Histamine-HisCl1 Receptor Axis Regulates Wake-Promoting Signals in *Drosophila melanogaster*

Yangkyun Oh, Donghoon Jang, Jun Young Sonn, Joonho Choe*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

### Abstract

Histamine and its two receptors, histamine-gated chloride channel subunit 1 (HisCl1) and ornithine transientless (Ort), are known to control photoreception and temperature sensing in *Drosophila*. However, histamine signaling in the context of neural circuitry for sleep-wake behaviors has not yet been examined in detail. Here, we obtained mutant flies with compromised or enhanced histamine signaling and tested their baseline sleep. Hypomorphic mutations in histidine decarboxylase (HDC), an enzyme catalyzing the conversion from histidine to histamine, caused an increase in sleep duration. Interestingly, hisCl1 mutants but not ort mutants showed long-sleep phenotypes similar to those in hdc mutants. Increased sleep duration in hisCl1 mutants was rescued by overexpressing hisCl1 in circadian pacemaker neurons expressing a neuropeptide pigment dispersing factor (PDF). Consistently, RNA interference (RNAi)-mediated depletion of hisCl1 in PDF neurons was sufficient to mimic the hisCl1 mutant phenotypes, suggesting that PDF neurons are crucial for sleep regulation by the histamine-HisCl1 signaling. Finally, either hisCl1 mutation or genetic ablation of PDF neurons dampened wake-promoting effects of elevated histamine signaling via direct histamine administration. Taken together, these data clearly demonstrate that the histamine-HisCl1 receptor axis can activate and maintain the wake state in *Drosophila* and that wake-activating signals may travel via the PDF neurons.

### Citation

Oh Y, Jang D, Sonn JY, Choe J (2013) Histamine-HisCl1 Receptor Axis Regulates Wake-Promoting Signals in *Drosophila melanogaster*. PLoS ONE 8(7): e68269. doi:10.1371/journal.pone.0068269

### Editor

Erik C. Johnson, Wake Forest University, United States of America

### Received

April 1, 2013; Accepted May 29, 2013; Published July 3, 2013

### Copyright

© 2013 Oh et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Funding

This work was supported by grants from the Brain Research Center of the 21st Century Frontier Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (MEST), the Republic of Korea, and from the National Research Foundation of Korea grant funded by MEST (No. 20110015442). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Competing Interests

The authors declared that no competing interests exist.

* E-mail: jchoe@kaist.ac.kr

### Introduction

Although sleep is known to be crucial for the physiology and life of an animal [1], the precise regulatory mechanisms that govern sleep are not yet fully understood. Sleep is regulated by the circadian rhythm and the homeostatic systems, which control the timing and need for sleep [2]. In mammals, hypothalamic neurons constitute one of the major control centers of sleep and wakefulness; in the hypothalamus, wake-promoting neurons and sleep-promoting neurons create a feedback loop that modulates sleep and wakefulness [3–7].

In mammals, histamine, a monoamine synthesized from histidine by histidine decarboxylase (HDC), is a major neurotransmitter that regulates learning, immune reactions [8], and sleep-wake behavior [9,10]. Histaminergic neurons control the wakefulness in the hypothalamus, especially in the tuberomammillary nucleus (TMN). There are four mammalian histamine receptors belonging to the family of rhodopsin-like G-protein-coupled receptors [11]: H1, H2, H3 and H4. The H1 receptor, which is coupled to the phospholipase C pathway for the activation of calcium signals [12], is known to play an important role in cognitive function and activation of the waking state [13,14]. H1 receptor antagonists have been used to treat allergic symptoms, but often show drowsiness as a common side effect; some (e.g., doxylamine succinate and diphenhydramine) are even used to treat insomnia. The H2 receptor, which activates cAMP signaling by activating adenylate cyclase, has memory modulating effects, but has little impact on sleep/wake regulation [13,15]. The H3 receptor acts as an auto-receptor in presynaptic histaminergic neurons and controls histamine turnover through feedback inhibition of histamine synthesis and release. The H4 receptor is located on presynaptic terminals and can affect the sleep/wake cycle as well as learning and memory by controlling histamine synthesis and release [16–18]. Finally, the H4 receptor is highly expressed in bone marrow and white blood cells and mediates several immune responses [19].

The fruit fly, *Drosophila melanogaster*, is an emerging model system for sleep research [20,21]. It has a simple central brain system that has streamlined the identification of novel sleep regulators and the mechanisms of sleep regulation [22]. The potassium channel, *Shaker*, was the first novel sleep-related gene found in *Drosophila* [23], and cAMP and protein kinase A (PKA) have been shown to regulate sleep in this model organism [24]. The mushroom body (MB) neurons, lateral ventral neurons (LNvs), pars intercerebralis (PI) neurons and dorsal fan-shaped body (FB) neurons constitute the brain regions known to be involved in regulating sleep in *Drosophila* [25–31], while the sleep-regulatory function of several monoamines, including dopamine, serotonin and octopamine, have also been elucidated [32–35].

