NEUROPHYSIOLOGY

A sleep-like state in *Hydra* unravels conserved sleep mechanisms during the evolutionary development of the central nervous system

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Sleep behaviors are observed even in nematodes and arthropods, yet little is known about how sleep-regulatory mechanisms have emerged during evolution. Here, we report a sleep-like state in the cnidarian *Hydra vulgaris* with a primitive nervous organization. *Hydra* sleep was shaped by homeostasis and necessary for cell proliferation, but it lacked free-running circadian rhythms. Instead, we detected 4-hour rhythms that might be generated by ultradian oscillators underlying *Hydra* sleep. Microarray analysis in sleep-deprived *Hydra* revealed sleep-dependent expression of 212 genes, including cGMP-dependent protein kinase 1 (PRKG1) and ornithine aminotransferase. Sleep-promoting effects of melatonin, GABA, and PRKG1 were conserved in *Hydra*. However, arousing dopamine unexpectedly induced *Hydra* sleep. Opposing effects of ornithine metabolism on sleep were also evident between *Hydra* and *Drosophila*, suggesting the evolutionary switch of their sleep-regulatory functions. Thus, sleep-relevant physiology and sleep-regulatory components may have already been acquired at molecular levels in a brain-less metazoan phylum and reprogrammed accordingly.

INTRODUCTION

Sleep-like states have been defined in a wide range of animal taxa (*1*). The neural mechanisms underlying sleep are substantially conserved among vertebrates, arthropods, and nematodes. However, it remains elusive how the molecular commonality of sleep regulation has been shaped during the evolutionary process of the central nervous system (CNS). In this sense, Cnidaria is ideally positioned in the phylogenetic tree of the animal kingdom (*2*). Cnidarians lack a centralized nervous system and thus are thought not to have a brain. Their diffuse nerve net innervates most parts of the body, likely representing the ancestral organization of the nervous system. Daily periodic quiescence has been observed in cnidarian species. For example, soft corals display 24-hour pulsation-rest rhythms in their tentacle movement (*3*). The box jellyfish *Tripedalia* is active during the day, whereas *Copula* exhibits nocturnal behaviors (*4*). The upside-down jellyfish *Cassiopea* also display sleep-like behaviors (*5*) comparable to those in other animals with a well-defined CNS. Their common features include (i) reversible behavioral quiescence, (ii) reduced responsiveness to stimuli (i.e., high arousal threshold), (iii) regulation by circadian clocks and sleep homeostasis, and (iv) pharmacological effects of sleep-inducing drugs such as melatonin and a histamine receptor antagonist. These findings support the hypothesis that the phylogenetic origin of sleep has preceded the evolution of a centralized nervous system (*5*). We further reasoned that comparative analyses of the sleep-regulatory mechanisms across animal species, including those with a poorly defined CNS, should elucidate how this essential physiology has evolved along with the development of the CNS.

RESULTS

A sleep-like state in brain-less *Hydra*

We used *Hydra vulgaris* as the cnidarian model of sleep (Fig. 1A). *Hydra* consists of only two cell layers (*6*) (Fig. 1B), displaying the simplest body plan among cnidarians. To detect any sleep-like state in *Hydra*, we video-recorded their movement under 12-hour light:12-hour dark (LD) cycles (Fig. 1C). We measured changes in the pixel value between two consecutive imaging frames at 5-s intervals. Any differences above a threshold were then regarded as movements in individual animals (Fig. 1D). We also calculated the ratio of frames with detectable movements in a 2-min bin (i.e., fraction movement) and defined "behavioral quiescence" if no movement was detected in more than 50% frames per 2-min bin (i.e., fraction movement <0.5). This frame subtraction analysis revealed that *Hydra* exhibited diurnal behaviors in LD conditions (Fig. 1E).

We further analyzed the quiescent state in *Hydra* based on the behavioral criteria of sleep (*7*). *Hydra* had a reversible episode of the behavioral quiescence because they spontaneously entered a quiescent period, while a light pulse at night readily interrupted their quiescent state (Fig. 1F and movie S1). The latency to light-induced arousal inversely correlated with the intensity of light stimuli at night (Fig. 1, G and H). Animals in the quiescent state for longer than 20 min showed a significant delay in their responses to the light pulse (Fig. 1H). A similar delay in sensory responsiveness was observed with the behavioral quiescence when we provided *Hydra* with reduced glutathione (GSH) as a feeding signal. *Hydra* forms tentacle balls as a sensory response to GSH (fig. S1A) (*8*). Latency to the sensory response substantially increased when GSH was given

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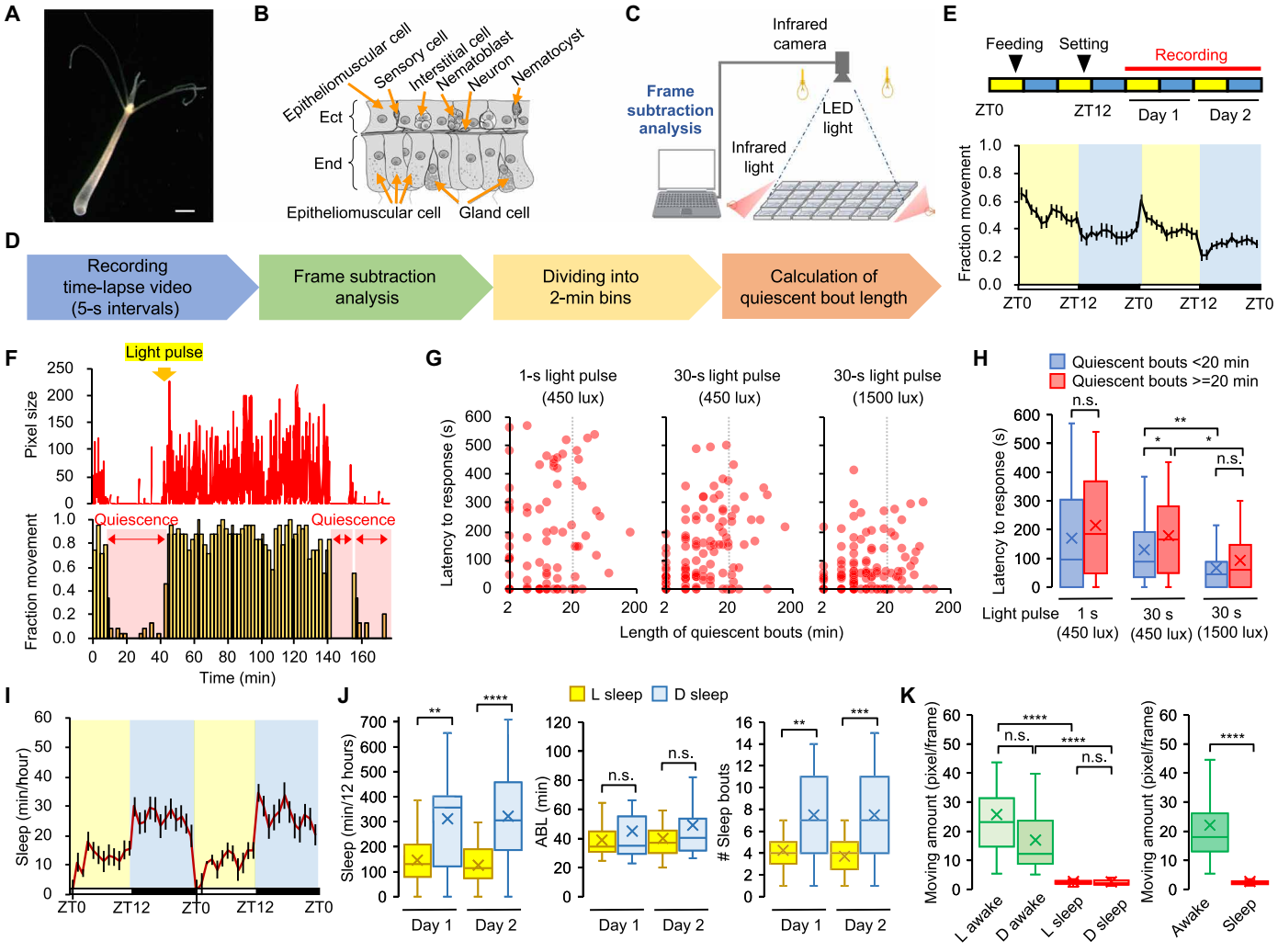

