Sleep–wake regulation and hypocretin–melatonin interaction in zebrafish

Lior Appelbaum[a,b], Gordon X. Wang[c], Geraldine S. Maro[d], Rotem Mori[e], Adi Tovin[e], Wilfredo Marin[a,b], Tohei Yokogawa[a,b], Koichi Kawakami[f], Stephen J. Smith[c], Yoav Gothilf[e], Emmanuel Mignot[a,b], and Philippe Mourrain[a,g,1]

[a]Center for Narcolepsy, Department of Psychiatry and Behavioral Sciences, [b]Howard Hughes Medical Institute, [c]Department of Molecular and Cellular Physiology, Beckman Center, and [d]Department of Biology and Pathology Stanford University, Palo Alto, CA 94305; [e]Department of Neurobiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel; [f]Division of Molecular and Developmental Biology, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan; and [g]Institut National de la Santé et de la Recherche Médicale, Inserm U784, Ecole Normale Supérieure, 75005 Paris, France

In mammals, hypocretin/orexin (HCRT) neuropeptides are important sleep–wake regulators and HCRT deficiency causes narcolepsy. In addition to fragmented wakefulness, narcoleptic mammals also display sleep fragmentation, a less understood phenotype recapitulated in the zebrafish HCRT receptor mutant (*hcrtr−/−*). We therefore used zebrafish to study the potential mediators of HCRT-mediated sleep consolidation. Similar to mammals, zebrafish HCRT neurons express vesicular glutamate transporters indicating conservation of the excitatory phenotype. Visualization of the entire HCRT circuit in zebrafish stably expressing *hcrt:EGFP* revealed parallels with established mammalian HCRT neuroanatomy, including projections to the pineal gland, where *hcrtr* mRNA is expressed. As pineal-produced melatonin is a major sleep-inducing hormone in zebrafish, we further studied how the HCRT and melatonin systems interact functionally. mRNA level of arylalkylamine-N-acetyltransferase (AANAT2), a key enzyme of melatonin synthesis, is reduced in *hcrtr−/−* pineal gland during the night. Moreover, HCRT perfusion of cultured zebrafish pineal glands induces melatonin release. Together these data indicate that HCRT can modulate melatonin production at night. Furthermore, *hcrtr−/−* fish are hypersensitive to melatonin, but not other hypnotic compounds. Subthreshold doses of melatonin increased the amount of sleep and consolidated sleep in *hcrtr−/−* fish, but not in the wild-type siblings. These results demonstrate the existence of a functional HCRT neurons-pineal gland circuit able to modulate melatonin production and sleep consolidation.

pineal gland | sleep consolidation

Hypocretin 1 and 2 (HCRT 1 and 2, also known as orexin A and B) are two neuropeptides originally isolated in rats, that are derived from a single gene precursor (*Hcrt/Orx*) (1, 2). HCRT preproprotein is exclusively expressed in neurons restricted to the lateral hypothalamus (LH) organized as a single compact cluster in each hemi-brain (1–3). HCRT neuron number may vary from a few thousand in a rodent LH to 50,000–80,000 in the human LH. This cluster organization is conserved in all mammals investigated (4). Despite its restricted expression, HCRT is a critical regulator of the sleep–wake cycle and is further implicated in food intake regulation, energy homeostasis, arousal, drug addiction, stress, and cardiovascular function. Interestingly, the complexity of HCRT physiological function is reflected in the diversity of HCRT anatomic projections and HCRT receptor expression sites in the central nervous system. From their discrete location in the LH, HCRT neurons send widespread projections throughout the brain and the spinal cord (3, 5). This broad fiber distribution is consistent with the diffuse expression patterns of the two HCRT G protein-coupled receptors (HCRTR1/OX1R and HCRTR2/OX2R) (2, 6).

HCRT deficiencies produce narcolepsy, a disorder characterized in mammals by excessive sleepiness during the normal wake periods, direct transitions from wake to REM sleep, and sudden loss of muscle tonus while still being awake (cataplexy) (4, 7). Moreover, HCRT is crucial to maintain wakefulness and has been extensively shown to have a wake-promoting role. HCRT neurons are active during wakefulness (8) and intracerebroventricular administration of HCRT induces a dose-dependent increase of time spent awake (9). Furthermore, HCRT also appears to play an important role in sleep regulation and consolidation. Individuals with narcolepsy and similarly affected dogs and mice also suffer sleep fragmentation (10, 11), even when pharmacologically kept awake during the daytime to increase homeostatic pressure for sleep (12).

