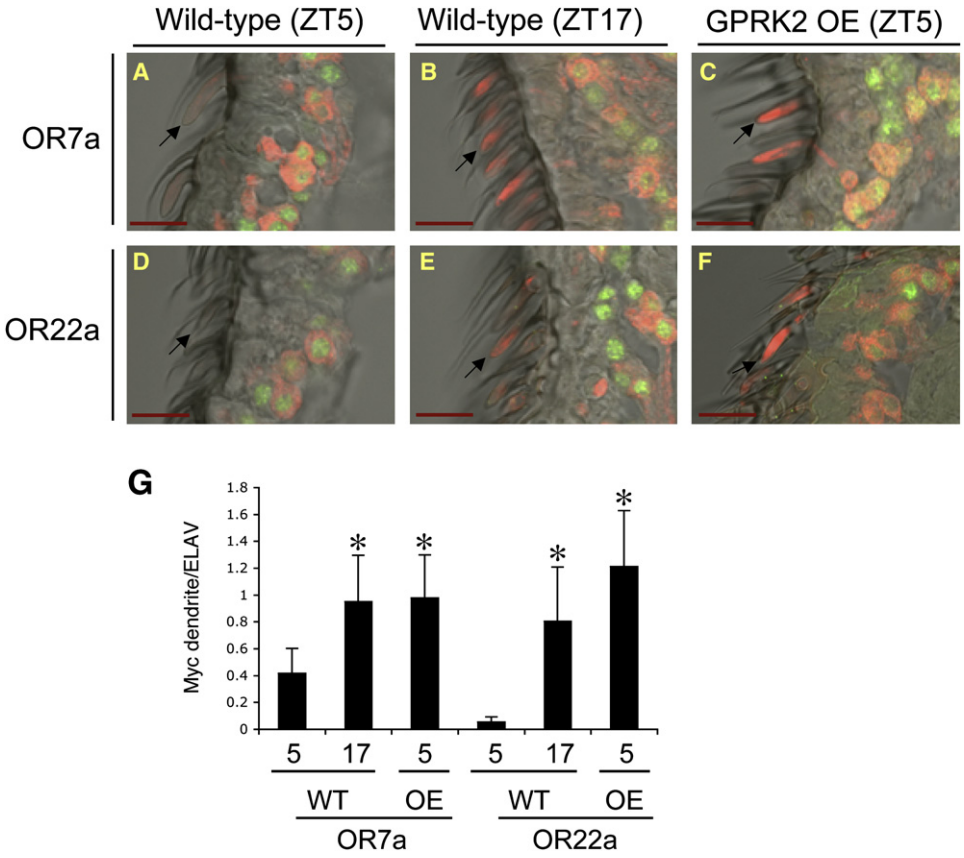

Figure 5. GPRK2 Levels Control the Accumulation of ORs in OSN Dendrites

(A–F) Myc antibody was used to detect *Or83b*-Gal4-driven UAS-Myc-tagged *Or7a* in wild-type antennae at ZT5 (A) and ZT17 (B) and in antennae from *Gprk2* overexpression (Gprk2 OE) flies at ZT5 (C) and to detect *Or83b*-Gal4-driven UAS-Myc-tagged *Or22a* in wild-type antennae at ZT5 (D) and ZT17 (E) and in antennae from *Gprk2* overexpression (GPRK2 OE) flies at ZT5 (F). Each sample was coimmunostained with ELAV, a nonrhythmically expressed nuclear antigen that serves as a reference to quantify Myc-OR levels. The images are representative data from seven to nine antennae for each genotype and time point. Arrows indicate large basiconic sensillae. Scale bars represent 5.0 μm.