Fig. 1. A sleep-like state in *Hydra*. (**A**) *H. vulgaris* (strain 105). The white bar indicates 1 mm. (**B**) Two-cell layers in *Hydra*. Ect, ectoderm; End, endoderm. (**C** and **D**) Behavioral recording and data processing (see Materials and Methods). LED, light-emitting diode. (**E**) Diurnal behaviors in *Hydra* under LD cycles. ZT, zeitgeber time (lights-on at ZT0; lights-off at ZT12). The last feeding was >24 hours (ZT8) before loading into the imaging chamber (ZT10). Data represent mean ± SEM ($n = 32$). (**F**) Light-induced reversibility of the quiescent state. The size of pixel changes between 5-s frames, and fraction movement in 2-min bins was traced in a single animal subject to a light pulse at ZT16. (**G** and **H**) Inverse correlation of quiescent bout length and latency to the light-induced arousal at ZT16 ($n = 16$ to 89). Box plots range from Q1 to Q3 quartile; crosses and horizontal lines indicate mean and median values, respectively; whiskers extend to minimum or maximum values of 1.5× interquartile range. (**I** and **J**) Daily sleep profiles represent mean ± SEM ($n = 32$). Box plots represent sleep amount, averaged sleep bout length (ABL), and total number of sleep bouts. (**K**) Quantification of *Hydra* movements using averaged pixel change between frames ($n = 32$). n.s., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by Aligned ranks transformation analysis of variance (ANOVA), Wilcoxon rank sum test [(H); Sleep and # Sleep bouts in (J); left in (K)]; by Kruskal-Wallis ANOVA, Dunn's multiple comparisons test [ABL in (J)]; or by Mann-Whitney *U* test [right in (K)].

to animals in the quiescence phase, not less than 20 min (fig. S1B). These results together indicate a high arousal threshold with a >20-min episode of the behavioral quiescence, operationally defining the length of a sleep bout in *Hydra*.

The daily averaged profiles of sleep bout length and sleep duration time in LD cycles indicated that *Hydra* displayed longer sleep at night (Fig. 1, I and J), consistent with their diurnal activity. As expected, *Hydra* showed a low amount of movements during sleep bouts, as assessed by the averaged pixel change per awake-bout frame or sleep-bout frame during the LD cycle (Fig. 1K). Also, a quantitative trace of their head positions using the Tracker software (https://physlets.org/tracker/) revealed that *Hydra* moved shorter distances at lower velocity during sleep bouts (fig. S1C). Feeding behaviors in *Hydra* affect general movement (*9*). Fed animals stay rather inactive until they complete digestion, absorption, and excretion, possibly masking any sleep-like quiescence. Given that we routinely fed *Hydra* cultures two to four times per week, we performed all our sleep analysis in nonfeeding conditions. Nonetheless, we confirmed that prolonged (up to 136 hours) or shorter (down to 16 hours) starvation did not make any significant change in *Hydra* sleep behaviors under our experimental conditions (fig. S1D), although starvation is thought to suppress sleep for foraging in a range of animals from flies to humans (*10*).

A previous study has defined six housekeeping behaviors in *Hydra* (fig. S2A and movie S2) (*11*). Our frame subtraction analysis reliably detected the pixel changes in frames corresponding to

"silent," "elongation," "body sway," and "contraction," among others (fig. S2B). However, ~35 and ~72% of "bending" and "tentacle sway" frames were not detected by the pixel size, indicating that our imaging analysis might underestimate these two types of *Hydra* movements. We manually scored individual housekeeping behaviors during the wake or sleep bouts throughout an LD cycle. This analysis showed that *Hydra* spent their sleep bouts in either a silent or a tentacle-swaying state (fig. S2C). Nonetheless, tentacle swaying was also observed during ~40% of their awake time, and thus, we concluded that silent behavior is relatively specific to the sleep-like state in *Hydra*.

Ultradian rhythms and homeostasis in *Hydra* sleep

It has long been suggested that both circadian rhythms and sleep homeostasis shape daily patterns of sleep-wake cycles (*12*). To examine any circadian regulation of *Hydra* sleep, we monitored their sleep behaviors under constant light (LL) or constant dark (DD), where the absence of any light transitions allowed endogenous clocks to run free. Although *Hydra* displayed overt rhythms in their daily sleep-wake cycles in LD conditions (Fig. 1I), these rhythmic behaviors disappeared in either LL or DD conditions (fig. S3, A to C). It is thus likely that *Hydra* lacks circadian clocks, but they display behavioral responses to light transitions (*13*). However, constant conditions revealed shorter periodic changes in *Hydra* behaviors. Autocorrelation analyses detected 4-hour rhythms evidently in DD fraction movement, as well as in DD sleep (fig. S3D). Those ultradian rhythms were less robust in LL sleep, possibly indicating dampening effects of LL.

To assess any behavioral changes in sleep-deprived *Hydra*, we applied gentle vibration to the culture chamber for the last 6 hours at night under LD cycles and then video-recorded *Hydra* movements starting from lights-on on the next day (Fig. 2A). This mechanical sleep deprivation (MSD) notably lengthened the sleep duration time during the subsequent L phase as rebound sleep. We also found that sleep-deprived *Hydra* had a shorter latency to sleep onset, likely indicating a homeostatic increase in sleep drive (Fig. 2A) (*14*). In contrast, daytime MSD did not lead to a sleep rebound in the following D phase (Fig. 2B). Those sleep-deprived animals instead displayed short D sleep, possibly indicating persistent effects of the daytime mechanical stress on *Hydra* behaviors (Fig. 2B). Because our imaging-based sleep analysis did not allow us to monitor the degree of sleep deprivation during mechanical stress, we further established a temperature-dependent sleep deprivation protocol in *Hydra* (Fig. 2, C and D). Animals were first entrained at low temperature (10°C) for the measurement of their baseline sleep and then subject to high temperature shifts (20°C) for the last 6 hours in either L or D phase. This transient increase in ambient temperature reduced L and D sleep comparably (Fig. 2E). However, a rebound sleep was evident only after D sleep deprivation (Fig. 2F), possibly indicating differential accumulations of the sleep pressure during the day and night. These behavioral features convincingly demonstrate a sleep-like state in *Hydra* and its homeostatic regulation, particularly during the D phase, consistent with their diurnal behaviors.