We, and others, have previously characterized the HCRT system in zebrafish (13–16), a simpler model to understand the anatomy and function of the HCRT neuronal network and its evolution across vertebrates. Unlike mammals, zebrafish have only one HCRT receptor gene (*hcrtr*) (16). Despite the close similarity of this receptor with the mammalian HCRTR2, zebrafish *hcrtr−/−* null mutants do not have a fragmented wake during the daytime or cataplexy-like behavior but only possess the sleep fragmentation phenotype (16). Moreover, in contrast to the mammalian HCRT system involving thousands of neurons in a complex neuronal circuit, the larval and adult zebrafish hypothalamus contain approximately 20 and 60 HCRT-positive neurons, respectively (13–16). In this study, we show a neuroanatomical and functional connection between the HCRT and melatonin systems, supporting a role for a HCRT/melatonin pathway in sleep consolidation.

Results

Zebrafish HCRT Neurons Are Mostly Glutamatergic. In mammals, the majority of HCRT neurons are glutamatergic, a phenotype consistent with the wake-promoting influence and neuroexcitatory nature of HCRT neuropeptides (17). However, HCRT signaling deficiency in zebrafish failed to impact wake behavior (16). This led us to examine the excitatory or inhibitory nature of the zebrafish HCRT neurons. We studied their fast neurotransmitter phenotype using double in situ hybridization (ISH) of *hcrt* with vesicular glutamate transporter genes *vglut1*, *vglut2a*, *vglut2b* (markers of excitatory glutamate), and glutamate decarboxylase gene *gad67* (marker of inhibitory GABA). Colocalization with *hcrt* was determined by confocal microscopy. We found that no HCRT neurons express *gad67* (Fig. 1*A*, close-up *A1*) suggesting that HCRT neurons
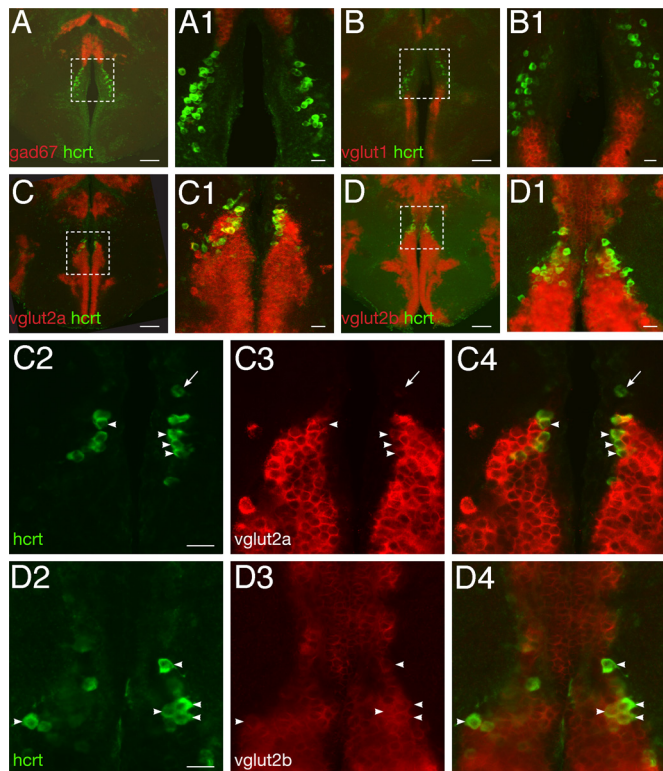
Fig. 1. Zebrafish HCRT neurons are glutamatergic. (*A–D*) and close-ups (*A1–D1*) Double fluorescent ISH between *hcrt* mRNA and fast neurotransmitter phenotype markers as visualized using confocal microscopy on adult brain sections (reconstructed stacks of 0.5- or 1-μm sections). (*C2–C4*) and (*D2–D4*): single-plane, high-magnification pictures of *hcrt* cells (green, *C2, D2*), *vglut2a* or -*b* cells (red, *C3, D3*), and merged views (*C4, D4*). Arrowheads indicate cells coexpressing *hcrt* and *vglut2a* or *vglut2b*. Note the frequent colocalization. Absence of coexpression is occasionally observed (arrow). [Scale bar, 100 μm (*A–D*), 20 μm (*A1–D1, C2–C4,* and *D2–D4*).]

are not GABAergic. Similarly, HCRT neurons in majority do not express *vglut1* (Fig. 1 *B* and *B1*), however occasional coexpression in a few ventralmost HCRT cells was observed. In contrast, a large majority of the HCRT neurons express one of the *vglut2* genes suggesting a glutamatergic phenotype (Fig. 1 *C* and *D*, and associated close-up panels *C1–C4* and *D1–D4*). Of note, *vglut2a*- or *2b*-negative HCRT neurons were sometimes observed dorsally (Fig. 1 *C2–C4*, arrow). These results are reminiscent of the rat, where

none of the HCRT neurons express *gad67*, a minority expresses *vglut1* and a majority expresses *vglut2* (17), suggesting a conservation of the developmental program underlying the HCRT cell identity across vertebrates. Functionally, the glutamatergic phenotype of HCRT neurons in zebrafish suggests a conservation of the excitatory nature of these neurons between fish and mammals.

