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Drosophila GPCR Han Is a Receptor for the Circadian Clock Neuropeptide PDF

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Summary

The pigment-dispersing factor (PDF) is a neuropeptide controlling circadian behavioral rhythms in *Drosophila*, but its receptor is not yet known. From a large-scale temperature preference behavior screen in *Drosophila*, we isolated a P insertion mutant that preferred different temperatures during the day and night. This mutation, which we named *han*, reduced the transcript level of *CG13758*. We found that Han was expressed specifically in 13 pairs of circadian clock neurons in the adult brain. *han* null flies showed arrhythmic circadian behavior in constant darkness. The behavioral characteristics of *han* null mutants were similar to those of *pdf* null mutants. We also found that PDF binds specifically to S2 cells expressing Han, which results in the elevation of cAMP synthesis. Therefore, we herein propose that Han is a PDF receptor regulating circadian behavioral rhythm through coordination of activities of clock neurons.

Introduction

A wide variety of animals displays circadian rhythms ranging from daily fluctuation in cellular physiology to locomotor behaviors. These rhythms are governed by endogenous molecular oscillators that can be entrained by environmental stimuli such as daily light-dark (LD) (Panda et al., 2002) or temperature cycles (Crosthwaite et al., 1997). The molecular oscillators enable the organism to anticipate environmental transitions (e.g., day and night transition) and perform biological activities at advantageous times.

In general, circadian oscillators consist of a set of clock genes that generate autoregulatory transcriptional and translational feedback loops. In *Drosophila*, the core circuitry consists of two interlocked transcriptional feedback loops that involve dClock (dClk), Cycle (Cyc), Period (Per), Timeless (Tim), and Vrill (Vri) (Allada et al., 2001; Edery, 2000; Williams and Sehgal, 2001; Cyran et al., 2003; Glossop et al., 2003). Clk and Cyc heterodimerize to activate *per* and *tim* transcription along day and night cycles (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). Per and Tim feed back directly upon Clk and Cyc to inhibit *per* and *tim* transcriptions (Darlington et al., 1998; Lee et al., 1998). Clk itself is also expressed rhythmically and its expression is inhibited by Clk/Cyc (Bae et al., 1998; Darlington et al., 1998; Glossop et al., 1999) and by Vri and PAR domain protein 1 (Pdp1) (Cyran et al., 2003; Glossop et al., 2003). The expression of Vri and Pdp1 are also dependent on Clk (Blau and Young, 1999; McDonald and Rosbash, 2001). Synchronization of rhythmic expression of these genes along the daily cycle of sunlight involves the blue light photoreceptor, Cryptochrome (Cry) (Helfrich-Förster et al., 2001).

Six clusters of clock neurons have been characterized in each hemisphere of the adult *Drosophila* brain (Kaneko and Hall, 2000). These include: four large ventrolateral neurons (l-LNv), five small ventrolateral neurons (s-LNv), six dorsolateral neurons (LNd), approximately 15 dorsal neurons of group 1 (DN1), two dorsal neurons of group 2 (DN2), and approximately 40 dorsal neurons of group 3 (DN3) (Kaneko and Hall, 2000). However, compared to the molecular network of circadian oscillators, little is known about the features of brain clock neurons and the function of each clock neuronal group.

Among the clock neurons, LNvs are known as behavioral pacemaker neurons (Kaneko, 1998; Renn et al., 1999), and their importance as circadian oscillators has been established through various observations. Notably, clock gene expression within LNv is sufficient to drive behavioral rhythms in constant darkness (DD) (Frisch et al., 1994). The *disco* mutant also supports the role of LNv. LNv neurons do not develop properly in this mutant and the circadian rhythmic behavior is not maintained (Helfrich-Förster, 1998). The l-LNvs have large arborizations at the periphery of the medulla and send projections to the contralateral LNvs through the posterior optic tract (POT), whereas the s-LNvs project dorsally to the superior protocerebrum (Helfrich-Förster and Homberg, 1993). In DD, the Per and Tim oscillation is maintained for more than five days in the s-LNvs, but rapidly fade away in l-LNvs, suggesting different roles for the two LNv neuronal groups in circadian clock function (Veleri et al., 2003).

Flies exhibit two distinct periods of high locomotor activity during a 12 hr light and dark cycle. One occurs at the time of "light on" and the other at "light off," indicating that flies can anticipate the time of morning and evening during the day-night cycle. The morning and evening oscillators are connected through intercellular networks, each of which is known to be controlled by a discrete neuronal group, LNv and LNd, respectively (Stoleru et al., 2004; Grima et al., 2004). Specific deletion of LNvs destroys the ability of flies to anticipate when the light will come on (though still knowing when light is off) and selective ablation of LNds has the opposite effect (flies are unable to anticipate the time of "light off," though still knowing when the light is on) (Stoleru et al., 2004; Grima et al., 2004).

One of the clock genes expressed in LNvs is the neuropeptide pigment-dispersing factor (PDF). PDF is known as pigment-dispersing hormone; which was

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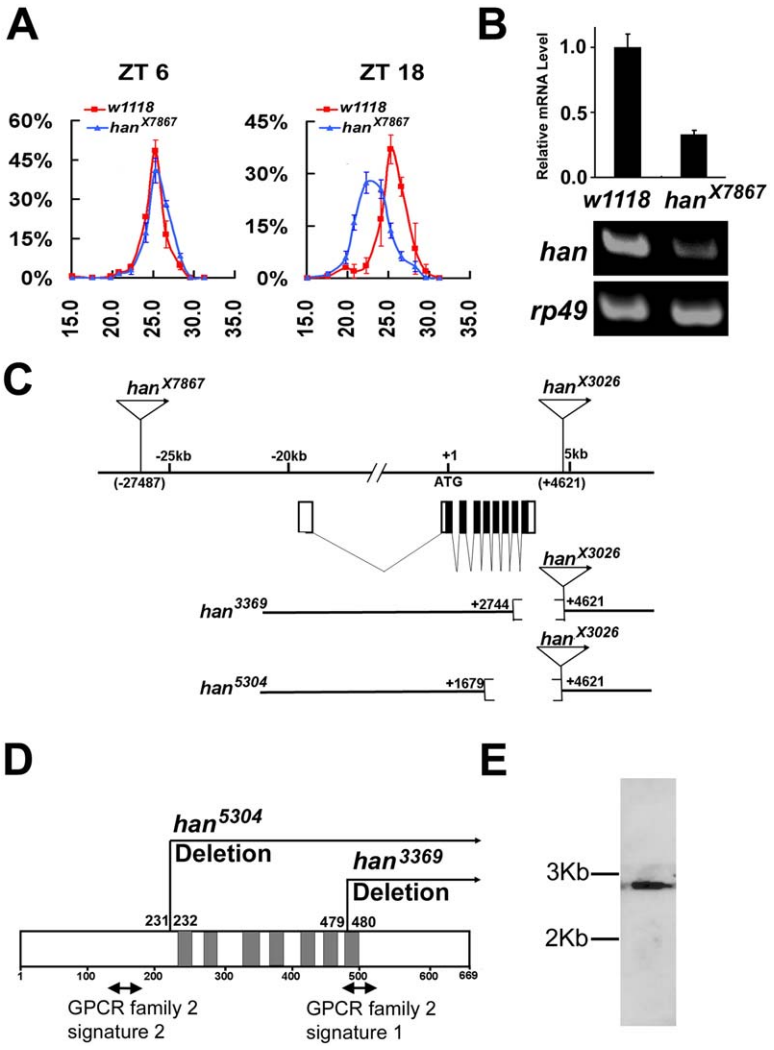


Figure 1. A *han* Allele Prefers Differential Temperatures during Day and Night and the *han* Locus Encodes a GPCR

(A) Temperature preference behavior of w^{1118} and han^{X7867} during day (left) and night (right). Temperature in degrees Celsius is indicated on the x axis and the percentage of flies populated in each temperature range is indicated on the y axis. During the daytime (ZT6), han^{X7867} flies prefer 25ºC as the wild-type control w^{1118} flies (left) do. However, they prefer colder temperature (23ºC) during the nighttime (ZT18), while the wild-type flies still prefer 25ºC (right). All flies were entrained for 12:12 LD cycles for 4 days before assay. Error bars represent SEM.