In *Drosophila*, histamine acts as a neurotransmitter for photoreception and temperature sensing [36–40], and pharmacological tests have shown that it acts as a wake-activator [20]. Consistent...
with this function, administration of hydroxyzine, a histamine-receptor antagonist, was shown to increase sleep and reduce its latency [20]. However, the specific sleep/wake-controlling function of histamine and the hisCl1 and ort genes, which encode histamine-gated chloride channels that act as histamine receptors [41,42], have not yet been examined in detail.

Additionally, histamine is expressed in the eyelet axons and 18 cell bodies in protocerebrum which are designated HP1–4 and 2 cell bodies in the subesophageal ganglion (SOG) which are designated HSI [40,43]. The HP 3 neurons innervate the lobular and lateral protocerebrum in each hemisphere [43]. Even though the central body which containing mushroom body is devoid of histamine neuron fibers, histamine neurons are detected adjacent to the circadian clock neurons such as LNvs or dorsal neurons (DN) and HisCl1 receptor was identified in large LNvs [40,43,44]. According to these previous studies, we supposed that histaminergic signaling may be involved in sleep and circadian behavior in Drosophila.

Here, we used hypomorphic mutants and sleep profiles to reveal that the histamine-synthesizing HDC enzyme and the HisCl1 receptor have wake-promoting function, whereas the Ort receptor does not appear to have any sleep/wake regulatory function. Pharmacological and genetic approaches confirmed the wake-activating function of HDC and HisCl1-mediated histamine signaling. Furthermore, we identified PDF neurons as the source of the wake-activating function of the HisCl1 receptor pathway. Thus, although both HisCl1 and Ort play critical roles in photoreception and temperature sensing [39,40], only the HisCl1 pathway appears to play a wake-promoting role. This study is the first to report the sleep/wake regulatory function of histamine receptors in Drosophila. Understanding the histaminergic wake-activating system in Drosophila can provide helpful clues for human sleep research.

Results

Defects in the hdc Gene Cause Extended Sleep

To test the function of histamine in the regulation of sleep and wakefulness in Drosophila, we tested loss-of-function histamine signaling mutants. First, we tested two hypomorphic mutants of the hdc gene, hdcP211 and hdcP218, which express lower levels of the hdc gene compared to wild-type flies [w1118] [39]. Daytime sleep durations of the outcrossed hdc mutants were significantly longer than those of wild-type flies (Figure 1A, B). The daytime sleep-length extension was approximately 200 min in hdc mutants, and nighttime sleep durations were also increased but to a lesser degree than daytime (Figure 1C). The waking activity of the mutants was similar to that of wild-type flies (Figure 1D), suggesting that the longer sleep in hdc mutants was not due to their inactivity, but rather due to an actual increase in their sleep duration. The number and average duration of daytime sleep episodes in hdcP211 and hdcP218 increased compared to wild-type flies. However, those of nighttime sleep episodes were not changed (Figure S1A-D). This may be due to a ceiling effect, since wild type flies sleep for most of the nighttime. These results show that histamine acts as a wake-promoting factor and has a critical role in maintenance of the waking state.

Previous studies demonstrated that histamine and its receptors are involved in photoreception of Drosophila, and histamine signaling mutants have been found to exhibit visual system defects [39]. To exclude the possibility that a defect in the visual system contributed to the changes in sleep patterns observed in the hdc mutants, we evaluated the sleep phenotypes of hdc mutants in constant darkness. We reasoned that if defects in the visual system affected sleep in hdc mutants, then altered sleep phenotypes should not be found in constant darkness. However, the hdc mutant flies also showed increased sleep duration in constant darkness (Figure 1E, F). These results show that defects in the visual system do not cause sleep extensions observed in the hdc mutants.

Moreover, trans-heterozygotic mutants of hdcP211 and hdcP218 showed increased sleep durations similar to those of the homozygous hdcP211 and hdcP218 mutants (Figure S2A, B), whereas heterozygotes of hdcP211 and hdcP218 had a sleep duration similar to controls. These results suggest that neither the genetic background nor additional mutations contributed to the alteration of sleep patterns in the hdc mutants. Although these data were obtained from female flies, male hdc mutants also had longer sleep durations compared to wild-type flies (Figure S3A).

The HisCl1 Receptor is Involved in Sleep-regulatory Mechanisms

To understand the role of histamine signaling in sleep regulation, we investigated the sleep-regulatory function of two histamine receptors. First, we tested the sleep phenotypes of hisCl1 deletion mutants, hisCl1134 (containing a 1.7-kbp deletion) and hisCl1384 (containing a 1.0-kbp deletion from the 5’-end) [40]. These hisCl1 mutant flies showed elevated sleep durations (Figure 1G). Increase of both daytime and nighttime sleep was significant, but increase of the daytime sleep was greater than increase in nighttime sleep. The daytime sleep durations were increased by 150 min in hisCl1 deletion mutants compared to wild-type flies [w1118] (Figure 1H, I). The waking activities of the mutants were unchanged (Figure 1J), indicating that our results reflected an increased sleep duration rather than inactivity. The number and average duration of daytime sleep episodes were increased in hisCl1 mutant flies (Figure S1E, G), further suggesting that the downstream signaling of the HisCl1 receptor is involved in sleep initiation and maintenance. However, the number and average duration of nighttime sleep episodes did not show a significant change (Figure S1F, H). As mentioned previously, this would be ceiling effect of nighttime sleep.