Architecture of the Larval and Adult Zebrafish HCRT System. We isolated the zebrafish *hcrt* gene promoter (14) and generated a specific stable *hcrt:EGFP* transgenic line (Fig. S1) to perform high resolution imaging of the zebrafish HCRT circuit, and its potential connectivity with sleep/wake-regulating brain regions. Two-photon microscopy analysis of living *hcrt:EGFP* larvae revealed the ipsilateral track projecting to the spinal cord (14, 15) and bilateral projections proceeding anteriorly through the telencephalon toward both olfactory bulbs (OB, Fig. 2*A* and close up in *B*), reminiscent of the rat dorsal descending and ventral ascending pathways respectively. In addition, contralateral projections were observed ventrally to the HCRT cell bodies (Fig. 2 *C* and *D*, arrow) and in the commissura anterior to the OB (Fig. 2*B*, arrow).

In adult *hcrt:EGFP* zebrafish (Fig. 3), HCRT fibers are found in the dorsal telencephalic, homologous to the mammalian, and in the subpallium midline (Fig. 3*A*), similar to the rat dorsal and ventral ascending pathways respectively (3). As in mammals, the highest density of axonal projections is detected in the hypothalamic periventricular region (Fig. 3 *B–D*) and HCRT cell bodies are organized as two compact clusters facing each other without direct contralateral projection to their counterpart (Fig. 3 *D* and *D1*). Commissural projections are clearly visible along at least four levels, the habenular commisure (Fig. 3*B1*), the commissura tecti, the commissura posterior, and through the most ventral enclosure of the periventricular hypothalamus (Fig. 3*D*). Fibers were also observed around the tectal ventricule in the periventricular gray zone of the optic tectum (Fig. 3 *C* and *D*), a structure homologous to the superficial layers of the superior colliculi in mammals where HCRT fibers have also been described in rats (3). Finally, posterior projections are sent dorsally and ventrally in the subventricular region of the rhombencephalon (Fig. 3*E*) similar to that observed in rat (3, 5).