Neurotransmitters for *Hydra* sleep

Melatonin is a sleep-promoting hormone in various animal taxa, including in humans (*5*, *15*, *16*). The incubation of melatonin with *Hydra* culture medium increased daily sleep amount and the number of sleep bouts in a dose-dependent manner (Fig. 3A and fig. S4A). However, we could not observe any strong correlation between melatonin concentration and the averaged pixel change per awake-bout frame (referred to as waking activity hereafter) (Fig. 3A). It was

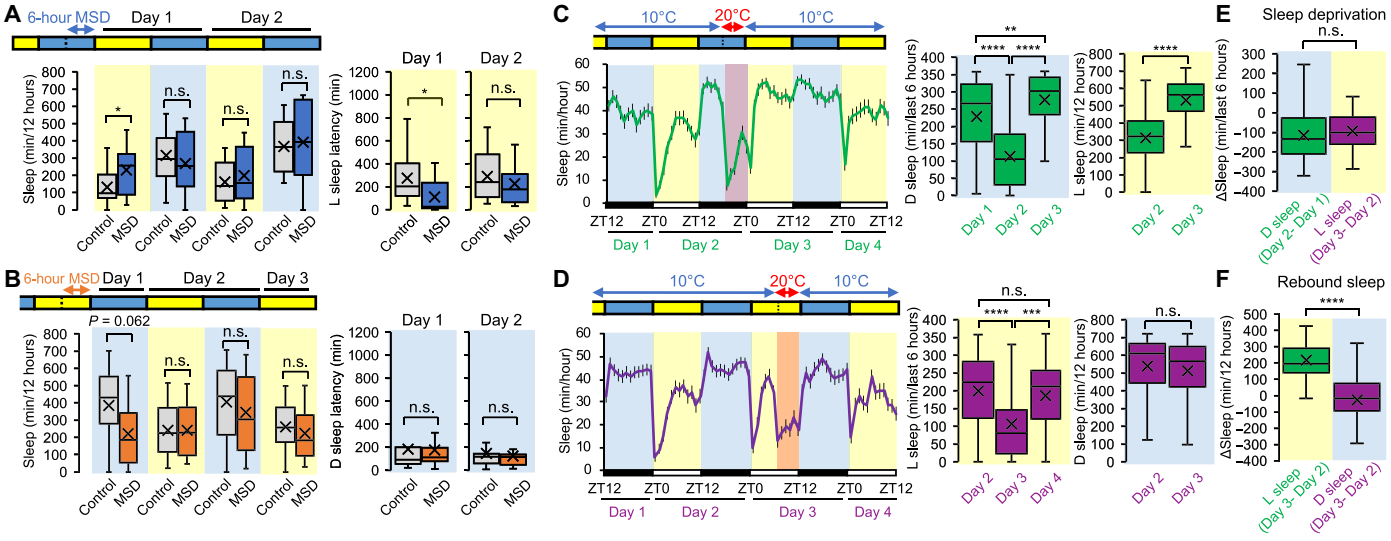

Fig. 2. Homeostasis in *Hydra* sleep. (**A** and **B**) Sleep rebound after MSD for the last 6 hours in the D phase (A, blue), but not in the L phase (B, orange), under LD cycles. Box plots represent sleep amount and latency to sleep onset after light transitions ($n = 13$ to 16). Yellow and blue bars indicate light and dark phases, respectively. *$P < 0.05$ by one-tailed Student's t test (Sleep) or by Mann-Whitney U test (Sleep latency). (**C** and **D**) Sleep rebound after sleep deprivation by a 6-hour shift to high ambient temperature in the D phase (C, green), but not in the L phase (D, purple), under LD cycles ($n = 51$). Green and purple colors indicate data from sleep-deprived animals during the D and L phase, respectively. **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by repeated-measures ANOVA, Friedman's test (Sleep min/last 6 hours) or by Wilcoxon matched-pairs signed rank test, one-tailed (Sleep min/12 hours). (**E**) Comparison of the amount of sleep loss by high ambient temperature in the D phase (green) or L phase (purple). n.s., not significant by unpaired t test with Welch's correction, two-tailed. (**F**) Comparison of the amount of rebound sleep after nighttime (green) or daytime (purple) sleep deprivation. ****$P < 0.0001$ by Mann Whitney test, two-tailed.

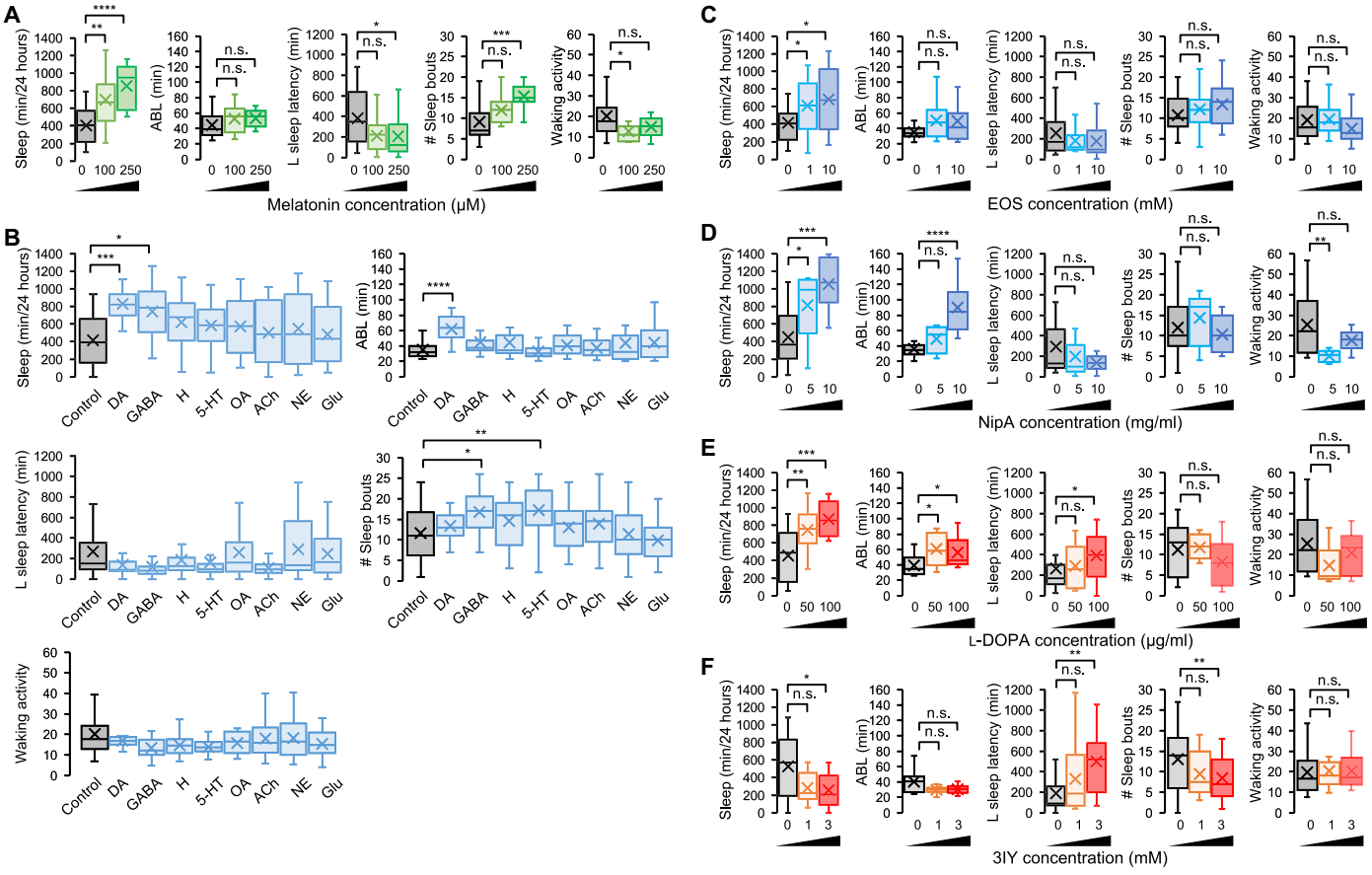

Fig. 3. Pharmacological manipulations of *Hydra* sleep. (**A**) Effects of melatonin administration on daily sleep amount, ABL, L sleep latency, the number of sleep bouts, and waking activity (averaged pixel change per awake-bout frame) under LD cycles ($n = 12$ to 26). (**B**) Effects of neurotransmitter administration (100 μM) on LD sleep ($n = 15$ to 50). DA, dopamine; GABA, γ-aminobutyric acid; H, histamine; 5-HT, serotonin; OA, octopamine; ACh, acetylcholine; NE, norepinephrine; Glu, glutamate. (**C** and **D**) Effects of GABA transaminase inhibitor (EOS) ($n = 18$ to 28) or GABA transporter inhibitor (NipA) on LD sleep ($n = 9$ to 21). (**E** and **F**) Effects of a DA precursor (L-DOPA) ($n = 9$ to 17) or a tyrosine hydroxylase inhibitor (3IY) on LD sleep ($n = 14$ to 26). *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, and ****$P < 0.0001$ by one-way ANOVA, Holm-Sidak's multiple comparisons test [Sleep in (A) and (E); # Sleep bouts in (B to (D)]; by Kruskal-Wallis ANOVA, Dunn's multiple comparisons test [Sleep in (C) and (D); ABL in (A), (B), and (E); Latency in (A) and (C) to (F); # Sleep bouts in (A) and (F); Waking activity in (A) and (C) to (E)]; by Welch's ANOVA, Dunnett's T3 multiple comparisons test [# Sleep bouts in (E)]; or by Aligned ranks transformation ANOVA, Wilcoxon rank sum test [Sleep in (B) and (F); ABL in (C), (D), and (F); Latency and Waking activity in (B)].