(B) The CG13758 transcript is reduced in han^{X7867} to one-third of that in w^{1118} wild-type control.

(C) Genomic structure of *han* locus and *han* mutant alleles. Positions of P element insertions and the deleted regions in various *han* alleles are denoted. The genomic position corresponding to the translation start site of *han* is denoted as the +1 position. Open boxes and closed boxes in the *han* transcript represent noncoding exons and coding exons, respectively.

(D) Illustration of the Han protein and its deleted regions in *han* mutants. Han contains seven transmembrane (TM) domains (gray boxes) with two GPCR Family 2 signatures. All of the TMs and C-terminal domains are deleted in han^{5304}, while one of two GPCR Family 2 signatures and the C-terminal portion from the seventh TM is deleted in han^{3369}.

(E) Northern blot analysis of adult head total RNA probed with a 1.5 kb riboprobe of 5′ han cDNA. Note that there is only one transcript of 2.9 kb.

named for its function in crustaceans, but it does not appear to play such a role in insects (Rao and Riehm, 1993). Instead, PDF has been proposed to control clock output in insects (Helfrich-Förster et al., 1998). *pdf* null mutants rapidly lose behavioral rhythmicity in DD, much like flies that have LNvs ablated (Renn et al., 1999). This observation suggests that PDF, which is produced by LNvs, might be an important molecular mediator for behavioral rhythmicity. The amount of PDF in the termini of dorsally projected axons of LNvs has been shown to oscillate along day and night cycles (Park et al., 2000). Recently, it has been proposed that PDF might coordinate interactions between pacemaker neurons in *Drosophila* brain to modulate circadian rhythms (Lin et al., 2004). Despite the importance of PDF in circadian biology, its receptor has not yet been identified.

Here, we report a *Drosophila* circadian gene, named *han*. Our data suggest that Han is a receptor for PDF.

Results

Isolation of *han* Mutants

Drosophila normally prefers an environmental temperature of 25ºC at all times during the day and night cycle. As described elsewhere (Lee et al., 2005), we performed a large-scale behavior screen for over 27,000 independent P-element insertion mutant lines to identify genes involved in temperature sensing and recognition. The P element carries a GAL4-inducible EP (enhancer and a basal promoter) (Rorth, 1996). All autosomal EP lines were derived from a single w^{1118} background male fly with an EP insertion on the X chromosome, and all X-chromosomal EP lines were also generated from a single male fly, but one with an EP insertion on the second chromosome. Therefore, we believe that the EP lines used in the screen had minimal variation in genetic background.

From this screen, we isolated a mutant line that preferred 23ºC only during the middle of the night, yet preferred the normal 25ºC temperature during the daytime (Figure 1A). We named this mutant allele han^{X7867} (*han* refers to the Korean letter for cold). This mutant contains an EP element 8.5 kb upstream of CG13758 (Figure 1C), of which the cytological location is 3A4-6. To find out if the differential temperature preference phenotype of han^{X7867} during day and night is caused by a deficit in CG13758 expression, we compared the level of CG13758 mRNA by RT-PCR analysis in han^{X7867} and

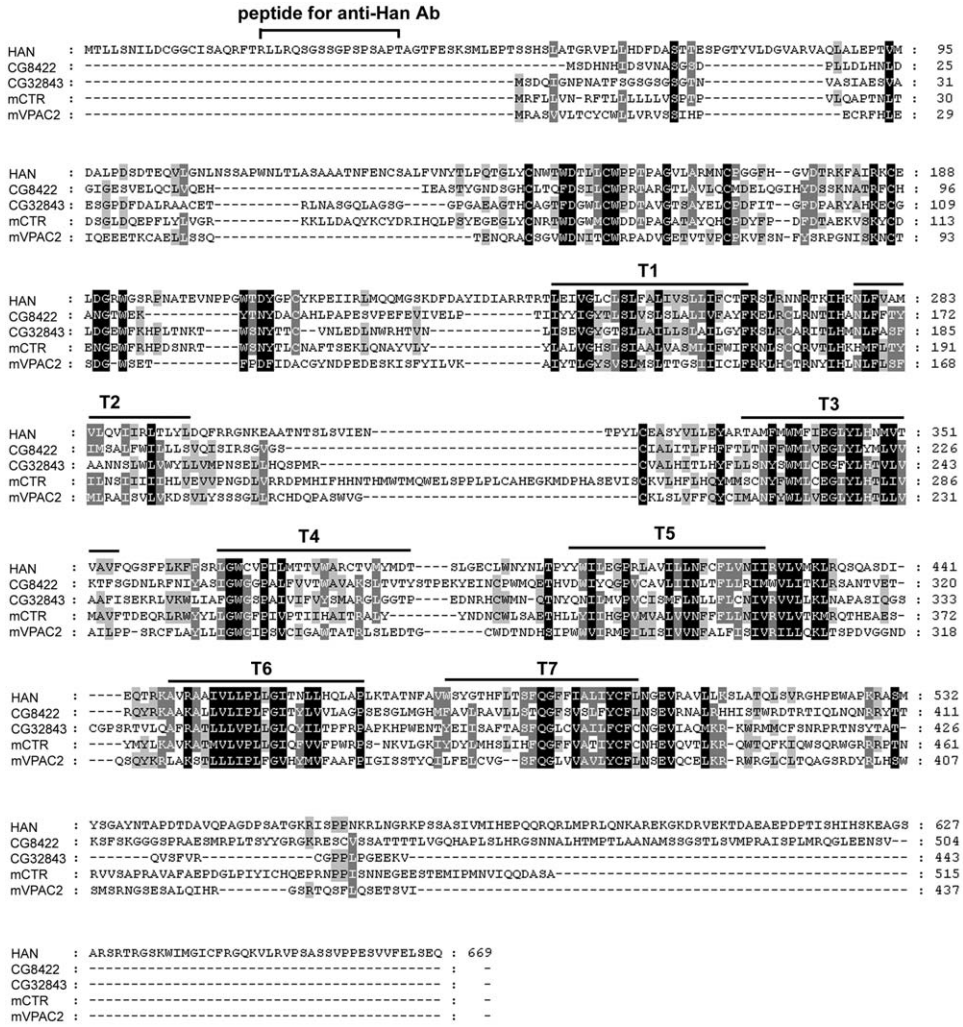


Figure 2. Alignment of the Amino Acid Sequence of Han with Two Other *Drosophila* GPCRs that Have Amino Acid Sequence Similarity with Han
The amino acid sequences of mouse calcitonin receptor (mCTR) and mouse $VPAC_2$ receptors (mVPAC2) are also aligned. T1–T7 indicate transmembrane domains. The amino acid sequence indicated by bracket was used to generate the Han peptide antibody.

w^{1118} wild-type flies. Three independent experiments with separate RNA preparations from han^{X7867} and w^{1118} adult heads revealed that CG13758 transcript levels in han^{X7867} were only 32% of that level found in wild-type w^{1118} control flies (Figure 1B).

CG13758 encodes a seven transmembrane protein that has G protein-coupled receptor family 2 signatures (Figure 1D). Northern blot analysis with riboprobes prepared with a 1.5 kb *han* 5′ cDNA fragment revealed a *han* transcript of 2.9 kb in adult heads (Figure 1E).