We next examined the distribution of HCRT receptor expression to confirm the projection architecture observed in our *hcrt:EGFP* transgenics. Consistently, the fiber distribution matched the HCRT receptor mRNA expression profile (Fig. 3, compare panels *A* vs. *F*, *B* vs. *G*, *C* vs. *H*, *D* vs. *I*, and *E* vs. *J*), except in the hypothalamus. Indeed, abundant projections around the HCRT cell bodies do not have corresponding receptor expression (see Fig. 3 *D* vs. *I*) suggesting those processes are likely dendritic. Overall, our stable
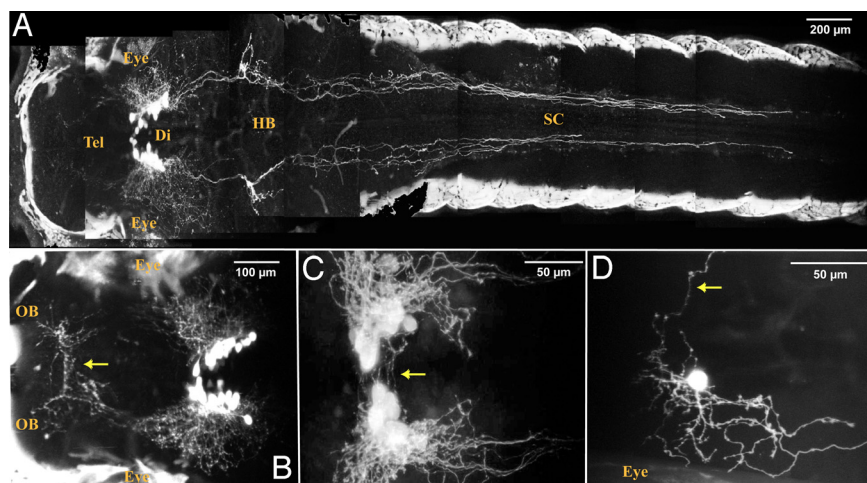
Fig. 2. A stable *hcrt:EGFP* transgenic line reveals the entire HCRT circuit of a living larva. (*A–C*) Two-photon imaging of a 7dpf stable transgenic larva with head to the left, dorsal views. (*A* and close-up *B*) Composite picture showing the HCRT cell bodies in the diencephalon (Di) and their processes in the hindbrain (HB) and spinal cord (SC) and in the telencephalon (Tel) directed toward the olfactory bulbs (OB). In *A* and *B*, white areas on both sides of the larva correspond to skin autofluorescence. Commissural projections are observed ventrally to the HCRT cell body clusters (*C*, arrow) and in the anterior telencephalon (*B*, arrow, commissura anterior). (*D*) Mosaic expression of a non-integrated *hcrt:EGFP* transgene allowing the observation of a single HCRT neuron harboring both commissural (arrow) and ipsilateral processes.
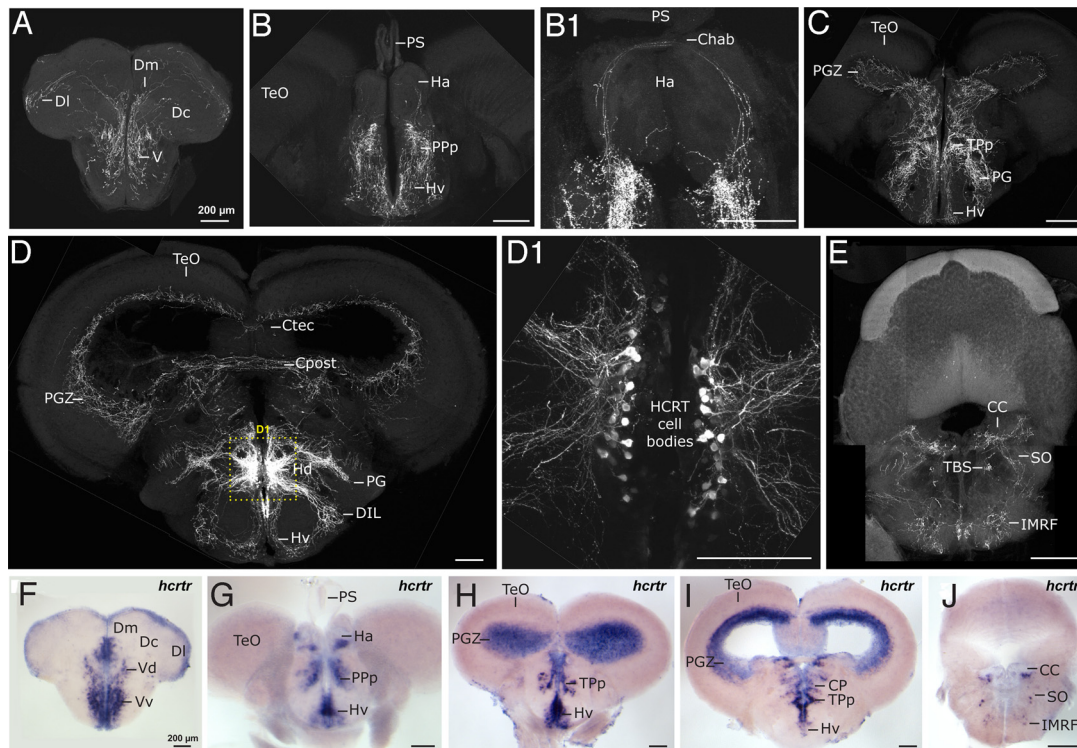
Fig. 3. Adult zebrafish HCRT circuit. (*A–E*) Confocal imaging of 100 μm transversal brain sections from a stable *hcrt:EGFP* transgenic adult fish (reconstructed stacks of 0.5- or 1-μm sections). Note the compact organization of the HCRT cell bodies in the periventricular hypothalamus (*D* and dashed-box close up in *D1*). (*F–J*) *hcrtr* mRNA ISH pattern in equivalent brain sections to *A–E*. Note the similar distribution of HCRT fibers and *hcrtr* mRNA. Compare sections of telencephalon (*A* vs. *F*), telencephalon-midbrain boundary (*B* and *B1* vs. *G*), anterior diencephalon and mesencephalon (*C* vs. *H*), mid diencephalon and mesencephalon (*D* vs. *I*), rhombencephalon (*E* vs. *J*). Chab, commissura habenularum; Ctec, commissura tecti; Cpost, commissura posterior; CC, crista cerebellaris; CP, central posterior thalamic nucleus; D, dorsal telencephalic area; Dm, medial zone of D; Dc, central zone of D; Dl, lateral zone of D; DIL, diffuse nucleus of the inferior lobe; Ha, habenula; Hv, ventral zone of the periventricular hypothalamus; Hd, dorsal zone of the periventricular hypothalamus; IMRF, intermediate reticular formation; PG, preglomerular nucleus; PGZ, periventricular gray zone of the optic tectum; PPp, parvocellular preoptic nycleus, posterior part; PS, pineal stalk; SO, secondary octaval population; TBS, tractus bulbospinalis; TeO, optic tectum; TPp, periventricular nucleus of posterior tuberculum; V, ventral telencephalic area; Vv, ventral nucleus of V; Vd, dorsal nucleus of V.