thus less likely that melatonin administration made the animals sick or sluggish to cause long sleep. Given that melatonin also promotes sleep in the jellyfish *Cassiopea* (*5*), these results suggest its conserved role between cnidarians and mammals. The *Hydra* genome also encodes orthologous genes that support the transmission of sleep-relevant neurotransmitters in other species (*2*). These include tyrosine hydroxylase, 3,4-dihydroxyphenylalanine (DOPA) decarboxylase, and dopamine receptors for dopamine biosynthesis and transmission, and glutamic acid decarboxylase, vesicular γ-aminobutyric acid (GABA) transporter, GABA receptors, and GABA transaminase for GABA biosynthesis, transmission, and degradation (table S1). We confirmed that most of these homologs were also conserved in other cnidarian species (table S1). Moreover, previous studies have validated their physiological relevance in the GSH-induced feeding response and the pacemaker activity implicated in *Hydra* body contraction (*17*). We, therefore, examined the possible roles of these neurotransmitters in *Hydra* sleep.

The administration of the inhibitory neurotransmitter GABA potently increased daily sleep amount and the number of sleep bouts (Fig. 3B and fig. S4B). Also, the pharmacological increase in synaptic GABA levels by chemical inhibitors of the GABA-metabolizing transaminase [ethanolamine-O-sulfate (EOS)] or the GABA reuptake transporter [nipecotic acid (NipA)] promoted *Hydra* sleep (Fig. 3, C and D, and fig. S4, C and D), consistent with general sleep-promoting effects of GABA. To our surprise, the administration of dopamine, a well-established arousal neurotransmitter that suppresses sleep in higher animals (*18*), promoted *Hydra* sleep as potently as did GABA (Fig. 3B). This observation was further confirmed by the sleep-promoting effects of a dopamine precursor [L-3,4-dihydroxyphenylalanine (L-DOPA)] (Fig. 3E and fig. S4E) and by the wake-promoting effects of 3-iodo-tyrosine (3IY), a chemical inhibitor of tyrosine hydroxylase that mediates the rate-limiting conversion of tyrosine to L-DOPA for dopamine biosynthesis (Fig. 3F and fig. S4F). Sleep-promoting effects of GABA and dopamine transmission were evident generally on D sleep (fig. S4, B to F), although cumulative effects of their pharmacological manipulations could explain these observations. Long sleep phenotypes caused by the pharmacological elevation of GABA and dopamine

levels could not be attributable to their general effects on waking activity (Fig. 3, B to F).

Given that GABA and dopamine have opposing effects on the duration of the GSH-induced feeding response in *Hydra* (*17*, *19*), our findings indicate that GABA and dopamine transmissions specifically interact in different behaviors. These results further support the ancestral origin of sleep regulation by GABA and dopamine, among other neurotransmitters. We reason that the dopaminergic sleep-regulatory pathway may have switched its mode from sleep-promoting to wake-promoting during the evolutionary development of the CNS (see Discussion).

Sleep-relevant *Hydra* genes

To elucidate the molecular basis of *Hydra* sleep behaviors, we compared the gene expression profiles of control and sleep-deprived animals. The microarray-based quantification identified 148 and 64 genes that were up-regulated and down-regulated, respectively, upon MSD (Fig. 4A and table S2). Using quantitative polymerase chain reaction (PCR), we further validated 52 and 35 genes that were up-regulated and down-regulated, respectively, upon MSD (Fig. 4B). Among these sleep-relevant genes, we identified 41 *Hydra* genes that displayed a ≥40% sequence similarity to their homologs in humans, mice, and *Drosophila* (Fig. 4C). The Gene Ontology (GO) enrichment analysis of this set of homologous genes did not reveal any enrichment of sleep-relevant terms in each animal species (table S2). However, the common homologs among the three animals included the voltage-gated potassium channel *Shaker* that was initially identified as a sleep-promoting gene from a forward genetic screen in *Drosophila* (*20*) and the cyclic guanosine monophosphate (cGMP)–dependent protein kinase 1 (PRKG1) that showed sleep-promoting functions in worms, flies, and mammals (*21*–*23*). Whole-mount in situ hybridization revealed that *Hydra* PRKG1 expression was enriched around the hypostome (Fig. 4D), possibly indicating a role in predation. The administration of a PRKG1 inhibitor (KT5823) suppressed *Hydra* sleep in a dose-dependent manner (Fig. 4E). A PRKG1 activator (8-pCPT-cGMP) consistently promoted sleep while not compromising waking activity at the highest dosage tested (Fig. 4F). Accordingly, PRKG1 may represent one of the ancestral genes of sleep regulation.

Given these findings, we hypothesized that *Hydra* genes identified by our gene expression profiling might have as-yet-unidentified sleep-regulatory functions. Because a large-scale genetic approach