We generated additional alleles by P element-mediated male-specific recombination to analyze further the roles of *han* in temperature preference and the circadian cycle (Preston et al., 1996; Chen et al., 1998). An EP-insertion line han^{X3026}, which has an EP element inserted 1.2 kb downstream of CG13758 (Figure 1C), was used as the parental line to generate deletions. Among the many deletion lines obtained from this effort, we identified two deletion mutant alleles han^{5304} and han^{3369} (Figure 1C). The han^{5304} allele has a large deletion that excludes all of the transmembrane domains and the C terminus of Han (Figure 1D). The han^{3369} allele has a smaller deletion that excludes the seventh transmembrane domain and the C terminus (Figure 1D). The han^{5304} and han^{3369} mutants are viable and do not show any atypical morphological phenotypes.

We examined whether the temperature preference during daytime was different from the temperature preference during nighttime in these *han* mutants and han^{X7867}. Unexpectedly, han^{5304} and han^{3369} flies did not prefer different temperatures during day and night (data not shown). They constantly preferred 23.5°C, which is little colder than the temperature preferred by wild-type flies. We do not yet know what role Han plays in thermosensation or in temperature-preference determination, if any.

Han has amino acid sequences similar to those in the mammalian calcitonin (CT) receptor and the $VPAC_2$ receptor (Figure 2). It has been shown that mammalian CT and $VPAC_2$ receptors are expressed in the suprachiasmatic nuclei (SCN) in mice, where circadian pacemaker neurons are located (Nakamoto et al., 2000;

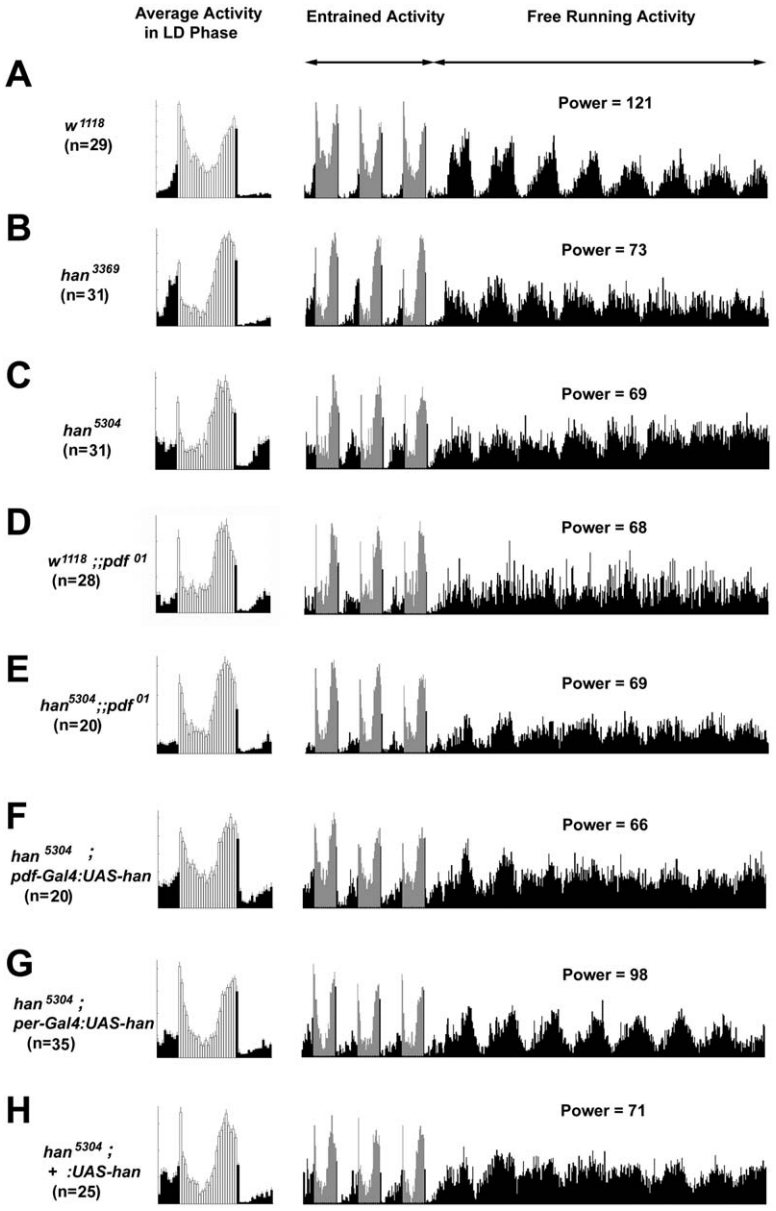


Figure 3. Circadian Locomotor Activities of *han* and *pdf* Mutants

The locomotor activity records shown are population averages. The y axes indicate relative levels of locomotor activity. White and black bars designate day and night phases, respectively.

(A–E) Comparison of locomotor activities of w^{1118}, han^{3369}, han^{5304}, w^{1118};; pdf^{01}, and han^{5304};; pdf^{01} double mutants. Activities in four LD cycles were pooled to show the average activity in LD phase (left). The 3 days of entrained activities in LD and the 8 days of free running activities in DD are shown at the right. The han^{5304} and w^{1118};; pdf^{01} mutants display the common stereotypic feature that the evening peak is strikingly advanced and the morning peaks are markedly reduced in the LD entraining period. The activities of han^{5304}, pdf^{01}, and han^{5304};; pdf^{01} double mutants are also similarly arrhythmic in the free running condition (DD).

(F) Locomotor activity of han^{5304} mutant flies, which express the *UAS-han* transgene under the control of *pdf-Gal4*. Han expression in the PDF expressing neurons (l-LNv and s-LNv) in han^{5304} mutant does not rescue the arrhythmic phenotype of han^{5304} in the free-running condition activity. The advancement of the evening peak is not rescued. Notably, however, morning anticipation in the LD phase was considerably rescued.

(G) Expression of Han in Per-expressing neurons in the han^{5304} mutant. Han expression in Per-expressing neurons rescued abnormal circadian behavioral phenotypes of the han^{5304} mutant.

(H) UAS-*han* transgene without Gal4 does not change the behavioral phenotype of the han^{5304} mutant. The method of analyzing rhythmic power is outlined in the Experimental Procedures. n = number of flies monitored.

Harmar et al., 2002). Furthermore, it has been demonstrated that the $VPAC_2$ receptor is essential for circadian clock activity in mice (Harmar et al., 2002). There are two additional GPCRs in *Drosophila* that show high sequence similarity with mammalian CT and $VPAC_2$ receptors. They are CG8422 and CG32843 (Figure 2). The genes for CG8422 and CG32843 are located at 50F8-9 and 49F15-50A1 of the second chromosome, respectively.

han Is Required for Circadian Behavior

We suspected that Han might be involved in circadian clock or rhythm processing because of the similarity of its amino acid sequence to the $VPAC_2$ receptor and because han^{X7867} showed different temperature preferences during day and night. To examine this possibility, we examined the rhythmic locomotor activity behaviors of *han* mutants. We placed *han* mutant flies in a *Drosophila* activity monitor and recorded their movement for 5 days in a 12 hr light:12 hr dark (LD) cycle and then for 8 days in constant darkness (DD).

Drosophila normally shows active behaviors in the morning and early evening during LD cycles and during the first few days of DD cycles (Figure 3A). Indeed, we observed that 24% of the daily activity of w^{1118} wild-type control flies occurred during the first two hours in the morning (Table 1). In contrast, the han^{3369} and han^{5304} flies showed reduced behavioral activity in the morning during LD cycles and during the first day of the DD cycle (Figures 3B and 3C and Table 1); the average activity during the first two hours after "light on" was 10% of the total daily activity in LD (Table 1). More than 60% of the han^{3369} and han^{5304} flies showed less than 10% of their total daily activity during the first two hours in the morning (Table 1). We also observed that han^{5304} flies did not demonstrate any gradual increase of activity before lights were turned on. These data indicate that *han* mutants have a deficit in morning anticipation.