(G) Quantification of Myc-ORs from 15–20 OSNs for each genotype and time point. Relative Myc-tagged OR levels were calculated as the ratio of myc immunofluoresence intensity in the dendrite to ELAV immunofluoresence intensity in the nucleus (Myc dendrite:ELAV). Error bars indicate ± SEM. Asterisks denote a significant ($p < 0.0001$) difference in Myc dendrite:ELAV ratio of OR7a and OR22a immunostaining between wild-type (WT) at ZT5 and *Gprk2* overexpression (OE) at ZT5 or wild-type at ZT17.

the wild-type trough level (Table 1). These results suggest that $Gprk2^{pj1}$ is not a null allele and raise the possibility that a *Gprk2* null mutant will lack EAG responses altogether. Such a result would demonstrate that *Gprk2* is required for olfactory responses per se. In contrast, constitutive overexpression of *Gprk2* produces constant high EAG responses in both wild-type and cyc^{01} flies, demonstrating that high levels of GPRK2 can effect high amplitude EAG responses independent of other clock-dependent factors. Taken together, these results argue that Gprk2 levels control the amplitude of EAG responses. If so, this would imply that low levels of GPRK2 present in the $Gprk2^{6936}$ mutant do not cycle in abundance.

Given that GPRK2 levels regulate the amplitude of EAG responses, what is the mechanism through which GPRK2 controls EAG response amplitude? The traditional targets of GPRKs are GPCRs [19]. In the mammalian olfactory system, GPRK3 desensitizes ORs by triggering their internalization [25]. Our results suggest that *Drosophila Gprk2* is necessary for EAG responses, and, taken together with *C. elegans Gprk2* function, they indicate that GPRKs play a different role in invertebrate olfaction than in vertebrate olfaction. The subcellular localization of ORs is high in dendrites of basiconic sensilla at ZT17 and low at ZT5, but the abundance of ORs in these dendrites at ZT5 can be driven to high levels by increasing GPRK2 expression (Figure 5). These results support a model in which the circadian clock generates a rhythm of *Gprk2* expression, which in turn generates rhythms in the amplitude of EAG responses by promoting OR accumulation (and consequently odor-gated cation-channel formation) in OSN dendrites from basiconic sensillae. GPRK2-dependent rhythms in the amplitude of spontaneous spikes are also seen in OSNs [42], thus demonstrating that the clock controls basic (i.e., odor-independent) properties of the OSN membrane. It is possible that the rhythmic localization of odor-gated cation channels to OSN dendrites accounts for rhythms in the amplitude of spontaneous spikes. Our results can't exclude the possibility that cyclic expression of other genes also contribute to rhythms in EAG responses. We have tested mRNA cycling for several genes that could potentially modulate EAG responses, including *arrestin 2*, *Gprk1*, and *kurtz* arrestin [20, 43, 44], and find that *arrestin 2* mRNA levels cycle, but neither *Gprk1* or *kurtz arrestin* mRNA levels cycle (data not shown). Given that microarray analysis was done on fly heads depleted of antennae, microarray analysis of antennae

may reveal other rhythmically expressed genes that contribute to EAG rhythms.

Myc-tagged ORs did not accumulate to high levels in the dendrites of trichoid sensilla at ZT17 (data not shown). Trichoid sensilla have different functions than basiconic sensilla; T1 trichoid sensilla detect the pheromone 11-cis-vaccenyl acetate (cVA), whereas the basiconic sensilla recognize food and plant odors [45, 46]. It could be that the circadian clock regulates OSN activity differently in basiconic sensilla and trichoid sensilla, although we can not exclude the possibility that detection of Myc-tagged ORs in dendrites failed because of low expression levels in trichoid sensillae, poor permeability of Myc antibody into trichoid sensilla, or the long, thin geometry of trichoid sensillae.

In summary, we show here that *Drosophila Gprk2* mRNA and protein expression is under clock control in antennae. The levels of GPRK2 protein determine the amplitude of EAG responses to ethyl acetate in basiconic sensillae; high levels generate high-amplitude EAGs, and low levels produce low-amplitude EAGs. This result suggests that GPRK2 directly or indirectly enhances OR activity, in contrast to the inhibition of olfactory signaling by Gprk3 in mammals. Given that the most severe *Drosophila Gprk2* mutant still produces low-amplitude EAG responses, a complete loss of *Gprk2* function may lack EAG responses altogether and be required for olfaction. High levels of GPRK2 enhance OR localization to dendrites of basiconic sensillae and support a model in which rhythms in GPRK2 levels drive rhythms in OR localization to dendrites that ultimately mediates rhythms in EAG responses.