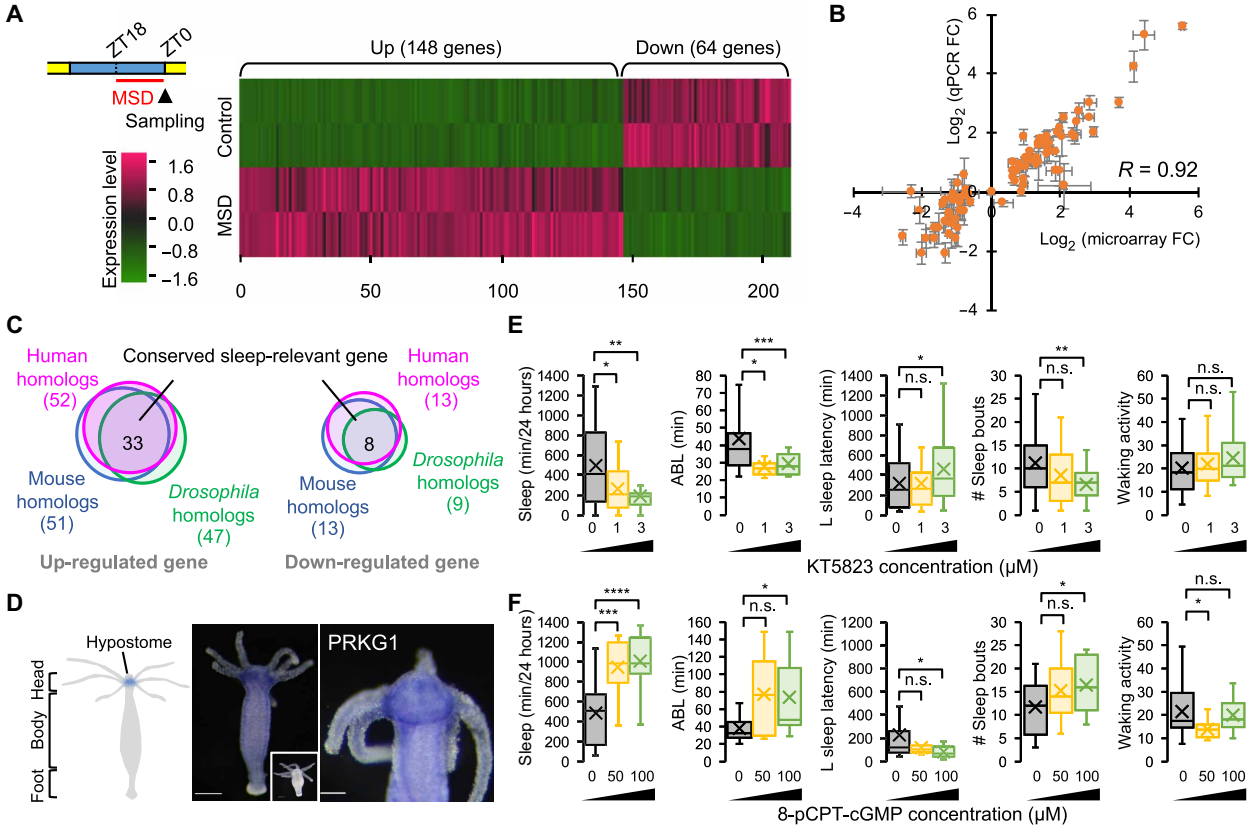

Fig. 4. Genetic mechanisms underlying *Hydra* sleep. (**A**) Microarray analysis of differentially expressed genes (DEGs) in mechanically sleep-deprived *Hydra* (MSD). Mean and SD were set to 0 and 1, respectively, and expression levels were normalized accordingly. (**B**) Pairwise comparison of $\log_2$-transformed fold changes (FCs) in gene expression by MSD (88 genes, correlation coefficient $R = 0.92$). Data represent mean ± SEM ($n = 2$ for microarray; $n = 3$ for quantitative PCR). (**C**) Diagram of the DEG homologs. The numbers of homologous genes were indicated. (**D**) Whole-mount in situ hybridization of *Hydra* PRKG1. A sense probe in the thumbnail served as a negative control. White bars indicate 250 μm (left) and 100 μm (right), respectively. (**E** and **F**) Effects of a PRKG1 inhibitor (KT5823) ($n = 25$ to 66) or a PRKG1 activator (8-pCPT-cGMP) ($n = 9$ to 26) on LD sleep. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by one-way ANOVA, Holm-Sidak's multiple comparisons test [Sleep and # Sleep bouts in (F)]; by Kruskal-Wallis ANOVA, Dunn's multiple comparisons test [Latency in (E) and (F); Waking activity in (E)]; or by Aligned ranks transformation ANOVA, Wilcoxon rank sum test [Sleep and # Sleep bouts in (E); ABL in (E) and (F)].

was relatively limited in *Hydra*, we examined the possible roles of their homologous genes in a *Drosophila* model of sleep behaviors. Several of these candidate genes, including both up-regulated and down-regulated ones in sleep-deprived *Hydra*, increased daily sleep amount when their expression was pan-neuronally depleted by transgenic RNA interference in *Drosophila* (Fig. 5A and table S3). These wake-promoting genes included *CG9005* (family with sequence similarity 214 member A), *CG10082* [inositol hexakisphosphate kinase 1 (IP6K1)], *CG12795* (zinc finger AN1-type containing 2B), *sprouty* (sprouty RTK signaling antagonist 2), *Ornithine aminotransferase* (OAT), *rolling pebbles* (tetratricopeptide repeat, ankyrin repeat, and coiled-coil containing 2), *Goosecoid* (goosecoid homeobox), *Sterol carrier protein X-related thiolase* (sterol carrier protein 2), *Rho GTPase activating protein at 102A* (Rho GTPase activating protein 6), *lethal (2) essential for life* (crystallin alpha B), and *CG31743* (carbohydrate sulfotransferase 11). The pan-neuronal depletion of some of these genes reduced waking activity (i.e., activity count per waking minute) (fig. S5), and the impaired locomotor activity might thus have contributed to the long sleep phenotypes. Nonetheless, low waking activity in CG9005-depleted flies, for example, did not cause any D sleep phenotypes (fig. S5), suggesting that waking activity and sleep behaviors may not necessarily be coupled. These results support our hypothesis above, although it remains to be determined whether these *Drosophila* genes are expressed differently in sleep-deprived flies or play a role in sleep homeostasis.

Sleep regulation by ornithine metabolism

Ornithine is a nonessential amino acid but is involved in the urea cycle (Fig. 5B) (*24*, *25*). The mitochondrial enzyme OAT mediates a metabolic step that subsequently converts ornithine into proline or glutamate. OAT deficiency thus leads to an increase in plasma ornithine levels, causing gyrate atrophy that results in retinal degeneration in humans (*25*). Consistent with the long sleep observed in OAT-depleted flies, we found that the oral administration of ornithine to wild-type flies increased daily sleep amount and shortened the latency to sleep onset (Fig. 5, C and D). The sleep-promoting effects of dietary ornithine were robust in mated females but not in virgins or male flies, implicating a female-specific post-mating mechanism in ornithine-dependent sleep regulation (*26*).

Hydra OAT expression was enriched in nematoblasts (Fig. 5E and table S1), progenitors of nematocytes that are responsible for prey capture and protection from predators. Unexpectedly, we