Table 1. Circadian Behavioral Characteristics of *han* and *pdf* Flies

		LD					DD Day 3 – DD Day 8			
			Morning Activity		Evening Activity			Rhythmicity		
Genotype	Number of Flies Tested	% of Flies with Low Morning Activity[a]	Magnitude[b]	P Value	Phase (ZT) of the Highest Activity	P Value	% of Rhythmic Flies	Power	*p*	Period[c]
w^{1118}	29	7	24.2 ± 1.3	—	11.0 ± 0.1	—	90	121 ± 9	—	23.3 ± 0.2
han^{3369}	31	61	10.4 ± 0.8	3×10^{-12}	9.8 ± 0.2	3×10^{-8}	16	73 ± 3	8×10^{-6}	22.8 ± 0.4
han^{5304}	31	61	10.4 ± 1.3	6×10^{-10}	9.6 ± 0.2	4×10^{-10}	10	69 ± 3	4×10^{-6}	22.7 ± 0.2
w^{1118};;pdf^{01}	28	61	11.2 ± 1.6	6×10^{-8}	9.6 ± 0.2	2×10^{-9}	7	68 ± 3	4×10^{-6}	22.3 ± 0.3
han^{5304};;pdf^{01}	20	60	13.8 ± 1.5	3×10^{-6}	9.7 ± 0.2	1×10^{-7}	10	69 ± 3	4×10^{-5}	22.5 ± 0.5
han^{5304};*pdf-gal4*: UAS-*han*	20	35	15.0 ± 1.0	6×10^{-6}	10.1 ± 0.3	0.001	10	66 ± 3	1×10^{-5}	22.3 ± 0.3
han^{5304};*per-gal4*: UAS-*han*	35	11	20.8 ± 1.3	0.1	10.7 ± 0.2	0.2	63	98 ± 5	0.3	23.0 ± 0.2
han^{5304};+: UAS-*han*	25	52	13.3 ± 1.3	3×10^{-7}	9.6 ± 0.2	7×10^{-8}	12	71 ± 3	7×10^{-6}	22.3 ± 0.2

[a] The percentage of the flies for which activity during the first 2 hours after "light on" is less than 10% of daily total activity.
[b] The activity that occurred during the first 2 hours after "light on" as the percentage of the total daily activity.
[c] Periods were calculated only with the data for the rhythmic flies of the tested flies.
p values were determined by comparing each genotype with w^{1118} using Student's t tests. ± values indicate the standard error of the mean.

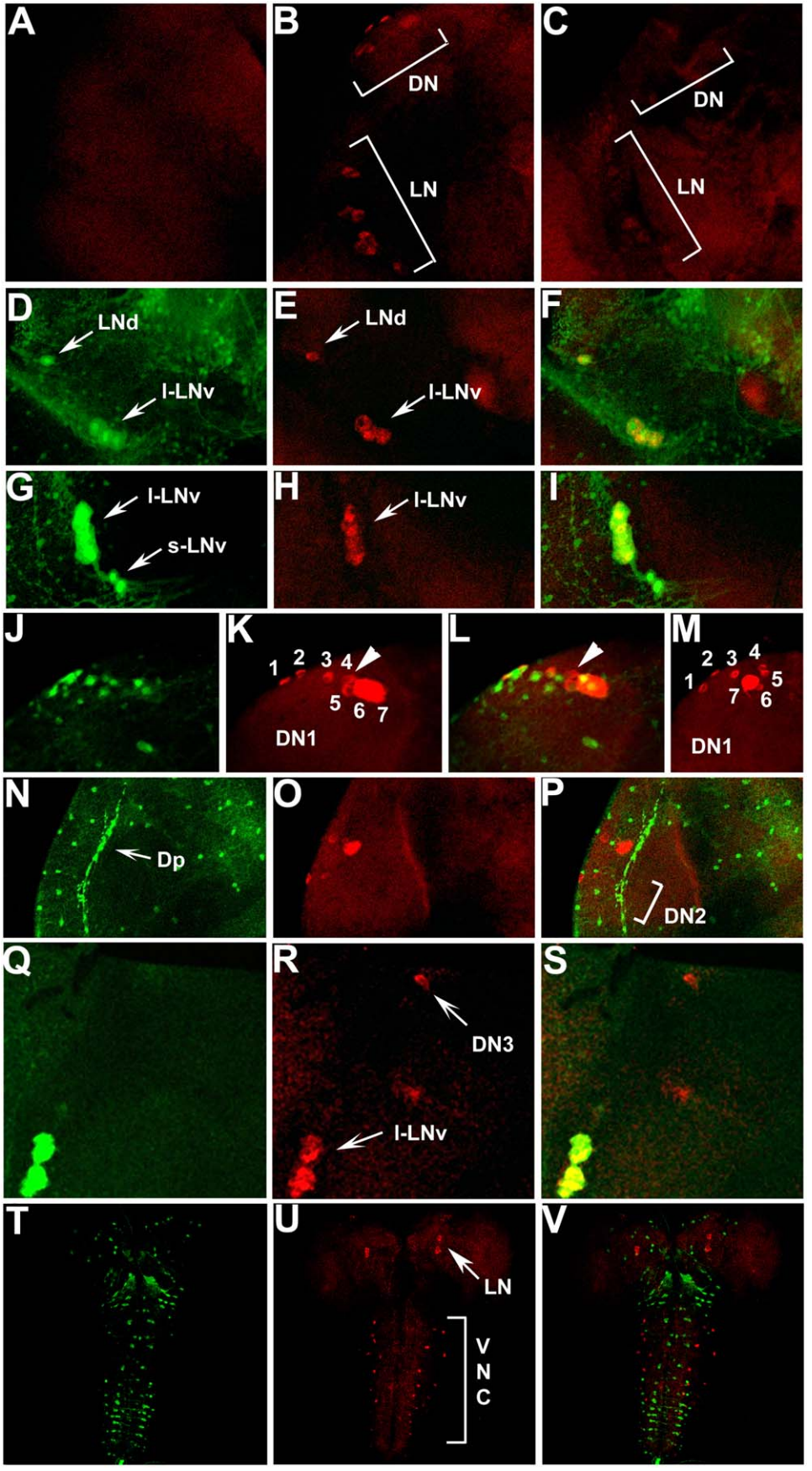
The *han* mutants also showed an abnormality in evening behavioral activity. They began evening activity earlier than wild-type flies and the activity continued until lights were turned off in LD cycles (Figures 3B and 3C). The highest evening activities were observed at ZT9.8 and ZT9.6 in han^{3369} and han^{5304}, respectively, while it was observed at ZT11.0 in w^{1118} control flies (Table 1). These findings suggest that evening anticipation is advanced in *han* mutants.

The han^{3369} and han^{5304} mutant flies also showed abnormal circadian rhythmic behavior in the DD condition. They showed residual circadian rhythmic behavior in the first few days, but they gradually lost the rhythmic activity as the DD condition continued (Figures 3B and 3C and Table 1). The rhythmic powers of the han^{3369} and han^{5304} mutants were 73 ± 3 and 69 ± 3 in DD, while w^{1118} control flies showed 121 ± 9 rhythmic power (mean power ± SEM) (Table 1). The *han* mutants also appeared to have shortened rhythmic periods of 22.8 hr and 22.7 hr in han^{3369} and han^{5304}, respectively, while a period of 23.3 hr was observed in w^{1118} (Table 1). The rhythmicity during DD was observed only in 10%–15% of individual flies tested in han^{3369} and han^{5304} genotypes, precluding definitive conclusions about the short-period phenotype. However, we believe that this phenotype truly corresponds to *han* because we have observed similar behaviors consistently in several independent experiments conducted on different days (data not shown).