Since HisCl1 also functions in light perception [37], we assessed sleep phenotypes of hisCl1 mutants in constant darkness to exclude the possibility that defects in the visual system affected their sleep patterns. The hisCl1 mutant flies showed increased sleep durations compared to wild-type flies in constant darkness (Figure 1K, L). This result means that defects in the visual system do not cause the sleep extensions observed in the hisCl1 mutants.

To exclude the possible genetic background effects, we evaluated sleep durations in heterozygotes and trans-heterozygotes of the hisCl1 mutants. The sleep durations of heterozygotes of the hisCl1 mutants were similar to those of wild-type flies, but were longer in the trans-heterozygotes of hisCl1134 and hisCl1384 (Figure S2C, D), indicating that the genetic background did not affect the experimental outcome. Additionally, the male flies showed similar sleep pattern to those of female flies (Figure S3B). Collectively, these findings indicate that the histamine receptor, HisCl1, can activate wakefulness and that the activation pathway may be shared with the HDC enzyme. Thus, our results suggest that the histamine-HisCl1 receptor axis can activate and maintain wakefulness in Drosophila.

The Ort Receptor is Not Involved in Sleep-regulatory Mechanisms

To further explore the sleep-regulatory effects of histamine receptors, we tested the role of the Ort receptor using the mutant CSor1, which has a 569-bp deletion encompassing the second and
Data are represented as mean type (n = 27) in constant darkness (DD), but it is not significant enough. All flies were 4 to that of wild-type flies (L:D). (Figure 1B, C) The hdc mutants, hdcP211 (n = 24) and hdcP218 (n = 45), have longer sleep durations compared to control flies (w1118, n = 68). (D) The hdcP211 and hdcP218 have waking activity levels similar to that of the control (w1118). (E, F) The daytime and nighttime sleep duration of hdcP211 (n = 20) flies is increased compared to wild-type (n = 27) in constant darkness (DD). (G) Sleep profiles of hisCl1 mutants in 12 hr:12 hr light dark (L:D). (Figure 2B, C) The hisCl1 mutants, hisCl1384 (n = 50) and hisCl1313 (n = 57), have longer sleep durations compared to control flies. (J) The hisCl1 and hisCl1384 have waking activity levels similar to that of control flies (w1118). (K) The daytime sleep durations of hisCl1384 (n = 17) are increased compared to that of wild-type flies (w1118, n = 27) in constant darkness (DD). (L) The nighttime sleep duration of hisCl1384 (n = 17) flies is longer than that of wild-type (n = 27) in constant darkness (DD), but it is not significant enough. All flies were 4–6-day-old females. The results were averaged over two days. Data are represented as mean ± s.e.m. (**, p < 0.01; Student’s t test). doi:10.1371/journal.pone.0068269.g001

Histamine Signaling

The PDF Neurons are Essential for the Wake Activation of Histamine Signaling

To confirm the wake-activating function of the histamine-HisCl1 receptor axis, we suppressed the expression of histamine signaling genes using RNAi lines. Knockdown of hdc or hisCl1 by targeted expression of either hdc-RNAi or hisCl1-RNAi using pan-neuronal elav-Gal4 significantly increased sleep duration, whereas the knockdown of Ort receptor using ort-RNAi did not (Figure 3A, B). Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that transcript level in RNAi expressing flies was indeed lower than those of heterozygotic controls (Figure S4A–C). These results reconfirm that HDC and HisCl1 receptors have wake-promoting function, but the Ort receptor does not.

Histamine is mainly expressed in 10 cell bodies in each hemisphere of the Drosophila brain [43]. Moreover, our anti-histamine staining reconfirmed a previous study showing histamine-expressing cells being located near PDF neurons (Figure 3C) [44]. HisCl1 receptor was reported to be expressed in l-LNvs [40], suggesting that PDF neurons can receive histaminergic wake-activation signals, which could arise via the secretion of histamine from the HP2 or HP3 cell bodies in each hemisphere [43]. To clarify that the HisCl1 receptor in PDF neurons has a wake-promoting function, we reduced hisCl1 gene expression in PDF neurons using hisCl1-RNAi via pdf-Gal4. As predicted, the daytime and nighttime sleep duration was increased in hisCl1-knockdown flies (Figure 3D, E). However, knockdown of hisCl1 by targeted expression of hisCl1-RNAi in ort-expressing neurons using ort-Gal4 did not extend the sleep duration (Figure 3D, E).

Next, we performed genetic restoration experiments in which we restored the expression of hisCl1 gene in the mutant background using a UAS-hisCl1 line. To verify the overexpression of hisCl1 gene via the Gal4/UAS system, the transcript level of hisCl1 was monitored by reverse transcription-polymerase chain reaction (RT-PCR) (Figure S4D). The expression of UAS-hisCl1 using either tim-Gal4 or pdf-Gal4 in hisCl1 mutant flies restored the
increased daytime sleep duration back to the wild-type level. In contrast, expression of either UAS-hisCl1 via dilp2-Gal4 or UAS-ort via pdf-Gal4 did not restore the increased daytime sleep duration of hisCl1 mutant flies (Figure 3F). Together, these results strongly support that histamine has a wake-promoting function, specifically via the HisCl1 receptor in PDF neurons.