hcrt:EGFP transgenic line documents considerable conservation of the HCRT circuit architecture across vertebrates and is therefore a valuable tool for identifying HCRT circuitry involved in sleep–wake regulation.

HCRT Neurons Project to the Pineal Gland Where *hcrtr* Is Expressed. Through 2-photon imaging of *hcrt:EGFP* larvae, we identified axons emerging from both HCRT cell clusters and forming an arch reaching the most dorsal region of the midbrain-telencephalon boundary (Fig. 4*A*, dorsal view and *B*, frontal view). These fibers were located superficially, just underneath the skull, suggesting an innervation of the pineal gland. In all vertebrates, the pineal gland influences daily and annual physiological changes mediated by the secretion of melatonin at night (18, 19). In zebrafish, melatonin mediates the circadian clock output and also promotes sleep (20, 21). To demonstrate that HCRT neurons project into the pineal gland, we crossed *hcrt:EGFP* and *aanat2:EGFP* transgenic fish. The *aanat2:EGFP* fish expresses EGFP specifically in melatonin producing photoreceptors of the pineal gland (22). In the resulting double transgenic fish, HCRT axons were indeed found to project into the pineal gland (Fig. 4*C*). This observation was confirmed in adult brains, where, in addition to the commissural innervation of the habenula (Ha, Fig. 3*B1*), HCRT neurons innervate the pineal gland stalk (PS, Fig. 4 *D* and *E*). This HCRT-pineal connection is an exceptional case of a direct neuropeptidergic innervation of the fish pineal gland. The importance of this projection was substantiated by the detection of *hcrtr* mRNA in the pineal gland of both

larvae (Fig. 4 *F–H*) and adults (Fig. 4 *I–K*), strongly suggesting that pineal gland activity could be modulated by HCRT circuitry.

HCRT Increases Melatonin Production in Cultured Pineal Glands. To test whether HCRT peptides affect melatonin production in the pineal gland, we used a well-established perfusion assay of adult zebrafish pineal glands (*SI Materials and Methods* and Fig. S2). Pineal glands were removed and placed in a flow-through perfusion system for 3 days. As expected, isolated pineal glands of fish displayed rhythmic production of melatonin under light–dark (LD) and dark–dark (DD) cycles (Fig. 5*A* and *B*). Custom-made mature zebrafish HCRT-1 peptide (10^{-6} M) (16) was applied to individual pineal glands for 2 h during the second night at ZT (zeitgeber time) 21–23, when melatonin level is declining. HCRT-1 application led to a significant increase in melatonin production (up to 70%, $P < 0.05$, $n = 8$), which lasted for the duration of HCRT application (Fig. 5*B*), indicating a stimulating effect of HCRT-1 on melatonin production. In contrast, minimum essential medium (MEM) application did not affect melatonin production in control pineal glands ($n = 12$, Fig. 5*A*). These results demonstrate that the zebrafish pineal gland can respond to HCRT excitatory input and suggest the existence of a functional HCRT/pineal gland neuronal circuit in zebrafish.