Behavioral characteristics of *han* mutants are similar to those of *pdf* mutants (Figure 3D), which were previously identified as circadian rhythm mutants (Renn et al., 1999). The pdf^{01} mutant has low morning activity and advanced evening peak activity during LD cycles and gradual loss of rhythmicity during DD (Figure 3 and Table 1). This led us to hypothesize that Han and PDF might act in the same signaling pathway that controls circadian rhythmic behaviors. This idea was supported further by the behavioral analysis of *han* and *pdf* double mutant flies. The han^{5304};; pdf^{01} mutants showed the same behavioral characteristics as han^{5304} or pdf^{01} single mutant flies (Figure 3E). They showed weak morning activity and advanced evening anticipation in LD and gradual loss of rhythmicity during DD cycles (Figure 3E and Table 1). ANOVA analysis of rhythmicities of han^{5304}, pdf^{01}, and han^{5304};; pdf^{01} mutant flies indicated that the rhythmicities of these three genotypes of flies were similar ($p = 0.982$).

The data from the behavior assay of *han* mutants raised the possibility that Han might exert its role in pacemaker neurons. To test this, we artificially expressed Han in pacemaker neurons of han^{5304} mutant flies, using the Gal4/UAS targeted expression technique (see Brand and Perrimon, 1993). The *pdf-Gal4* drives expression of transgenes under the control of yeast Upstream Activator Sequences (UAS) in LNv neurons (Renn et al., 1999). We observed that artificial Han expression in the LNv neurons, achieved by combining the *UAS-han* transgene with *pdf-Gal4* in a han^{5304} mutant background, did not rescue the gradual arrhythmic phenotype in DD (Figure 3F and Table 1).

It has been demonstrated that LNv and LNd control the morning and evening activities, respectively (Stoleru et al., 2004; Grima et al., 2004). We observed that artificial Han expression in the LNv neurons in han^{5304} alleviated the advanced evening anticipation phenotype partially but not completely (Figure 3F and Table 1). These data suggest that Han signaling in LNv may have a partial role in controlling the evening oscillator. Notably, restricted LNv expression of Han in han^{5304} flies partially but not completely rescued the weak morning activity, which is almost absent in han^{5304} flies (Figure 3F and Table 1). This observation implies that Han signaling, not only in LNv but also in other neurons, is necessary for the proper function of morning oscillators. This finding complements the recently reported finding that morning behavioral activities are governed by LNv neurons (Stoleru et al., 2004; Grima et al., 2004). Taken together, these findings lead us to conclude that expression of Han in LNv neurons is not sufficient to generate Han-mediated circadian locomotor activity completely.


A
B
DN
LN
C
DN
LN
D
LNd
l-LNv
E
LNd
l-LNv
F
G
l-LNv
s-LNv
H
l-LNv
I
J
K
1 2 3 4
5 6 7
DN1
L
M
1 2 3 4
5
7 6
DN1
N
Dp
O
P
DN2
Q
R
DN3
l-LNv
S
T
U
LN
V
N
C
V

We examined whether the circadian behavioral defects of *han*^{5304} mutants, which could not be rescued by artificial Han expression in LNv neurons, would be rescued by Han expression in Per-expressing clock neurons. Per is expressed in LNd, DN1, DN2, and DN3 neurons in addition to LNv neurons (Kaneko and Hall, 2000). We expressed the *UAS-han* transgene with *per-Gal4* in the *han*^{5304} background. When Han was expressed with *per-Gal4*, all of the circadian behavioral deficits of *han*^{5304} were rescued to a level comparable to wild-type (Figure 3G and Table 1). Only 11% of the flies showed low morning activity, and 21% of daily activity occurred during the first two hours after lights were turned on. These results are comparable to the *w*^{1118} wild-type control, in which only 7% of flies showed low morning activity and 24% of the average daily activity of all tested flies occurred during the first two hours after lights were turned on. The advanced evening anticipation phenotype was also restored in *han*^{5304};;*per-gal4*:*UAS-han* flies. The highest evening activity occurred at ZT9.6 in *han*^{5304} flies, but shifted back to ZT10.7 in *han*^{5304};;*per-gal4*:*UAS-han* flies, which is similar to the activity of *w*^{1118} (ZT11.0) (Table 1). The arrhythmic locomotor behavior during the free running condition of DD was also rescued by Han expression in Per-expressing neurons (Figure 3G). The rhythmic power was 98, which is 81% of the wild-type rhythmic power, and the period was 23 hr in *han*^{5304};;*per-gal4*:*UAS-han* flies (Figure 3G; Table 1). These data imply that Han-mediated signaling is required in LNv neurons and other clock neurons that express Per but not PDF. However, the phenotypic rescue of *han* mutants by artificial expression of Han with *per-Gal4* was not 100% complete although close to wild-type. This may be because the *per-Gal4* that we used may not drive UAS-transgene expression in all Per-expressing cells or because other neurons that express Han, but not Per, are also required for complete circadian rhythmic behavior.

han Is Expressed in Clock Neurons

We generated antibodies against the peptide sequence of the Han N terminus, which is shown in Figure 2. The specificity of the Han antibody was verified in two ways. First, the antibody was preincubated overnight with a 10-fold molar excess of the antigen peptide prior to immunostaining. As shown in Figure 4A, the antibody solution preabsorbed with the Han peptide did not give any detectable stain in clock neurons, while Han antibody without preabsorption gave specific staining in DNs and LNs (Figure 4B). Second, we compared the staining results in *w*^{1118} wild-type and *han* mutant brains. As shown in Figure 1D, *han*^{5304} expresses truncated N-terminal polypeptides of Han, which appear to be unstable, but are recognized by the Han antibody (Figure 1C and data not shown). To create a situation in which a minimal amount of truncated Han is present, we combined the *han*^{5304} chromosome with the *Df(1)HC194* chromosome, resulting in the *han*^{5304}/*Df(1)HC194* heterozygote. *Df(1)HC194* is a deficiency chromosome, uncovering the cytological map region of 3A1 to 3C3-4 and excluding the *han* locus completely. As shown in Figure 4C, the *han*^{5304}/*Df(1)HC194* heterozygotes showed very weak Han staining in DNs and LNs in contrast to the strong staining in wild-type brain (Figures 4B and 4C). These immunostaining data provide compelling evidence that the Han antibody used in this study specifically recognizes Han.

We stained wild-type adult fly brains with the Han antibody to identify neurons in which Han has a role in regulating circadian rhythmic behavior. We used the *UAS-EGFP* transgene driven by *per-Gal4* (Figures 4D–4F and 4J–4L) or *pdf-Gal4* (Figures 4G–4I and 4N–4P), or we used the PDF antibody (Figures 4Q–4S) to mark the clock neurons. *per-Gal4* drove expression of Gal4 proteins in the following neurons: four of the l-LNv; one of six LNd (Figure 4D); DN1 (Figure 4J); DN2; and DN3 (data not shown). On the other hand, *pdf-Gal4* drove expression of Gal4 proteins in all l-LNv and s-LNv neurons (Figure 4G).

From immunostaining analysis with the Han antibody, we found that Han was expressed specifically in 13 neurons in each hemisphere of wild-type adult brains (Figure 4). Han was expressed in all four l-LNv neurons (Figures 4E and 4H), but not in s-LNv neurons (Figures 4G–4I). It is also expressed in one of the six LNd neurons (Figure 4E). We also found Han expression in seven neurons in the DN1 area (Figures 4J–4M) and in one neuron in the DN3 area (Figures 4Q–4S). Among the seven neurons expressing Han in the DN1 area, one neuron did not express Per at any detectable level and one neuron expressed Per at a low level, while the remaining five neurons showed relatively high Per expression (Figures 4J–4M). None of the DN2 neurons expressed Han (Figure 4P).