Sleep Duration in hdc Mutants is Reduced by Histamine Treatment

To activate histamine signaling through pharmacological means, we administered the exogenous histamine to wild type flies. To determine the optimal level of exogenous histamine, we fed wild-type flies 100 mM and 250 mM of histamine and examined their sleep behaviors. When flies were fed 100 mM of histamine, the daytime sleep duration was decreased but was not significant enough. In contrast, treating with 250 mM of histamine caused significant daytime and nighttime sleep reductions (Figure 4A–C), but did not cause any difference in food preference between histamine-containing and non-containing food (data not shown). Moreover, administration of 250 mM histamine decreased the sleep duration in ort mutants but not in hisCl1 mutants (Figure 4D–I). Additionally, administration of 250 mM of histamine could not shorten the sleep duration in flies with a pan-neuronal knockdown of hisCl1 (Figure S5), indicating that histamine administration could activate the waking state only through the HisCl1 receptor.

When 250 mM histamine was fed on day 3, sleep duration was reduced by 150–200 min and this sleep reduction occurred during both daytime and nighttime (Figure 5A, B). On the first day of recovery (day 4), histamine-fed w1118 flies showed sleep rebounds due to the prior day’s sleep loss. On the second day of recovery (day 5), the histamine-fed w1118 flies showed a sleep pattern similar to that of the histamine-unfed w1118 flies (Figure 5A, B). By day 5 (second day of recovery), the sleep duration was once again extended in histamine-fed hdcP218 flies. These results suggest that histamine administration causes hdcP218 flies to exhibit a wild-type sleep pattern.

As expected, histamine administration reduced the number of sleep episodes in the tested lines. When histamine treatment
activated histamine signaling in \textit{hdcP218} flies, the increase in the number of daytime sleep episodes was restored back to the wild-type level (Figure 5C). After 2 days of recovery from histamine administration, the number of daytime sleep episodes in \textit{hdcP218} flies increased back to the level seen in \textit{hdcP218} flies before histamine administration. By day 4 (first day of recovery), the number of daytime sleep episodes of histamine-fed \textit{w1118} flies surpassed the number of daytime sleep episodes of histamine-fed \textit{ort-RNAi} flies, indicating that the recovery of sleep bout number was faster than the recovery of sleep duration. Taken together, these data show that histamine administration activates and maintains the wake-promoting signal which does not cause any irreversible defects in the neuronal system of \textit{Drosophila}.

**Histamine Administration Reconfirms that the PDF Neurons are Important for Wake-activation by Histamine Signaling**

To further identify the brain regions that modulate wake-activation by histamine signaling, we fed histamine to \textit{UAS-hid,rpr} expressing flies; these flies express \textit{reaper} and \textit{hid}, which can ablate specific brain regions when placed under the control of different \textit{Gal4} drivers [46,47]. We reasoned that if the ablated brain regions are important in wake-activation by histamine, then histamine treatment should not change the sleep durations when specific brain regions were ablated. If they are not involved, however, histamine administration should produce a sleep-reduction phenotype similar to that seen in wild-type flies. As a control, histamine was administrated to heterozygotes of the \textit{UAS-hid,rpr} line (\textit{UAS-hid,rpr/+}). Histamine-fed \textit{UAS-hid,rpr} heterozygotes...
Figure 4. Histamine treatment dose-dependently decreases sleep duration and it decreases sleep duration of ort mutant but not of hisCl1 mutants. (A–C) Wild-type flies (w1118) were exposed to food containing 100 mM (n = 14) and 250 mM (n = 21) histamine, or to histamine untreated food (n = 28). The sleep duration is dose-dependently reduced in histamine-fed flies compared to unfed flies. (D–F) CS;ort1 flies fed with histamine-HisCl1 Axis Regulates Wakefulness
Histamine-HisCl1 Axis Regulates Wakefulness

showed a reduction in sleep duration comparable to that of histamine-unfed UAS-hid,spex heterozygotes during both daytime and nighttime (Figure 6A, D, E), suggesting that the genetic background did not affect the wake-activating function of histamine administration in these flies. Next, 250 mM histamine was fed to ort neuron-ablated flies. We found out that the sleep duration of the histamine-fed line was significantly decreased during both daytime and nighttime, providing a pharmacological confirmation that ort neurons (and thus Ort receptors) are not important in wake-activation by histamine signaling (Figure 6B, D, E).