***aanat2* Expression Is Down-Regulated in *hcrtr−/−* Pineal Gland.** We next tested whether this putative circuit is functional in vivo and analyzed whether the absence of HCRT input in *hcrtr−/−* background could affect pineal gland melatonin production. To do so,
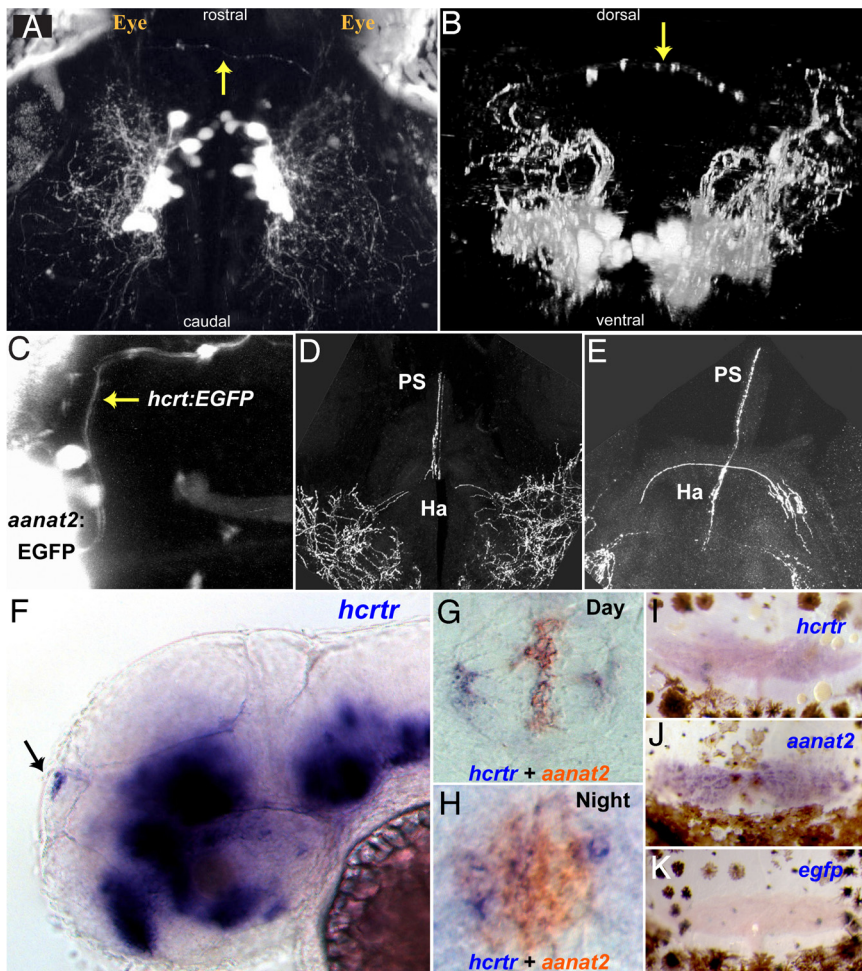
Fig. 4. The HCRT-pineal gland circuit. (*A* and *B*) Dorsal and frontal views of the brain of a 7 dpf *hcrt:EGFP* transgenic larva imaged by two-photon microscopy. HCRT axons (arrows) projecting toward the pineal gland are observed. (*C*) A dorsal image of 6 dpf transgenic larva carrying two transgenes; an EGFP reporter driven by *hcrt* (*hcrt:EGFP*) and the pineal-specific *aanat2* (*aanat2:EGFP*) promoters, demonstrate direct axon projection (arrow) to the pineal gland. (*D* and *E*) Close-ups of two adjacent transversal *hcrt:EGFP* adult brain sections showing HCRT projections to the habenula and the pineal gland stalk. (*F*) Lateral and (*G* and *H*) dorsal views of whole-mount in situ hybridization of 2-dpf embryos. (*F*) *hcrtr* mRNA is expressed in several regions of the brain (16) including the pineal gland (arrow). Double ISH experiment with *aanat2* demonstrates that *hcrtr* is expressed in the pineal gland during the day (*G*) and the night (*H*). Similarly, in adult animals, *hcrtr* is expressed in the pineal gland (*I*). *aanat2* (*J*) and *egfp* (*K*) probes were used as positive and negative controls, respectively. Adult pineal glands (*I–K*) were removed with the upper skull and skin hence presence of brown melanophores cells in the preparations.

we studied the level of expression of AANAT the key enzyme of melatonin production (19). In zebrafish pineal glands, *aanat2* mRNA level is rhythmic, and is a robust indicator of melatonin production (23). *aanat2* mRNA levels from six wild-type sibling and six *hcrtr*−/− adult pineal glands collected at 1:00 AM (ZT16) were measured using quantitative PCR. Strikingly, we observed a significant reduction of 31% of *aanat2* expression level in *hcrtr*−/− pineal glands ($P < 0.05$, Fig. 5*C*), indicating that melatonin production is decreased in absence of HCRT signal. This result demonstrates the existence of a functional HCRT-pineal gland circuit and may explain the fragmented sleep phenotype of *hcrtr*−/− fish in the dark.