Experimental Procedures

Fly Strains and *P* Element Excisions

The wild-type strain was Canton S. Mutant and transgenic strains used in these studies include per^{01} [47], tim^{01} [48], cyc^{01} [33], *Or83b*-Gal4 [49], *Or22a*-Gal4 [50], UAS-GFP [51], UAS-Myc-*Or22a* [50], and UAS-Myc-*Or7a* [52]. Two *P* element inserts in exon 1 of *Gprk2*, P{PZ}$Gprk2^{06936}$ ($Gprk2^{6936}$), and P{EPgy2}CG11337^{EY09213} ($Gprk2^{EY09213}$) are hypomorphic mutants [27]. For the generation of additional *Gprk2* mutants via imprecise excision, P{EPgy2}CG11337^{EY09213}, a w^+ transposable element insert in the 5′ UTR of *Gprk2*, was excised as described [53]. A total of 22 w^- excision lines were produced, including $Gprk2^{pj1}$, which contains a rearrangement that causes a more severe loss of *Gprk2* function (see Two GPRK2 Isoforms Are Expressed in Olfactory Sensory Neurons).

Transgenic Flies

For the generation of UAS-*Gprk2* transgenic flies, *Gprk2* EST LE65371 (coding region and 5′ and 3′ UTRs) was first digested with BamHI and ligated into pBlueScript SK(+). The resulting pBS-*Gprk2* plasmid was digested with EcoRI and XbaI and subcloned into the pUAST transformation vector [54] and used to generate transgenic animals at the Duke University Non-mammalian Model Systems Flyshop. Transgenic UAS-*Gprk2* flies were balanced with *w*; Sco/CyO; TM2/TM6b.

Antibody Generation and Western Blotting

A C-terminal fragment of *Gprk2* (Met563-Ser714) was amplified from complementary DNA (cDNA) via polymerase chain reaction (PCR) (5′ primer: 5′-AATTAATCATATGCTGGAGCCACCCTTTGTG-3′; 3′ primer: 5′-CAGG AAACAGCTATGAC-3′). The PCR amplification products were digested with NdeI and XhoI, subcloned into pET28b (EMD Biosciences), and expressed as a histidine-tagged protein in the *E. coli* BL21 (DE3) after induction with 0.3 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG). His-tagged GPRK2 protein was purified with nickel column chromatography (QIAGEN) according to the manufacturer's instructions. The protein preparation was then used for antibody production in Guinea pigs (Cocalico Biologicals).

For western blots, 200–300 third antennal segments were dissected from flies entrained to at least three 12 hr light: 12 hr dark (LD) cycles and homogenized in protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.3% Triton X-100, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Western blots were prepared with 400 ng of protein for each sample as described [55]. Western blots were probed with anti-GPRK2 diluted 1:5000, anti-V5 diluted 1:5000, or anti-actin (Sigma-Aldrich) diluted 1:200 and visualized with enhanced chemiluminescence (ECL) (Amersham). Western blots were repeated at least three times with independent samples. The levels of GPRK2 α and β isoforms were quantified relative to the α isoform at ZT5, which was set to 1.0.

Immunostaining

Flies were collected after entrainment to at least three LD cycles. Consecutive 14 μm cryosections were collected, fixed in 4% formaldehyde in PBST (0.1% Triton X-100 in PBS) and blocked in 10% goat serum for 1 hr as described [50]. Cryosections were incubated overnight with primary antibody at 4°C, rinsed with PBST, incubated for 3 hr with secondary antibody at room temperature, rinsed with PBST, and mounted on a glass slide with Vectashield (Vector Laboratories) mounting medium. For cryosections coimmunostained with embryonic lethal, abnormal vision (ELAV) antibody, after the first primary and secondary antibodies were incubated and washed as above, ELAV antibody was incubated for 3 hr at room temperature, rinsed with PBST, incubated for 3 hr with secondary antibody at room temperature, and rinsed with PBST before mounting. Specimens were imaged with an Olympus FV1000 confocal microscope. The primary antibodies used were rat anti-ELAV (Developmental Studies Hybridoma Bank) diluted 1:200, mouse anti-Myc (Sigma-Aldrich) diluted 1:500, and Guinea pig anti-GPRK2 diluted 1:500. The secondary antibodies used were Cy3-conjugated anti-mouse, Cy3-conjugated anti-rat, Cy3-conjugated anti-Guinea pig, Alexa 488-conjugated anti-rat, and Alexa 488-conjugated anti-Guinea pig diluted 1:200. Levels of GPRK2 were quantified as the ratio of GPRK2 signal intensity in the cell body to ELAV intensity in the nucleus in 15-20 OSNs with Image J (National Institutes of Health [NIH]). Levels of Myc-tagged ORs were quantified as the ratio of Myc signal intensity in the dendrite to ELAV intensity in the nucleus in 15–20 OSNs with Image J (NIH).