The circadian rhythms of both hdc and hisCl1 mutants were normal in constant darkness (Table S1). These results suggest that PDF neurons may regulate sleep and circadian rhythms using independent signaling pathways. Moreover, The expression of PDF was unchanged in either hdc or hisCl1 mutants and administration of 250 mM histamine decreased the sleep duration in the loss-of-function mutant of the pdf gene (pdf01) (data not shown). Thus, we reasoned that if PDF neurons are important in wake activation, then histamine administration should not reduce sleep duration in the pdf-Gal4/UAS-hid,spex line, in which the ablation of PDF neurons was confirmed by PDF staining (data not shown). Our results revealed that administration of 250 mM histamine to PDF neuron-ablated flies could not reduce their daytime and nighttime sleep duration, confirming that PDF neurons are important for wake-activation by histamine (Figure 6C–E).

Because using UAS-hid,spex to ablate cells can cause several defects during developmental stages, we used an UAS-shibire line in which the targeted Gal4 expressing synapses are conditionally blocked only at a high temperature [48–50]. Using the UAS-shibire line along with pdf-Gal4, we showed that conditional synaptic blocking in PDF neurons inhibited the sleep reduction caused by 250 mM administration of histamine (Figure S6). This further confirms that PDF neurons can regulate histaminergic wake-promoting signals.

Discussion

Using genetic and pharmacological methods to manipulate histamine signaling, we show that the HisCl1 receptor and its downstream signaling cascade regulate wake-evoking behavior in Drosophila, while Ort receptor does not show any sleep/wake regulatory function. Histamine promotes activity via the HisCl1 receptor. Reduced histamine in HDC mutants or loss of the HisCl1 receptor both show reduced activity and enhanced sleep. Additionally, the relevant signaling pathway downstream of the HisCl1 receptor may function in the PDF neurons. Finally, we demonstrate that the histamine-HisCl1 receptor axis can activate and maintain wakefulness in PDF neurons.

These data show the complete functional segregation of the two histamine receptors for the first time. Ort receptor is expressed in large monopolar cells (LMC), postsynaptic to photoreceptors in the lamina and is a major target of photoreceptor synaptic transmission in Drosophila. In contrast to Ort, HisCl1 receptor is not expressed in postsynaptic neurons of photoreceptors. It is expressed in lamina glia and shapes the LMC postsynaptic response of Ort signaling [37]. Both Ort and HisCl1 receptor are involved in temperature-preference behaviors [40], but the major independent function of HisCl1 receptor remains elusive. In this study, we found out that sleep regulation is a novel and independent function of HisCl1 receptor. Additionally, this finding is an important clue in understanding the functional evolution of the two histamine receptors in Drosophila.

We propose that wake-activation by histamine signaling in Drosophila is similar to that found in mammals. We found out that hdc mutant flies have increased sleep durations compared to controls and a previous study showed that HDC-knockout mice have increased paradoxical sleep compared to controls [10]. This suggests that the HDC enzyme has a common wake-promoting function in mammals and flies. However, the structures of histamine receptors differ between flies and mammals; the histamine receptors of Drosophila are histamine-gated chloride channels, whereas the mammalian histamine receptors belong to the rhodopsin-like G-protein-coupled receptor family [11,45]. Currently, researchers are working to identify a metabotropic histamine receptor in Drosophila [43,51]. Despite the structural differences of the mammalian and Drosophila receptors, they share a wake-activating function. This functional homology may be the result of evolution and gives us a hint to find out the metabotropic histamine receptors in Drosophila.

Surprisingly, functional conservations between flies and mammals are also found among the histamine receptor subtypes. The HisCl1 receptor has a wake-activating role, whereas the Ort receptor does not. This result parallels differences in the wake-activation roles of the H1 and H2 receptors in mammals: the H1 receptor can activate wakefulness, but the H2 receptor cannot [14,15]. Thus, our data provide a more detailed understanding of the potential functional relationship between the HisCl1 and H1 receptors. A functional connection between the Ort receptor and the H2 receptor is also possible, since the two have little effect on sleep/wake regulation in their corresponding model systems. No auto-receptor of histamine has yet been found in Drosophila, suggesting that there may not be a Drosophila homolog for the mammalian H2 receptor. Further research should shed greater light on the evolutionary relationship between the histamine receptors of flies and mammals.

Histamine signaling modulates the maintenance of wakefulness and controls light sensing, and we speculate that a number of interactions are possible between these two different pathways. Previous studies on light-perception mechanisms showed that histamine mutants exhibit light-sensing defects [36–39]. However, we found out that the sleep duration was increased in histamine signaling mutants compared to wild-type flies in constant darkness (Figure 1F and 2F). Thus, the perception of light in the context of evoking wakefulness is independent of vision-related light perception in Drosophila. Further research will be required to definitively establish the relationship between light perception and sleep regulation.

Previous studies revealed that the PDF neurons promote wakefulness in Drosophila [28,52]. Our findings show that histamine signaling acts as a wake-promoting pathway in PDF neurons. The HisCl1 receptor is a chloride channel, which would be expected to inhibit the function of the neurons. However, since previous studies showed that chloride channels can activate the function of the neurons [53,54], hence the HisCl1 receptor might be an activator of the PDF neurons. The downstream signaling of histamine-HisCl1 receptor in PDF neurons should be further
studied using genetic manipulation and electro-physiological methods.