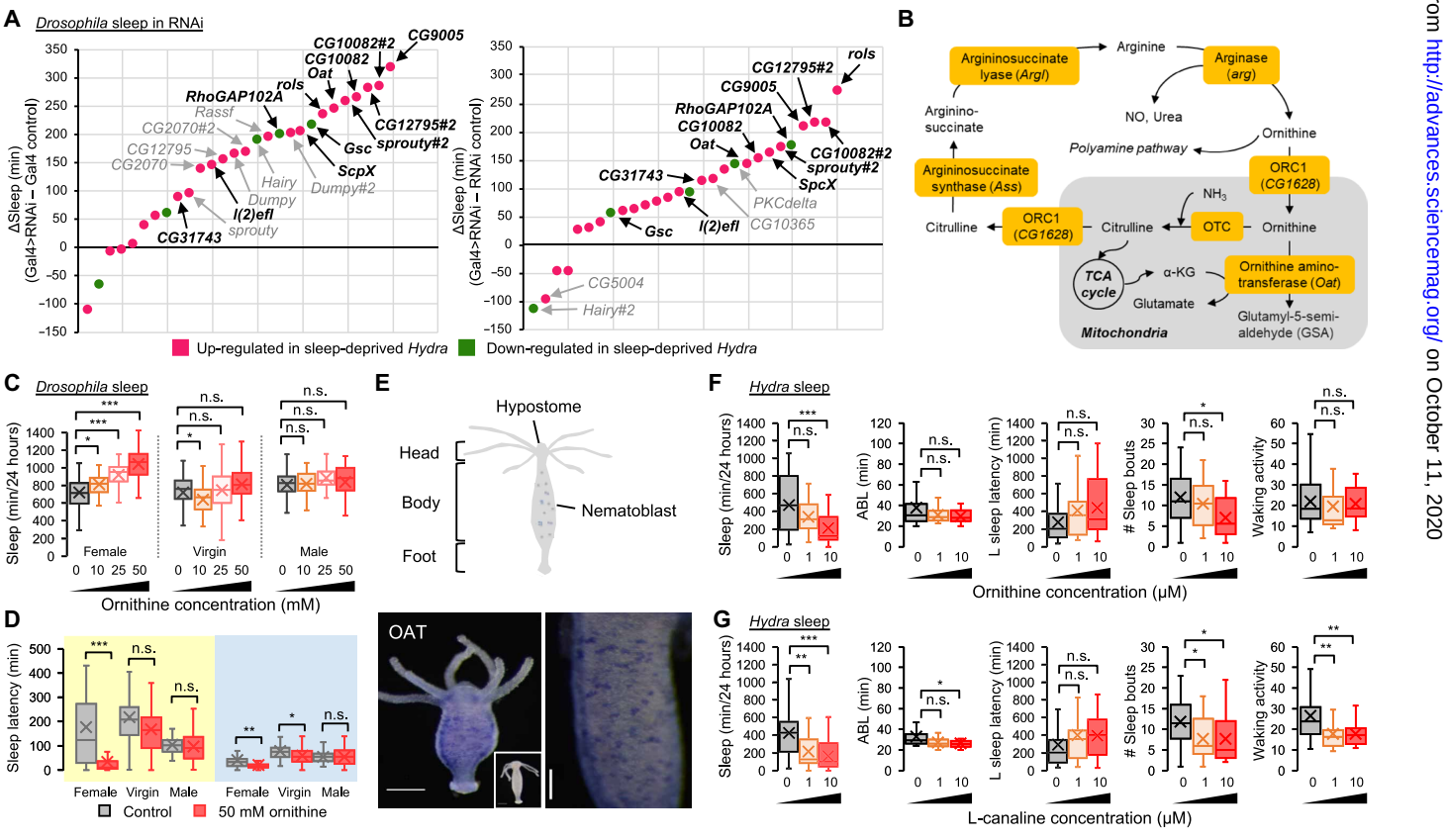

Fig. 5. Sleep regulation by ornithine metabolism. (**A**) Effects of the pan-neuronal depletion of individual DEGs on daily sleep amount in *Drosophila* ($n = 15$ to 219). Gene names in black/bold or gray colors indicate RNA interference (RNAi) lines that display significant sleep phenotypes compared to both transgenic controls (Gal4 control and RNAi control) or to only either one of the two, respectively. (**B**) Biochemical pathways of the urea cycle. (**C** and **D**) Effects of dietary ornithine on *Drosophila* sleep in LD cycles ($n = 36$ to 52). Raw data were collected individually from mated female, virgin female, and male flies. Yellow and blue colors indicate light and dark phases, respectively. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Kruskal-Wallis ANOVA, Dunn's multiple comparisons test (sleep amount); by Mann-Whitney *U* test (latency in female and virgin); or by Welch's *t* test (latency in male). (**E**) Whole-mount in situ hybridization of *Hydra* OAT. A sense probe in the thumbnail served as a negative control. White bars indicate 250 μm (left) and 100 μm (right), respectively. (**F** and **G**) Effects of ornithine administration ($n = 10$ to 33) or an OAT inhibitor (L-canaline) ($n = 19$ to 30) on *Hydra* sleep in LD cycles. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Kruskal-Wallis ANOVA, Dunn's multiple comparisons test [Sleep and # Sleep bouts in (G); ABL and Latency in (F) and (G); Waking activity in (F)] or by Aligned ranks transformation ANOVA, Wilcoxon rank sum test [Sleep in (F); Waking activity in (G)].

observed that dietary ornithine suppressed *Hydra* sleep in a dose-dependent manner (Fig. 5F). The wake-promoting effects of dietary ornithine were more evident in female *Hydra* strains, although the baseline sleep duration was longer in females than in males (fig. S6). Moreover, the pharmacological inhibition of OAT similarly suppressed *Hydra* sleep (Fig. 5G). Given the sleep-promoting effects of dietary ornithine in mammals, including humans (*27*, *28*), our data support the ancient origin of ornithine as a sleep-regulatory molecule. It might also be relevant to the lethargy associated with urea cycle disorders or pregnancy (*29*, *30*). Future studies should determine how ornithine metabolism responds to sleep needs and which specific effector pathway is downstream of sleep-modulatory ornithine in individual animal species.

Sleep-dependent cell proliferation

Previous studies have shown that sleep deprivation suppresses cell proliferation in mammals and *Drosophila* (*31*, *32*). We reasoned that, if a sleep-like state in *Hydra* is physiologically relevant, sleep deprivation might impose restrictions on this fundamental physiology in individual cells. Thus, we measured cell proliferation in *Hydra* using 5-bromo-2′-deoxyuridine (BrdU) incorporation and compared it between control and sleep-deprived animals (Fig. 6A). Although *Hydra* exhibited diurnal activity in LD cycles, control animals displayed no significant difference in the relative levels of BrdU incorporation during the L or D phase (fig. S7). However, MSD remarkably reduced the number of proliferative cells (Fig. 6, B to D). It was unlikely that *Hydra* suppressed cell proliferation due to mechanical stress because pharmacological sleep deprivation using 3IY (a tyrosine hydroxylase inhibitor) or KT5823 (a PRKG1 inhibitor) similarly impaired the BrdU incorporation (Fig. 6, C and D). We further found that sleep-dependent cell proliferation occurred throughout the body column in *Hydra*, likely reflecting a systemic response to sleep needs (Fig. 6, B and D). We reason that cell proliferation in *Hydra* is homeostatically regulated in LD cycles, but sleep deprivation substantially disrupts this regulation. These results validate the physiological significance of *Hydra* sleep. Cellular effects of sleep and the origin of sleep function may thus trace back to ancestral organisms with poorly defined nervous systems.

DISCUSSION

Our demonstration of the sleep-like state in *Hydra* and the commonality of sleep-regulatory genes, neurotransmitters, and physiology provide important insights into how ancestral sleep has evolved with developing CNS and how sleep-regulatory pathways have been reorganized accordingly. While the two-process model for shaping daily sleep has been widely accepted (*12*), free-running circadian rhythms are not readily detectable in *Hydra* behaviors. This observation