In summary, Han was expressed in all four l-LNv neurons, one LNd neuron, seven DN1 neurons, and one DN3 neuron. All of these neurons except one neuron

Figure 4. Han Is Expressed in Clock Neurons

Han proteins visualized with Han antibody are shown in red. GFP signals driven by *per-Gal4* (D, F, J, and L), *pdf-Gal4* (G, I, N, and P), or *DDC-Gal4* (T and V) are shown in green. PDF proteins visualized with PDF antibody staining are also shown in green (Q and S). The third panels in each row, except panel C, are the merged images of the two left panels. (A–C) Verification of Han antibody specificity. Han antibody preincubated with 10-fold molar excess concentrations of the Han N-terminal peptide, with which the antibody was generated, does not stain any clock neurons (A). The antibody preincubated with only the buffer clearly stains clock neurons (B). The staining signals with Han antibody are dramatically reduced in female brains of *han*^{5304}/*Df(1)HC194* mutant flies (C). (D–I) Han is expressed in one of the LNd neurons and all four l-LNv neurons but not in s-LNv neurons. (J–M) Han is expressed in seven neurons in DN1 area, which are numbered. There is a neuron that expresses Han but not Per (arrowhead). (J)–(L) and (M) show the DN1 regions in two independent samples. (N–P) DN2 neurons do not express Han. The DN2 region is marked with a bracket, based on the topological location of PDF axonal terminals (green, N and P) and DN1 neurons expressing Han (O and P). Dp indicates dorsal projection of s-LNv axons. Unidentified spot-like GFP signals appeared when brains of *pdf-Gal4:UAS-EGFP* flies were fixed with Zamboni's fixative, but it was not observed when the samples were fixed with paraformaldehyde fixative without picric acid. (Q–S) Han is expressed in one of the DN3 neurons. (T–V) Han is expressed in approximately 13 pairs of neurons in the ventral nerve cord (VNC) of third instar larval brain. These neurons do not overlap with dopaminergic and serotonergic neurons (shown in green). DN1 neurons and some of the LNvs, which also express Han, are out of focal plane. Dorsal is upward in all panels except in panels (T)–(V).

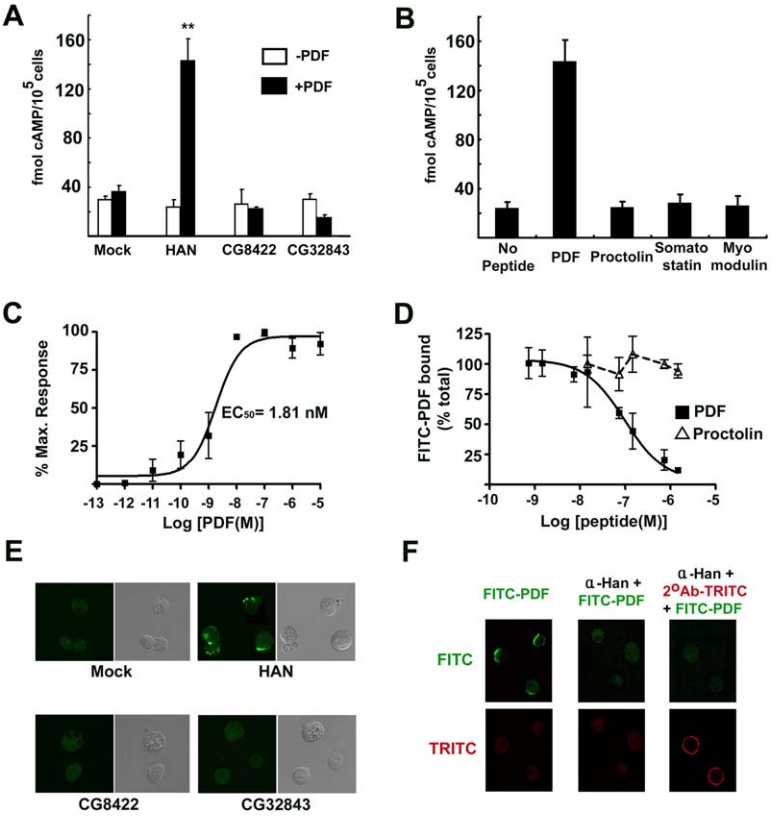


Figure 5. PDF Binds to Han and Activates cAMP Synthesis

(A) cAMP measurement in the S2 cells that are transiently transfected with Han-, CG8422-, or CG32843-expressing plasmids without (open bars) or after treatment with 1 μM PDF (solid bars). The transfection efficiency is 31% ± 6%. Only the S2 cells expressing Han showed high levels of cAMP when they were treated with PDF. Three independent experiments were performed, and the double asterisk indicates that the cAMP level was statistically different from nontreated controls ($p < 0.005$). (B) Han-expressing S2 cells specifically respond to PDF. Intracellular cAMP levels were measured after treating Han-expressing S2 cells with 1 μM PDF, proctolin, somatostatin, or myomodulin. (C) PDF dose-response curve of Han-expressing S2 cells. Cells were treated with various concentrations of PDF, and the cAMP levels in three independent experiments were measured. (D) Competition binding assays of FITC-PDF and Han with unlabeled PDF. Binding of FITC-PDF to the S2 cells stably expressing the Han receptor was measured in the presence of increasing amounts of unlabeled PDF. Proctolin was used as a negative control peptide. The extent of binding was measured by fluorescence intensity. Values for five independent experiments are shown. In (A)–(D), error bars indicate the mean ± SEM. (E) Confocal microscopic images of S2 cells treated with FITC-PDF. S2 cells transfected with expression plasmids of Han, CG8422, CG32843, or pAc vector were incubated with FITC-PDF, and the cells were observed with the LSM510 confocal microscope after washing out unbounded FITC-PDF. Note that the cells transfected with Han-expressing plasmids only show a FITC signal, which indicates that PDF binds to Han but not to CG8422 or CG32843. (F) Han antibody blocks PDF binding to Han in S2 Cells. The reagents applied to S2 cells expressing Han are shown at the top of each column, in the sequence used. Two confocal channel images (FITC and TRIC) for each sample are shown in each column. FITC-PDF binds to the S2 cell membrane when added without Han antibody treatment (left column), while it does not bind if it is added after the application of Han antibody (middle column). Han is expressed and localized on the S2 cell membrane, but it does not bind FITC-PDF if Han antibody and rabbit IgG-TRITC secondary antibody are used to treat cells prior to the application of FITC-PDF (right column).

in the DN1 area also expressed Per. Therefore, all l-LNv neurons, about half of the DN1 neurons, and one neuron in each of the LNd and DN3 areas are probably regulated by Han-mediated signaling.

In addition to the clock neurons, Han was also expressed in approximately 13 pairs of neurons along the ventral nerve cord in third instar larvae (Figures 4T–4V). We do not know the identity of these neurons, but they do not overlap with dopaminergic or serotonergic neurons (Figure 4V). No specific staining pattern of Han was observed at the embryonic stage (data not shown).

PDF Binds and Activates Han

As described above, the characteristics of locomotor activity behaviors of *han* mutants in LD and DD cycles are very similar to those of *pdf* mutants. Moreover, Han is expressed in subsets of Per-expressing DN1 neurons in which the axons of the LNv neurons expressing PDF are projected. This led us to hypothesize that Han might be a receptor of the PDF neuropeptide.

To test this possibility, we synthesized oligopeptides of PDF in which the C terminus was amidated. This synthetic PDF was used to treat *Drosophila* S2 cells in which Han or two other *Drosophila* CT receptor homologs, CG8422 and CG32843, were transiently expressed by transfection. Han, CG8422, and CG32843 belong to Family B of GPCRs. This receptor family generally elevates the level of cytosolic cAMP upon activation (Poyner et al., 2002). Therefore, we expected that PDF treatment in the cells expressing the PDF receptor would activate the GPCR signaling cascade and stimulate cytoplasmic cAMP synthesis.