Orexin is a neuropeptide that acts as an important wake-activating neurotransmitter in mammals, as shown by the

Figure 5. The administration of histamine decreases the sleep duration in control flies and hdc mutants. (A, B) Sleep profiles and daytime sleep durations of histamine-fed and -unfed flies. Days 1 and 2 show baseline recordings prior to histamine treatment. At 9:00 a.m. of day 3, 250 mM histamine was fed to wild-type (w1118, n = 19) and hdcP218 flies (hdcP218, n = 18), while a control group (w1118, n = 18) was not fed histamine. At 9:00 a.m. of day 4, all flies were transferred to fresh histamine-untreated food. (C) Daytime sleep-bout numbers of histamine-fed and -unfed flies. All flies were 4–6-day-old females. Data are represented as mean ± s.e.m. (**, p<0.01; Student’s t test).

doi:10.1371/journal.pone.0068269.g005
demonstration that defects in orexin synthesis can cause narcoleptic symptoms in human and animals [6,55–57]. Orexin neurons activate wakefulness in the lateral hypothalamic area and the feedback loop between orexin neurons and monoaminergic neurons such as histaminergic and serotonergic neurons (tuberomammillary nucleus, TMN, and dorsal raphe nucleus, DR) controls wakefulness in the hypothalamus and the brain stem [6,7,55,58–60]. Histamine receptors are essential for wake-activation by orexin treatment [61], indicating that orexin and histamine signaling constitute an interactive wake-activating system in mammals. However, orexin has not been found in *Drosophila*. A previous study suggested that the PDF neuropeptide functions similar to those of orexin in *Drosophila* [52], potentially explaining many aspects of the wake-activation cascade in *Drosophila*. Histamine and orexin signaling constitute an interactive wake-activating system in mammals. Moreover, we demonstrate that histamine-signaling mutants cannot maintain wakefulness during the daytime, which is similar to the phenotype of orexin mutants in mammals. Hence, we propose that, in *Drosophila*, histamine may have a function similar to that of the mammalian orexin. Further research is required to establish the functional relationship between wake activation of histamine signaling in *Drosophila* and wake-promoting function of orexin and histaminergic system in mammals.

**Materials and Methods**

**Fly Strains**

All flies were reared on standard cornmeal-yeast-agar medium at 25°C under 12 h light/12 h dark conditions (LD cycle). The hisCl1134, hisCl1384, ort-Gal4, and isogenic control w1118 flies were graciously provided by Dr. Hong (KAIST, Republic of Korea, Daejeon) [40]. The pdf-Gal4 [62], tim-Gal4 [63], diap2-Gal4 [64], UAS-shibirets [48–50] lines were as described previously. The
Behavior Assays Following Pharmacological Treatments

Histamine diphosphate (Sigma, St. Louis, MO; catalog number, 53310) was dissolved in distilled water (DW) and mixed with 4% sucrose and 2% agar (100 mM and 250 mM). We recorded two days of baseline sleep before drug administration, and then moved flies from screening vials (4% sucrose and 2% agar) to the drug-treated vials (4% sucrose, 2% agar and histamine diphosphate) at 9:00 a.m. After one day of drug administration, the flies in the drug-treated vials were returned to normal screening vials, and behavioral recording was continued for two more days to monitor the sleep recovery of histamine-fed flies.

Immunohistochemistry (IHC)

For histamine antibody staining, tissues were fixed in 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma) dissolved in PBST, blocked with 3% normal goat serum in PBST, and then incubated overnight with anti-histamine (1:500 in blocking solution; Immunostar, Inc., Hudson, WI) at 4°C. The tissues were then washed with PBST and incubated with rhodamine-conjugated goat anti-rabbit (1:250; Jackson Immunoresearch). Tissues were mounted in a VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and examined by confocal microscopy (LSM510; Zeiss, Thornwood, NY).

Supporting Information

Figure S1 Sleep parameters of histamine-signaling mutant flies. (A, C) The daytime sleep-bout number and average duration of hdeP217 (n = 24) and hdeP218 (n = 45) flies are elevated compared to control flies (u1118, n = 68); (B, D) The nighttime sleep-bout number and durations of the hdeP217 and hdeP218 mutants are similar to those of the control (u1118). The daytime sleep-bout numbers and average durations of the hisCl1P218 and hisCl1P214 flies are elevated compared to control flies (u1118). The nighttime sleep-bout number and durations of the hisCl1P214 flies are similar to those of the control (u1118). All flies were 4–6-day-old females. The results were averaged over two days. Data are represented as mean ± s.e.m.** p<0.01; Student’s t test.

Figure S2 Trans-heterozygotes of either hde or hisCl1 mutants have increased sleep durations. (A, B) The heterozygous hdeP218 mutants, hdeP217/+ (n = 16) and hdeP218/+ (n = 16), have sleep durations similar to that of their wild-type control, u1118 (+/+, n = 68). The trans-heterozygous mutant of hdeP217 and hdeP218, hdeP217/+ and hdeP218, n = 38) shows an increased sleep duration similar to that of the homozygous hde mutants. (C, D) The heterozygous hisCl1 mutants, hisCl1P218/+ (n = 14) and hisCl1P214/+ (n = 15), have sleep durations similar to that of their wild-type control, u1118 (+/+, n = 68), but the trans-heterozygote of hisCl1P214 and hisCl1P214 (hisCl1P218/hisCl1P214, n = 35) shows a longer sleep duration. All flies were 4–6-day-old females. The results were averaged over two days. Data are represented as mean ± s.e.m.** p<0.01; one-way ANOVA.