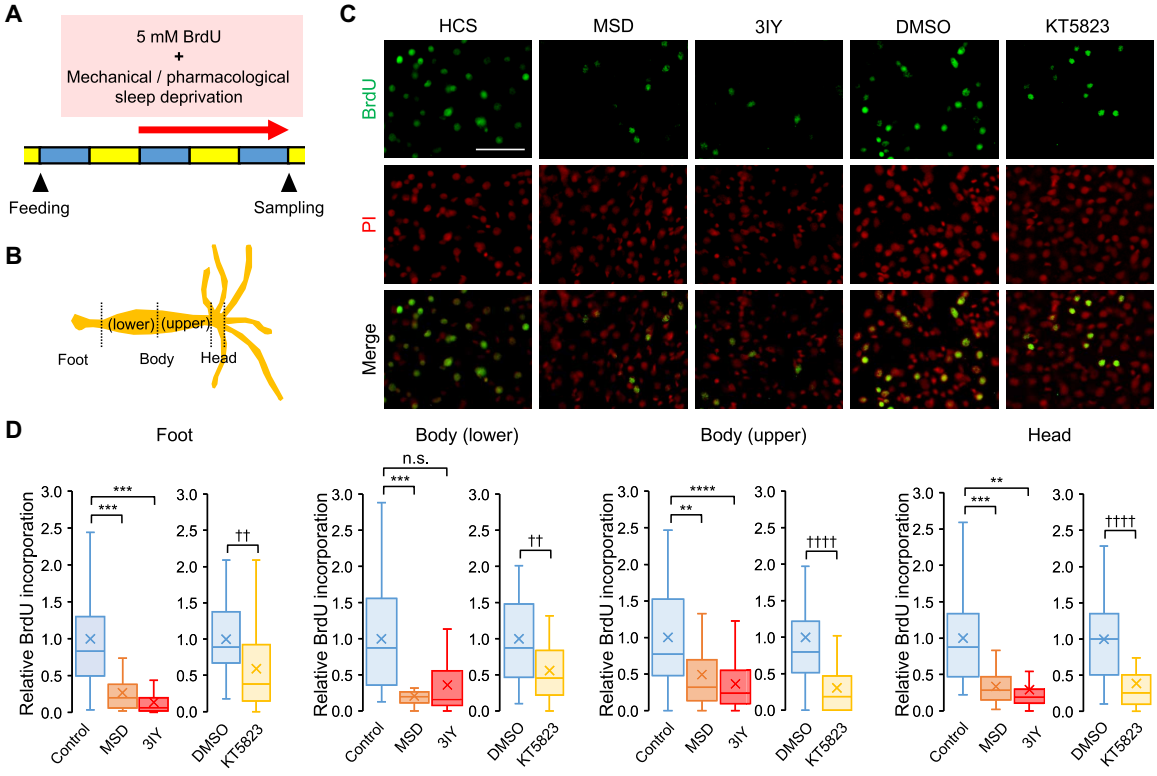

Fig. 6. Attenuation of cell proliferation by sleep deprivation in *Hydra*. (**A** and **B**) Experimental scheme of cell proliferation assay in sleep-deprived *Hydra*. Mechanical or pharmacological sleep deprivation was applied for 36 hours before immunostaining. BrdU incorporation was quantified from each body part. (**C**) Representative images of BrdU and propidium iodide (PI) labeling. The white bar indicates 50 μm. HCS, *Hydra* culture solution (control for MSD and 3IY); MSD, mechanical sleep deprivation; 3IY, 3-iodo-tyrosine (a tyrosine hydroxylase inhibitor; 3 mM); DMSO, dimethyl sulfoxide (vehicle control for KT5823, 0.1%); KT5823, a PRKG1 inhibitor (3 μM). (**D**) Quantitative analyses of BrdU incorporation. Fluorescence signals from anti-BrdU staining were quantified from six regions of interest per animal ($n = 5$ to 6 animals per condition). Data were normalized to each control group. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ by Kruskal-Wallis ANOVA, Dunn's multiple comparisons test (upper body), Aligned ranks transformation ANOVA, Wilcoxon rank sum test (the others). $^{††}P < 0.01$ and $^{††††}P < 0.0001$ by Mann-Whitney *U* test.

contrasts with circadian control of the quiescence state in the cnidarian jellyfish (*5*). Circadian clocks are not an essential prerequisite for sleep behaviors because animal species with no overt circadian rhythms (e.g., *Caenorhabditis elegans*) or circadian clock mutants in *Drosophila* and mammals exhibit sleep. Circadian rhythms have also been observed widely in nonanimal kingdoms, where sleep-like states are not recognized. Nonetheless, our discovery of 4-hour free-running rhythms in *Hydra* sleep may reflect an evolutionary intermediate for circadian clock-dependent sleep given that circadian rhythms emerge from coupled ultradian oscillators (*33*). We also reason that the ultradian rhythms in *Hydra* sleep could be an ancestral form of the sleep-stage cycling in mammals. In this sense, *Hydra* may represent one of the most primitive animal models for sleep.

Dopamine is a wake-promoting molecule conserved across animal species (*1*). We, however, showed that dopamine promotes *Hydra* sleep. This unexpected finding suggests that dopamine's sleep-regulatory function may depend on how dopaminergic circuits are incorporated into sleep-regulatory pathways of the developing CNS. Consistent with this idea, dopamine is one of the major arousal neurotransmitters in adult flies, whereas it is dispensable for sleep in developing larvae (*31*). We speculate that the functional flipping of specific sleep-regulatory pathways (e.g., dopamine and ornithine) may have occurred during the evolutionary development of CNS. On the other hand, sleep-promoting pathways involving melatonin, GABA, or PRKG1 may have persisted in this process.

Our evidence does not necessarily exclude the possible contribution of the diffuse nerve net to *Hydra* sleep. Emerging evidence, however, indicates the presence of sleep-wake cycles of cell-autonomous nature and sleep-regulatory mechanisms of non-neuronal origin in mammals and *Drosophila* (*1*). Likewise, dopamine may contribute to *Hydra* sleep via its indirect effects on peripheral tissues (e.g., metabolism, cell growth, and oxidative stress) (*34*). We predict that essential metabolism (e.g., ornithine-derived metabolic pathways) would play a key role in shaping these ancestral forms of sleep, and *Hydra* would act as an important node in the phylogenetic tree of sleep for validating this hypothesis. Future studies should further mine phylogenetic nodes to illustrate the evolutionary trace of sleep-regulatory mechanisms at high resolution and elucidate the origin of sleep.

MATERIALS AND METHODS

Animals

Hydra experiments were carried out with a standard *Hydra* strain (*H. vulgaris*, strain 105) without buds. Male and female were isolated from strain AEP. *Hydra* was routinely maintained in a *Hydra* culture solution [HCS; 1 mM NaCl, 1 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgSO_4$, 1 mM tris-(hydroxymethyl)-amino-methane; pH 7.4, adjusted with HCl] under 12-hour light (450 lux):12-hour dark (0 lux) (LD) cycles at 20°C. *Hydra* was fed with newly hatched *Artemia nauplii* two to four times per week. *Hydra* was subject to nonfeeding conditions for over 24 hours before behavioral recording unless otherwise indicated. *Drosophila melanogaster* was grown on standard cornmeal-yeast-agar medium (5.4% cornmeal, 1.3% yeast, 0.7% soy flour, 0.4% agar, 4.2% starch syrup, 0.4% propionic acid, and 0.8% methyl 4-hydroxybenzoate) at 22° to 25°C with 50 to 60% humidity. w^{1118} (BL5905, Bloomington Drosophila Stock Center) was set as a wild type to generate control flies heterozygous for each transgene or examine effects of dietary ornithine on *Drosophila* sleep.

Video recording of *Hydra* behaviors

The behavioral recording was performed as described previously (*13*) with minor modifications. Each *Hydra* was transferred from the LD-entrained culture to a silicone container (16 mm by 16 mm by 5 mm) filled with 1-ml HCS at zeitgeber time 10 (ZT10). After acclimation, video recording was initialized. *Hydra* was illuminated with infrared light (Sousin Digital) and visualized with an E3 CMOS (complementary metal-oxide semiconductor) camera (Visualix) through an infrared high-pass filter (FUJIFILM). Frames (1920 × 1200 pixels) were gained every 5 s using a Visualix ImageView software version 3.7 (Visualix). For pharmacological experiments, drugs were added to HCS 12 hours before the video recording for LD sleep analysis. HCS or dimethyl sulfoxide (≤0.1%) was used as vehicle control. After each recording, the survival rate of treated animals was compared to that of controls to exclude any toxic effects of drugs.

Analysis of *Hydra* behaviors

For the frame subtraction analysis, differences in grayscale values (256 gradations) between each pair of images were calculated for all pixels using ImageJ (*35*). Significant differences that exceed the automatically determined threshold were detected and regarded as movements. The ratio of frames that displayed any *Hydra* movement (i.e., fraction movement) was calculated using 2-min, 1-hour, or 24-hour windows. Data recordings from animals with the fraction movement of 24-hour window lower than 0.1 or larger than 0.7 were excluded from further analysis to triage data collected from dead animals or false-positive detections of movements (i.e., intense noise in the recording area). Behavioral quiescence was defined if no movement was detected in more than 50% frames per 2-min bin. The sleep bout was then defined if the duration of quiescence persisted for longer than 20 min (i.e., 10 × 2-min bin). Additional sleep parameters, including daily sleep amount, averaged sleep bout length, the number of sleep bouts, latency to sleep onset after light transitions, and the averaged pixel change per awake-bout frame (i.e., waking activity), were calculated accordingly. For pharmacological experiments, sleep parameters were calculated from the first-day recordings to avoid any effects from the altered drug concentrations by evaporation. The head positions were tracked using the video analysis and modeling tool Tracker (https://physlets.org/tracker/). After the length calibration, the *XY* coordinates of a hypostome region were manually pointed and recorded. Using the output data of *XY* coordinates from the Tracker program, moving distance and speed were calculated. Six housekeeping behaviors (*11*) were manually scored in individual frames throughout an LD cycle. For the autocorrelation analysis of ultradian rhythms, autocorrelation function was calculated for 50-hour data of fraction movement and sleep amount (per hour) using R (version 3.6.1).