The cAMP levels in S2 cells transfected with the vector vehicle alone were 30 ± 3.5 fmole/10^5 cells and 37 ± 5.0 fmole/10^5 cells without and with the treatment of synthetic PDF, respectively (Figure 5A). Surprisingly, the cAMP level in the cells transfected with Han-expressing plasmids was greatly increased by PDF treatment. The cAMP level was increased from 23 ± 6 fmole/10^5 cells to 144 ± 15 fmole/10^5 cells by administration of PDF (1 μM) for 30 min (Figure 5A). Accounting for the transfection efficiency of 31% ± 6% and assuming that all of the increased cAMP detected in the assay was synthesized in the cells harboring Han-expressing plasmids, the cAMP levels in the Han-expressing cells were estimated to be increased to

approximately 318 fmole/10^5 upon treatment with PDF. This means that PDF can increase the cAMP level in the cells expressing Han by almost 14-fold. This increase is specific for the cells expressing Han, because the cells expressing CG8422 or CG32843, which have amino acid sequence similarity to Han, did not show any increase of cAMP level in response to PDF treatment (Figure 5A). These results imply that PDF can bind to Han and activate cAMP synthesis. PDF treatment of S2 cells transfected with a CG32843 expression plasmid appeared to reduce cAMP level (Figure 5A). This raised a possibility that PDF might also bind to CG32843 and inhibit cAMP synthesis. To test this, we cotransfected S2 cells with Han-expressing plasmids and CG32843-expressing plasmids. The cAMP level of these cells after treatment with PDF was similar to cells cotransfected with Han-expressing plasmids and vector vehicle plasmids (data not shown). Therefore, it appears that CG32843 does not respond to PDF.

To test whether Han responds specifically to PDF, we transfected S2 cells with Han-expressing plasmids and treated them with 1 μM of proctolin, somatostatin, or myomodulin. These neuropeptides exert various effects in *Drosophila* (Anderson et al., 1988; Hewes and Taghert, 2001; O'Brien and Taghert, 1998). As shown in Figure 5B, none of these peptides could activate cAMP synthesis in the S2 cells expressing Han. This implies that Han specifically responds to PDF, but not to the other neuropeptides that we tested.

We measured cAMP synthesis in the Han-expressing S2 cells while increasing the concentration of PDF. PDF could activate Han at concentrations as low as 100 pM, and maximal cAMP synthesis was observed at 10 nM (Figure 5C). The half-maximal effective concentration (EC_{50}) of PDF for Han expressed in S2 cells was 1.8 nM (Figure 5C). This suggests that PDF can activate Han effectively at nanomolar concentrations.

The binding affinity of PDF to Han was calculated using the competition binding assay method described by Cvejic et al. (2004). We synthesized PDF peptides in which the N terminus was conjugated with FITC and the C terminus was amidated (referred to hereafter as FITC-PDF). We treated S2 cells stably expressing Han with 100 nM FITC-PDF along with various concentrations of unlabeled PDF and then measured the bound FITC-PDF with a fluorometer (Figure 5D). The displacement curves analyzed by the GraphPad Prism program gave the estimated equilibrium dissociation constant (Kd) of 31.2 nM (Figure 5D). Considering that various neuropeptides and their GPCRs in *Drosophila* have Kd values ranging from 150 nM to 480 nM (Park et al., 2002), the Kd of 31.2 nM for PDF and Han implies that PDF binds Han with relatively high affinity. In contrast to PDF, proctolin could not displace the bound FITC-PDF even at high concentrations (>1.2 μM) (Figure 5D).

These data strongly suggest that PDF binds specifically to Han and activates Han-mediated cAMP synthesis in vivo. The binding of PDF to Han was also confirmed by confocal microscopy. FITC-conjugated PDF peptides were applied to the S2 cells transfected with Han-expressing plasmids. As expected, these S2 cells exhibited FITC fluorescence around the cell membrane while other S2 cells transfected with vector plasmids, plasmids expressing CG8422, or plasmids expressing CG32843 did not show specific fluorescence (Figure 5E).

To examine whether PDF and Han are colocalized in S2 cells, we stained Han-expressing S2 cells with Han antibody and with FITC-PDF. However, FITC-PDF did not bind well to the S2 cells when the S2 cells were treated with Han antibody (Figure 5F). The Han antibody was raised against the N-terminal sequence of Han, which is present in the extracellular region. Therefore, it appears that PDF binding to its receptor site on the extracellular domain of Han was sterically hindered by Han antibody.

Taken together, the binding assay data described above provides compelling evidence that Han is a PDF receptor.

Discussion

PDF has been known as an important neuropeptide controlling circadian rhythmic behaviors in *Drosophila* (Renn et al., 1999). However, its receptor had not been identified prior to our study.

Here, we report a circadian rhythm gene, *han*. *han* mutants show circadian behavioral activity phenotypes similar to *pdf* mutants (Figure 3). The han^{5304} and pdf^{01} double mutants show the same behavioral characteristics as han^{5304} or pdf^{01} single mutant flies. These genetic data raised the possibility that Han and PDF may act in the same signaling pathway. Consistent with this hypothesis, we found that PDF specifically binds to Han in S2 cells with relatively high affinity (Figure 5). As expected for a Family B GPCR, PDF binding to Han resulted in elevated cytoplasmic cAMP synthesis (Figure 5). These data consistently point to Han as a receptor for PDF.

PDF is expressed in l-LNvs and s-LNvs and is secreted in the axon termini of these neurons. These neurons have large arborizations and send projections to contralateral and dorsal areas of the brain where LNd, DN1, DN2, and DN3 are present (Helfrich-Förster and Homberg, 1993; Veleri et al., 2003). On the other hand, the PDF receptor Han is expressed in 13 pairs of neurons in adult fly brains: four l-LNvs, one LNd, seven DN1s, and one DN3 (Figure 4). Therefore, it is reasonable to conclude that the PDF signals produced by l-LNvs and s-LNvs are transmitted to four l-LNvs, one LNd, seven DN1s, and one DN3 through Han receptors. LNv, LNd, DN1, and DN3 neurons play certain roles in the control of circadian rhythmic behaviors (Veleri et al., 2003) and, thus, PDF-Han signaling might also play some roles in coordinating interactions between these groups of clock neurons.

Comparing expression patterns of PDF reveals, interestingly, that Han is expressed only in l-LNv but not in s-LNvs. Both l-LNvs and s-LNvs express PDF. It has been suggested that s-LNv neurons are the most important master neurons of clock neurons in flies because strong oscillations of Per and Tim proteins are continuously sustained over five days in s-LNvs in DD (Veleri et al., 2003). Although neurite fibers of l-LNv and s-LNv are inter-connected, oscillation of Per and Tim proteins in l-LNv is not obvious a few days after the "light-off" in contrast to s-LNv neurons. Han-mediated PDF signaling may contribute to the coordinated

interaction of l-LNv with s-LNv neurons because Han is expressed in l-LNv neurons. These two groups of clock neurons have been shown to control morning oscillators while LNd neurons control evening oscillators (Stoleru et al., 2004; Grima et al., 2004). In both *pdf* and *han* mutants, the morning oscillators, as well as the evening oscillators, do not function properly (Figure 3). Flies cannot properly anticipate the time when light will be on or off. This implies that PDF-Han signaling in four l-LNv neurons and one LNd neuron, in which Han is expressed, is necessary for the proper function of the morning and evening oscillators.

Han is homologous to the mammalian CT receptor and the $VPAC_2$ receptor (Figure 2). The mouse $VPAC_2$ receptor is known to be important for the maintenance of circadian rhythmic behavior and core clock gene expression in mice (Harmar et al., 2002). Both the CT receptor and the $VPAC_2$ receptor are expressed in the mammalian clock center SCN (Nakamoto et al., 2000; Harmar et al., 2002). Therefore, it is possible that a similar mechanism to coordinate interactions between clock neurons via PDF-Han signaling may also be present in the mammalian brain.