Figure S3 Sleep patterns of male histamine-signaling mutant flies. (A) Sleep profiles of hde male mutants in 12 hr:12 hr light dark (LD). Male flies of the hde mutant lines, hdeP217 (n = 29) and hdeP218 (n = 29), have increased daytime sleep durations compared to control flies (u1118, n = 49). (B) Sleep profiles of hisCl1 male mutants in 12 hr:12 hr light dark (LD). The male flies of the hisCl1 mutant lines, hisCl1P218 (n = 38) and hisCl1P214...
durations are reduced in histamine-fed CS; ort1 flies (n = 28) compared to histamine-unfed flies (n = 18) and elav-Gal4/hdc-RNAi flies. Daytime and nighttime sleep durations are reduced in histamine-fed ort-RNAi/+ (n = 20) flies and elav-Gal4 hdc-RNAi flies (n = 18) compared to those in histamine-unfed ort-RNAi/+ (n = 18) and elav-Gal4 hdc-RNAi flies (n = 40) flies.

Figure S5 The administration of histamine does not reduce sleep duration in hscCl1 knockdown mutants. (A–C) Sleep profiles and sleep durations of histamine-fed and -unfed elav-Gal4/+ and elav-Gal4/hdc-RNAi flies. Daytime and nighttime sleep durations are reduced in histamine-fed elav-Gal4/+ (n = 13) and elav-Gal4/hdc-RNAi flies (n = 28) flies compared to histamine-unfed elav-Gal4/+ (n = 26) and elav-Gal4/hdc-RNAi flies (n = 32) flies. (D–F) Sleep profiles and sleep durations of histamine-fed and -unfed ort-RNAi/+ and elav-Gal4 ort-RNAi flies. Daytime and nighttime sleep durations are reduced in histamine-fed ort-RNAi/+ (n = 20) and elav-Gal4 ort-RNAi flies (n = 18) flies compared to those in histamine-unfed ort-RNAi/+ (n = 18) and elav-Gal4 ort-RNAi flies (n = 40) flies. (G–I) Sleep profiles and sleep durations of histamine-fed and -unfed hisCl1-RNAi/+ and elav-Gal4 hisCl1-RNAi flies. Daytime and nighttime sleep durations are reduced in histamine-fed hisCl1-RNAi/+ (n = 14) flies compared to those in histamine-unfed hisCl1-RNAi/+ (n = 15) flies. However, histamine-fed elav-Gal4 hisCl1-RNAi flies (n = 28) flies show similar sleep durations compared to histamine-unfed elav-Gal4 hisCl1-RNAi flies (n = 49), during both daytime and nighttime. (+) and (−) indicate the 250 mM histamine-fed and -unfed flies respectively. All flies were 4–6-day-old females. Data are represented as mean ± s.e.m. (**, p < 0.01; Student’s t test).

TI Figure S6 The administration of histamine does not decrease the sleep durations in PDF neuron-inhibited flies. (A, C, D) Histamine-fed pdf-Gal4/+ (n = 25) flies show a reduced sleep duration compared to the untreated control (pdf-Gal4/+; n = 27) at 29°C, during both daytime and nighttime. (B, C, D) Histamine administration does not decrease the sleep duration of pdf neuron-inhibited flies (pdf-Gal4/UA5-9shibire3; n = 61) compared to untreated controls (pdf-Gal4/UA5-9shibire3; n = 36) at 29°C, during both daytime and nighttime. All flies were 4–6-day-old females. Data are represented as mean ± s.e.m. (**, p < 0.01; Student’s t test).

Table S1 Histamine signaling mutants show normal locomotor activity rhythms in constant darkness.

Acknowledgments

We thank C. Lim for helpful discussions and suggestions, S-T Hong, W. Pak, C-H Lee, P. H. Taghert, H. Keshishian, R. Nusse and the Bloomington Stock Center for sharing their fly stocks, Y. J. Kim for generating the UAS-hscCl1 transformants.

Author Contributions

Conceived and designed the experiments: YO JC. Performed the experiments: YO DJ JYS. Analyzed the data: YO. Contributed reagents/materials/analysis tools: YO DJ JYS. Wrote the paper: YO JYS JC.