hcrtr−/− Larval and Adult Mutants Are Hypersensitive to the Sleep Inducing Effects of Melatonin. To further investigate whether melatonin signaling down-regulation is responsible for the *hcrtr*−/− mutant sleep phenotype, we next examined the effects of exogenous melatonin and other hypnotic drugs on *hcrtr*−/− fish and wild-type siblings. We treated larvae with melatonin and compounds from four different classes of hypnotics (barbiturate, benzodiazepine, anti-histaminergic, and α2 adrenergic agonist) and monitored their activity over 24 h under constant dim light conditions (Fig. 6 and Fig. S3). No significant differences were found under drug-free conditions between genotypes (Fig. 6*A*). However, with melatonin exposure, *hcrtr*−/− larvae were more sedated than the wild-type
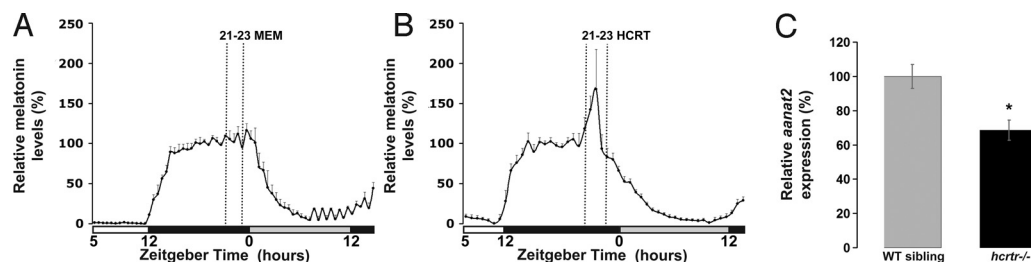
Fig. 5. HCRT modulates pineal gland melatonin production. (*A* and *B*) HCRT induces melatonin release from cultured zebrafish pineal glands. (*A*) Normal circadian rhythm of melatonin release of melatonin from zebrafish pineal glands cultured in constant darkness. MEM medium application does not affect melatonin production in 12 control pineal glands. (*B*) zebrafish HCRT-1 application (10^{-6} M) stimulates melatonin production (ZT 21–23) ($n = 8$, $P < 0.05$). The production lasts for the duration of HCRT application. (*C*) Quantitative PCR analysis of *aanat2* mRNA level in six wild-type sibling and 6 *hcrtr*−/− pineal glands collected during the night (ZT 16). Note the significant decrease (31%, $P < 0.05$) of *aanat2* expression in pineal glands devoid of functional HCRT input.
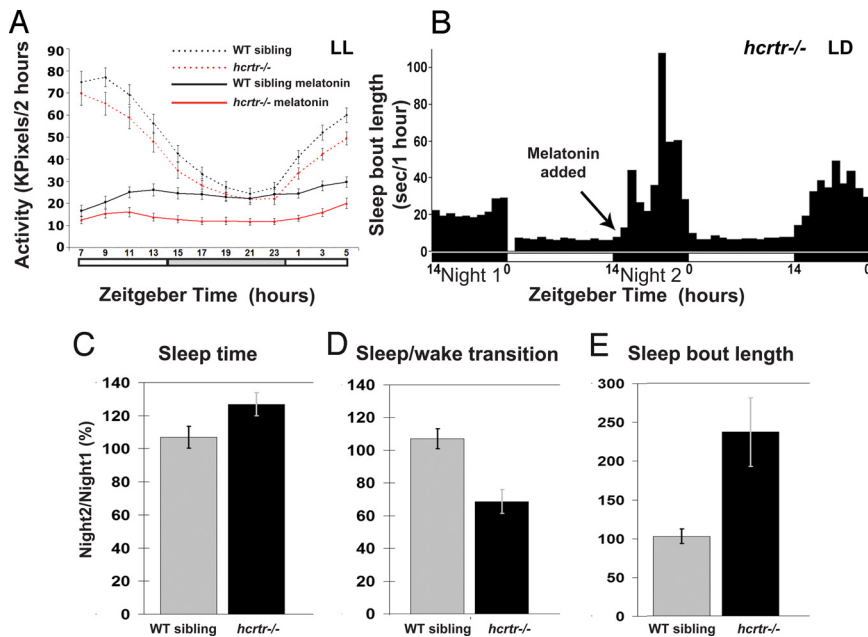
Fig. 6. *hcrtr−/−* fish are hypersensitive to the melatonin sleep-promoting effect. (*A*) Larvae were kept under constant dim light conditions (LL, <10lux) in the monitoring system (white bars represent the subjective day period). *hcrtr−/−* and wild-type sibling 5 dpf larvae demonstrate similar rhythmic activity that peak during the day. *hcrtr−/−* larvae were significantly more sensitive to melatonin's (1 μM) hypnotic effect. (*B–E*) Adult fish were kept under LD cycle (represented by white and black bars) in AFSRS [*SI Materials and Methods* and (16)] and fine sleep architecture was analyzed. (*B*) Representative sleep bout pattern of an adult *hcrtr−/−* mutant. Subthreshold dose of melatonin (1 μM) was added at the beginning of the second night (arrow) and sleep parameters were compared to first night. *hcrtr−/−* adults were more sensitive to melatonin as, after its administration, total sleep time increased (*C*), the number of sleep/wake transition decreased (*D*) and sleep bout length increased (*E*), as compared to wild-type siblings.