Measurement of arousal thresholds in *Hydra*

For the measurement of light-induced arousal, light stimulation was given at ZT16 on the second day of sleep recording. Three different intensities of the light-pulse (450 lux for 1 s; 450 lux for 30 s; 1500 lux for 30 s) were applied to analyze a dose-dependent response. Latency to the light response was defined as the duration time before detecting >1-pixel changes in five consecutive frames to minimize any false-positive measurement of *Hydra* movements. For the measurement of behavioral responses to GSH, *Hydra* was starved for 48 hours before loading onto the silicone container and incubated with 10 mM GSH between ZT4 and ZT7. This time

window allowed a manual application of GSH to individual animals in the imaging chamber while minimally perturbing *Hydra* sleep by the light transitions in LD cycles. Tentacle movements to the mouth were manually scored using ImageJ (*35*).

Sleep deprivation in *Hydra*

For MSD, a gentle vibration was applied to the silicone containers every 15 min using a microplate mixer (Sanko Junyaku) for the last 6 hours in either L or D phase. The silicone containers were then loaded onto the video-recording system, and their images were recorded for two additional LD cycles. For temperature-dependent sleep deprivation, *Hydra* movements were first video-recorded at 10°C in LD cycles and then the ambient temperature was transiently shifted to 20°C for the last 6 hours in either L or D phase.

Drosophila sleep analysis

Unless otherwise indicated, female flies were loaded individually into 65 by 5 mm glass tubes containing 5% sucrose and 2% agar (behavior food) on day 0 and entrained to LD cycles at 25°C. For oral administration of L-ornithine, ornithine was directly dissolved in the behavior food. Locomotor activity in individual flies was quantified by the number of infrared beam crosses per minute and recorded using the *Drosophila* Activity Monitor System (TriKinetics). The asleep bout was defined as an episode during which flies did not cross the infrared beam for 5 min or longer. Sleep behaviors were quantified accordingly using an Excel macro (*36*). Sleep parameters on day 4 were compared between control and experimental groups.

Quantitative transcript analysis

A custom-made *Hydra* microarray (Agilent Technologies; 4 × 44K) was used for differential gene expression profiling between control and sleep-deprived *Hydra*. Animals were entrained in LD cycles and then sleep-deprived by 6-hour mechanical stimuli before harvest at ZT0. Total RNAs were purified from 10 animals in duplicate using TRIzol Reagent (Thermo Fisher Scientific), and their gene expression analyses were performed as described previously (*13*). Up-regulated genes were defined by their z scores not less than 2.0 and fold changes not less than 1.5, while down-regulated genes were defined by z scores not more than −2.0 and fold changes not more than 0.66. For quantitative PCR analyses, RNA samples from control or sleep-deprived *Hydra* were prepared similarly as above. Complementary DNAs (cDNAs) were synthesized from total RNAs using Oligo(dT)$_{12\text{-}18}$ Primer (Invitrogen) with SuperScript III (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed with Mx3000P (Agilent Technologies) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). The PCR conditions were 5 min at 95°C, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. Translation elongation factor 1α (EF1α) was used as the internal control for normalization. Data were averaged from three independent experiments.

Phylogenetic analysis of sleep-relevant genes

Ortholog sequences were searched from OrthoDB release 10 (www.orthodb.org/) (*37*) using *D. melanogaster* genes as queries. The ortholog groups included *Homo sapiens, Mus musculus*, *D. melanogaster*, *C. elegans*, and six cnidarian species (*H. vulgaris*, *Nematostella vectensis*, *Exaiptasia pallida*, *Orbicella faveolata*, *Stylophora pistillata*, and *Acropora digitifera*). The representative sequences for each ortholog group, including those of *H. sapiens, M. musculus*, *D. melanogaster*, and *H. vulgaris*, were aligned using Muscle implemented in MEGA X (*38*). The percent identities of a pair of protein sequences were calculated using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). GO enrichment analysis was performed for differentially expressed *Hydra* genes that displayed high sequence similarity (sequence identity ≥40% and e value <0.001) to homologous genes in other model organisms (*H. sapiens*, *M. musculus*, and *D. melanogaster*) using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 (*39*, *40*).

Whole-mount in situ hybridization

Hydra was relaxed with 1% urethane and then fixed in 4% paraformaldehyde overnight at 4°C. Fixed samples were incubated with digoxigenin (DIG)–labeled RNA probes overnight at 55°C. After washes in 2× SSC, hybridized RNA probes were detected using alkaline phosphatase–conjugated anti-DIG antibody (Roche) and nitro blue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP) solution. Sense probes were used as negative controls. Images were acquired using the digital camera D5200 (Nikon) equipped with Leica MZ10F (Leica).

BrdU labeling

Hydra was maintained in HCS at 20°C under LD cycles and starved for 24 hours before BrdU labeling. Animals were incubated with 5 mM BrdU (Wako) for 12 hours in either L or D phase and then fixed in 4% paraformaldehyde for 4 hours at room temperature (RT). For BrdU labeling in sleep-deprived animals, animals were incubated with 5 mM BrdU for 36 hours in control or sleep-depriving conditions before fixation. After washing with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBST), fixed samples were treated with 2 N HCl for 20 min at RT. After washing three times at RT, samples were blocked with 5% normal goat serum in PBST for 30 min at RT. The samples were incubated with anti-BrdU antibody (Roche; 1:200) at 4°C overnight and then incubated with anti-mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific; 1:1000) at RT for 1 hour. After washing three times with PBST, samples were mounted in VECTASHIELD Mounting Medium with propidium iodide (Vector Laboratories). Images (3840 × 3072 pixels) were acquired using the digital camera DXM1200F (Nikon) equipped with the OPTIPHOTO microscope (Nikon). Quantitative image analysis was performed using ImageJ (*35*). After background subtraction, automatic thresholding was performed in each picture. Significant signals were detected by particle analysis (circularity is 0.2 to 1.0), and the number of particles was recorded. Six regions of interest (ROIs) (800 × 800 pixels) were randomly selected from each part of a given animal and used for the quantification. The ratio of BrdU-positive nuclei to propidium iodide–positive nuclei was calculated per ROI and normalized to that in each control.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software) or R (version 3.5.3). For the two-sample test, normality and homogeneity of variance were checked by the Shapiro-Wilk normality test and F test, respectively (significance levels are 0.05 in both tests). A two-tailed Student's t test or Welch's t test was then applied to compare unpaired two groups with normality. When the normality was rejected, the Mann-Whitney U test was applied. For the multiple comparison test, normality and homogeneity of variance were checked by the Shapiro-Wilk test and Brown-Forsythe test, respectively. To

compare unpaired multiple sample groups, one-way analysis of variance (ANOVA) followed by Holm-Sidak's multiple comparisons test or Welch's ANOVA followed by Dunnett's T3 multiple comparisons test were used for the dataset with normality. When the normality was rejected, Kruskal-Wallis ANOVA followed by Dunn's post hoc test or Aligned ranks transformation ANOVA followed by the Wilcoxon rank-sum test was applied. To compare paired sample groups, paired *t* test was applied for the dataset with normality, while Wilcoxon matched-pairs signed-rank test or Friedman's test followed by Dunn's post hoc test were applied for the dataset violating the assumption of normality. The details of statistical analyses, including the number of samples analyzed, the type of statistical tests performed for each experiment, and *P* values, were described in the appropriate figure legend.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/41/eabb9415/DC1

View/request a protocol for this paper from *Bio-protocol*.

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A sleep-like state in *Hydra* unravels conserved sleep mechanisms during the evolutionary development of the central nervous system

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