References

1. Siegel JM (2005) Clues to the functions of mammalian sleep. Nature 437: 1264–1271.
2. Beersma DG (1998) Models of human sleep regulation. Sleep Med Rev 2: 31–49.
3. Mignot E, Taheri S, Nishino S (2002) Sleeping with the hypothalamus: emerging therapeutic targets for sleep disorders. Nat Neurosci 5 Suppl: 1071–1075.
4. Szymusiak R, McGinty D (2008) Hypothalamic regulation of sleep and arousal. Physiol Rev 88: 1183–1241.
5. Leurs R, Traiffort E, Arrang JM, Tardivel-Lacombe J, Ruat M, et al. (1994) Guinea pig histamine H1 receptor. II. stable expression in chinese hamster ovary cells reveals the interaction with three major signal transduction pathways. J Neurochem 62: 519–527.
6. Schwartz JC, Arrang JM, Barbarg M, Pollard H, Ruat M (1991) Histaminergic transmission in the mammalian brain. Physiol Rev 71: 1–51.
7. Dai H, Kaneko K, Kato H, Fujii S, Jing Y, et al. (2007) Selective cognitive dysfunction in mice lacking histamine H1 and H2 receptors. Neurosci Res 57: 306–313.
8. Huang ZL, Mochizuki T, Qu WM, Hong ZY, Watanabe T, et al. (2006) Altered sleep-wake characteristics and lack of arousal response to H3 receptor antagonist in histamine H1 receptor knockout mice. Proc Natl Acad Sci U S A 103: 4607–4602.
9. Monti JM (1990) Sleep variables are unaltered by zolantidine in rats: are histamine H2 receptors not involved in sleep regulation? Brain Res Bull 25: 229–231.
10. Ebenshade TA, Brownwe KE, Bimer RS, Strakhova M, Cowart MD, et al. (2000) The histamine H3 receptor: an attractive target for the treatment of cognitive disorders. Br J Pharmacol 134: 1166–1181.
11. Leurs R, Bakker RA, Timmerman H, de Esch IJ (2005) The histamine H3 receptor: from gene cloning to H3 receptor drugs. Nat Rev Drug Discov 4: 107–120.
12. Toyota H, Dagovic K, Kochl M, Laposky AD, Weber C, et al. (2002) Behavioral characterization of mice lacking histamine H3 receptors. Mol Pharmacol 62: 389–397.
13. de Esch IJ, Thurmoul RL, Jongejan A, Leurs R (2005) The histamine H1 receptor as a new therapeutic target for inflammation. Trends Pharmacol Sci 26: 462–469.
14. Shaw DJ, Cirelli G, Greenspan RJ, Tomoni G (2000) Correlates of sleep and waking in Drosophila melanogaster. Science 287: 1834–1837.
15. Hendrickx JC, Funn SM, Panckeri KA, Chavkin J, Williams JA, et al. (2000) Rest in Drosophila is a sleep-like state. Neuron 25: 129–138.
16. Hendrickx JC, Schgal A, Pack AI (2005) The need for a simple animal model to understand sleep. Prog Neurobiol 61: 339–351.
17. Cirelli C, Bushey D, Hill S, Huber R, Krieger R, et al. (2005) Reduced sleep in Drosophila shaker mutants. Nature 434: 1087–1092.
18. Cirelli C, Williams JA, Panckeri K, Kirk D, Tello M, et al. (2001) A non-circadian role for cAMP signaling and CREB activity in Drosophila rest homeostasis. Nat Neurosci 4: 1094–1099.
20. Shang Y, Griffith LG, Roshash M (2008) Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci U S A 105: 19537–19542.

21. Donlea JM, Thüngam MG, Suzuki Y, Gottschalk L, Shaw PJ (2011) Inducing sleep by remote control facilitates memory consolidation in Drosophila. Science 332: 1571–1576.

22. Liu Q, Liu S, Kodama L, Driscoll Maria R, Wu Mark N (2012) Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila.Curr Biol 22: 2114–2123.

23. Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, et al. (2012) Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. Nat Neurosci 15: 1516–1523.

24. Iovchev M, Kodrov P, Wolstenholme AJ, Pak WL, Semenov EP (2002) Altered drug resistance and recovery from paralysis in Drosophila melanogaster with a deficient histamine-gated chloride channel. J Neurogenet 16: 1051–1062.

25. Crocker A, Shahidullah M, Levitan IB, Sehgal A (2010) Identification of a neural circuit that underlies the effects of octopamine on sleepwake behavior. Neuron 65: 670–681.

26. Yuan Q, Joiner WJ, Sehgal A (2006) A sleep-promoting role for the Drosophila serotonin receptor 1A. Curr Biol 16: 1051–1062.

27. Rulifson EJ, Kim SK, Nusse R (2002) Ablation of insulin-producing neurons in adult Drosophila melanogaster causes hyperphagia, obesity, and many other phenotypes. Cell 110: 269–279.

28. Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, et al. (2001) Arousal regulator of arousal in the fruit fly. J Neurosci 25: 7377–7384.

29. Groth AC, Fish M, Nusse R (2004) Construction of transgenic flies: growth and diabetic phenotypes. Science 296: 1118–1120.

30. Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A pdf gene regulates circadian rhythmicity in flies: growth and diabetic phenotypes. Science 296: 1118–1120.

31. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering gene expression in flies. Development 118: 401–415.

32. Hambleon MJ, White NE, Emery PJT, Kaiser K, Hall JC (1998) Molecular and behavioral analysis of four period mutants in Drosophila melanogaster encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics 149: 165–178.

33. Cirelli C (2003) Searching for sleep mutants of Drosophila melanogaster. Bioessays 25: 940–949.