qPCR

Flies (0–5 days old) were entrained for at least three LD cycles at 25°C and collected during LD. For each collection, RNA was purified from 150–200 dissected third antennal segments and used to generate cDNA for quantitative PCR (qPCR) analysis as described [13]. The following probe and primers for detection of *Gprk2* mRNA were designed with ABI primer Express software: 5′ primer, 5′-TGCTGGAGCCACCCTTTG-3′, 3′ primer, 5′-CGAGCAC ATCTTTGGCGTAA-3′; and probe 5′-CCAGACCCGCACGC-3′. The *Gprk2* probe was labeled with FAM (6-carboxyfluorescein) and 3′ labeled with TAMRA (6-carboxytetramethylrhodamine). So that the possibility of the contamination of genomic DNA amplification could be eliminated, the probe sequence was designed to across exon junctions in the cDNA sequence.

S2 Cell Experiments

So that V5-epitope-tagged GPRK2 in S2 cells could be inducibly expressed, the ORF of *Gprk2* from the UAS-*Gprk2* vector was amplified via PCR with the *Gprk2* forward (5′-GGTCGGAATTCATGGAATAGAGAAT-3′) and reverse (5′-GCTTACTCGAGCTTTCGACCGTCGTG-3′) primers, digested with EcoRI and XhoI, and inserted into the pMT/V5-His B vector (Invitrogen, Carlsbad, California) to generate pMT-*Gprk2*. For constitutive expression of GPRK2 from the *Gprk2* ORF in S2 cells, the UAS-*Gprk2* vector was amplified via PCR with the same *Gprk2* forward and reverse primers listed above, digested with EcoRI and XbaI, and inserted into the pAc5.1/V5-His B vector (Invitrogen, Carlsbad, California) to generate pAc-*Gprk2*. For constitutive expression of GPRK2 from the *Gprk2* ORF + 5′ and 3′ UTRs in S2 cells, the EcoRI-XbaI fragment from UAS-*Gprk2* was subcloned into pAc5.1/V5-His B to generate pAc-*Gprk2*+UTRs. All *Gprk2* constructs were verified by DNA sequencing. Five hundred nanograms of pAc-*Gprk2* or pAc-*Gprk2*+UTR plasmids were transfected into S2 cells and cultured as described [13]. Five hundred nanograms of either pMT-*Gprk2* or pMT vector alone was transfected into S2 cells and induced by the addition of $CuSO_4$ as described [56]. Transfected cells were harvested via centrifugation (3000 × g, 5 min, 4°C), homogenized in protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1 mM PMSF, 1 mg/ml Leupeptin, 1 mg/ml Pepstatin), and used for western-blot analysis.

EAG Measurements

EAG measurements were performed as described [57]. A 10^{-4} dilution of ethyl acetate was used in all EAG experiments.

Sequence Comparisons

Amino acid sequence comparisons between *Drosophila* GPRK2 and human GPRKs or *C. elegans* GPRK2 were performed with Clustal W analysis. Sequence identity was determined within the GPRK N-terminal domain, kinase domain, and C-terminal domain.

Statistical Analysis

Statistical analysis was done with MS-EXCEL (Microsoft) and Statistica (Statsoft). Analysis of variance (ANOVA) analysis was done with Statistica and MS-EXCEL. Post hoc comparisons were done with Scheffe's test ($\alpha = 0.05$). Student's t test was used to compare values at peak and trough time points.

Supplemental Data

Two figures are available at http://www.current-biology.com/cgi/content/full/18/11/787/DC1/.

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