Screening mutants showing abnormal thermal preference originally led to the isolation of *han*. han^{X7867} prefers a colder temperature than normal only during the night (Figure 1A). However, han^{3369} and han^{5304}, which is null, did not show clear differences in temperature preference during day and night. Instead, they consistently preferred a temperature of 23.5ºC, slightly colder than normal. We do not yet know if Han has a role in temperature sensation or thermoregulation in flies. Because the pdf^{01} mutant has been reported to show abnormal geotactic behavior (Toma et al., 2002), we also examined whether *han* mutants exhibit any defects in geotaxis by means of a simple climbing assay described in Leal and Neckameyer (2002). In this assay, we did not observe any significant geotactic phenotypes in *han* mutants (data not shown). Our observation does not exclude the possibility that some subtle geotactic phenotypes are present in *han* mutants, which might be detectable by more elaborate analysis with the sophisticated apparatus described by Toma et al. (2002). Han expression in neurons other than clock neurons such as those in ventral nerve cords in the larval brain suggests that Han may play roles other than regulating circadian rhythmic behaviors. The possible roles of Han in other physiological or behavioral phenomena such as temperature preferences or sensation need to be investigated further in connection with circadian rhythms.

Experimental Procedures

Fly Strains

The EP lines han^{X7867} and han^{X3026} were purchased from GenExel, Inc. The pdf^{01} mutant and *pdf-Gal4* flies were obtained from Jae H. Park (University of Tennessee), and *per-Gal4* was obtained from M. Rosbash (Brandeis University). To generate *UAS-han* flies, the *han* EST cDNA (RH51443 from BDGP) was cloned into the pUAST vector and microinjected into w^{1118} embryos. *DDC-Gal4*, *UAS-EGFP*, and *Df(1)HC194/FM7c* flies were obtained from the Bloomington *Drosophila* Stock Center.

Circadian Behavior Analysis

Locomotor activity of each fly was monitored using the Trikinetics infrared beam-crossing system, as described elsewhere (Nitabach et al., 2002). Actimetrics Clocklab software was used to analyze the circadian rhythmic power and period by chi square peridograms with significance set to $\alpha = 0.025$. Flies with a χ^2 statistic ≥ 20 over the level of significance were considered to be rhythmic. In calculating the average rhythmic power in the DD phase, power below the level of significance was assigned a circadian power of 55.7, which corresponds to χ^2 statistic = 20. Rhythmic power was assessed from the third DD phase because pdf^{01} and han^{5304} mutants show dramatic arrhythmicity about two days after "light off." The average activity in the LD phase of each group was assessed over a period of at least 4 days. All of the flies' individual normalized activity counts were averaged to create a single average activity.

Immunohistochemistry

Flies were dissected in PBS and their brains transferred immediately to Zamboni's Fixative (4% paraformaldehyde, 7.5% saturated picric acid in 0.1 M PB) for Han antibody staining. When antibodies other than anti-Han were used, 4% paraformaldehyde without picric acid was used as the fixative. After 1 hr incubation at room temperature, brains were washed several times with PBS and PBT (PBS with 0.5% Triton X-100 [pH, 7.4]) and blocked with 5% normal donkey serum in PBT. The polyclonal rabbit Han antibody was used at a 1:1000 dilution. FITC- or TRITC-conjugated donkey anti-rat or anti-rabbit IgG antibodies (Jackson Laboratory) were used at 1:200 dilutions as the secondary antibody. PDF antibody, described elsewhere (Park et al., 2000), was obtained from J.H. Park. All antibody incubation was performed at 4ºC. To verify the binding specificity, Han antibody was preincubated with 10-fold excess of antigen peptide at 4ºC overnight prior to immunohistochemical staining.

Temperature Preference Assay

The temperature preference of adult flies was measured as described elsewhere (Lee et al., 2005). A temperature gradient from 14ºC to 44ºC with a slope of 0.75ºC/cm was produced between cold and hot water chambers in an aluminum block (42 cm × 24 cm × 7 cm). A glass plate slightly coated with quinine sulfate, an aversive stimulus for *Drosophila*, was placed 5 mm above the surface of the aluminum block. Three separate lanes (each, 8 cm wide) were formed along the temperature gradient channel. Adult flies were placed under the glass plate and above the aluminum block for 25 min in darkness before the photographs were taken. All experiments were performed under conditions of constant 25ºC temperature and 40% humidity. The age of all flies tested was synchronized at 3–7 days.

cAMP Measurement and Peptide Binding

S2 cells were cultured and maintained in M3 medium (Sigma). Culture of S2 cells and transfection with dimethyldioctadecylammonium bromide (DDAB; Fluka) were done as described elsewhere (Han, 1996). The *han* cDNA RH51443 and the cDNAs of CG8422 and CG32843, isolated by RT-PCR from head mRNA and confirmed by DNA sequencing, were cloned into pAC vector. The pAc vector is described elsewhere (Han, 1996). The PDF peptides, the C termini of which were amidated, and the FITC-PDF peptide, with its N terminus conjugated with FITC and its C terminus amidated, were chemically synthesized by Peptron, Inc. Proctolin, somatostatin, and myomodulin were purchased from Sigma. S2 cells were transfected with appropriate plasmids and incubated for 36 hr at 25ºC before treatment with PDF peptides. PDF peptides were applied to the S2 cells for 30 min at a final concentration of 1 μM. Intracellular cAMP levels were measured with the cAMP Biotrak Enzymeimmunoassay System (Amersham Bioscience) according to the manufacturer's instructions. To test FITC-conjugated PDF peptide binding to the cells expressing Han, the transfected S2 cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. After washing twice with PBS, cells were incubated with 0.1 μM FITC-PDF in peptide binding buffer (0.1% saponin/1% normal goat serum in PBS) for 30 min at room temperature and washed twice with the peptide binding buffer. The images were obtained with a Zeiss LSM510 confocal microscope.

Competition Binding Assay

Han cDNA was cloned into the plasmid pMtHy, resulting in pMtHy-Han (Panin et al., 1997). Selection of stably transfected S2 cells

containing Han-expressing pMtHy plasmids with hygromycin B and induction with $CuSO_4$ was performed as described elsewhere (Panin et al., 1997). Competition binding assays were performed as described in Cvejic et al. (2004) with the following modification. S2 cells containing pMtHy-Han were induced with $CuSO_4$ to express Han and plated evenly onto poly-L-lysine-coated 96-well plates for 24 hr. After washing with M3 culture medium to remove the $CuSO_4$, cells were incubated with 100 nM FITC-PDF in the absence or presence of increasing concentrations of unlabeled PDF or proctolin for 30 min at room temperature. Following washing of unbounded FITC-PDF ligand, the amount of bound FITC-PDF was determined by measuring fluorescence intensity with a Fluoroskan instrument (Ascent-LABSYSTEMS). Total binding was determined in the absence of unlabeled ligand; binding is presented as the percentage of total binding. Competition binding curves and estimated Kd values for FITC-PDF were calculated using GraphPad Prism software.

RT-PCR and Northern Blot

Total RNAs prepared from w^{1118} and han^{X7867} adult heads were reverse transcribed with the Reverse Transcription System (Promega), and PCRs were performed with a forward primer (5′-GAACATTCTC GACTGCGGA-3′) and a reverse primer (5′-GTGGTAGTAGGACT CTAGC-3′) to detect *han* transcripts. A pair of *rp49* primers was also included in the reactions to detect *rp49* transcripts as an internal control. PCR products were separated on 1% agarose gel by electrophoresis and quantified with a densitometric program (TINA; Raytest Isotopen Messgeräte, Straubenhardt, Germany). Northern blotting was performed with standard procedures using a formaldehyde agarose gel and morpholinepropanesulfonic acid buffer. Riboprobes labeled with ^{32}P were synthesized using the 1.5 kb EcoRI/XhoI fragment of a *han* EST cDNA RH51443 as the template.

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