siblings ($P < 0.0001$, Fig. 6*A*). In contrast, *hcrtr−/−* and wild-type siblings showed no differences upon exposure to the other hypnotics (Fig. S3), demonstrating a specific hypersensitivity to melatonin and suggesting, in this mutant, an up-regulation of the actors downstream of melatonin, which might reflect a reduction of endogenous melatonin signaling (24).

We next analyzed the impact of melatonin application on the sleep dynamics of adult *hcrtr−/−* and wild-type sibling fish. Based on the hypersensitivity observed in larvae and to test whether a very small concentration of melatonin could abrogate the sleep fragmentation phenotype, we used a subthreshold dose of melatonin: the highest dose that could be administered to wild-type adults without inducing hypnotic effects; that is, 1 μM. Sleep time, sleep bout length, and sleep transition number were monitored under LD cycle in wild-type and mutant adult fish using our previously described Adult Fish Sleep Recording System (AFSRS) (16). For each individual fish, sleep patterns in the presence of melatonin were compared to records obtained during the previous night without exogenous melatonin. Although, as expected, wild-type fish were not affected by this low dose of melatonin, we found that *hcrtr−/−* fish were strongly sedated. They displayed an approximate 30% increase in sleep ($P < 0.01$) (Fig. 6*C*), and a strong increase of sleep consolidation indicated by a 40% decrease in the number of sleep–wake transitions ($P < 0.005$) (Fig. 6*D*), and a 100–150% increase in mean sleep bout length ($P < 0.005$) during the night (Fig. 6 *B* and *E*). Together, these data indicate that hypersensitivity to melatonin is a strong and specific feature of *hcrtr−/−* fish. Further, it demonstrates a behavioral-level of interaction between the HCRT and melatonin systems and suggests that the absence of functional HCRT pathway affects sleep-promoting melatonin signaling at a level yet to be discovered.

Discussion

In mammals, HCRT has a wake-promoting role (4). In addition to this function, evidence supports a major role of HCRT in the consolidation not only of wake state but also of sleep state. In human, dog, and mouse, HCRT deficiency is associated with both wake and sleep fragmentation (10, 11). Moreover, sleep fragmentation persists in narcoleptic patients even when pharmacologically kept awake during the daytime (12), excluding a simple wake rebound hypothesis. Furthermore, mice with HCRT neurons devoid of GABA-B receptors also display both fragmented sleep and

wake without reduction of total sleep amount (25). Finally, although studies have shown that HCRT neurons decrease firing during sleep, they do not completely cease to discharge during sleep (8). This is also reflected at the extracellular levels, where HCRT levels stay high during sleep (26) and could regulate it (27). We therefore propose that HCRT has a dual function on promoting wake and on consolidating sleep, thus explaining daytime sleepiness and insomnia in narcoleptic patients. In zebrafish, this dual function is very likely conserved. Ubiquitous HCRT overexpression in transgenic fish increases wakefulness (15), while *hcrtr−/−* mutants display sleep fragmentation (16). Surprisingly, *hcrtr−/−* mutants do not have wake fragmentation or cataplexy phenotype suggesting the existence of compensatory system(s) or wiring differences. Nevertheless commonalities observed in these experiments (15, 16) suggest that HCRT function is conserved in zebrafish and can be implicated in both wake promotion and sleep consolidation.

Here, we demonstrate conservation of glutamatergic phenotype of HCRT neurons across vertebrates, and we further show fish HCRT circuit homology with the mammalian system (3, 5), including the direct HCRT innervation of the pineal gland. In sheep (28), rat (29), and pig (30), HCRT fibers and/or HCRT receptors were indeed also found in the pineal gland, demonstrating conservation across diurnal and nocturnal vertebrates. In all vertebrates, diurnal or nocturnal, melatonin is secreted in the dark by the pineal gland and controls daily and seasonal changes in physiology (18, 19). Melatonin is also a well-established sleep inducer in zebrafish (21), albeit a weaker one in humans. In mammals, light acts via retinohypothalamic projections to entrain the central oscillator located in the suprachiasmatic nucleus (SCN). In turn, the SCN synchronizes rhythmic melatonin production through sympathetic innervation of the pineal gland. In contrast, in teleosts, phototransduction, oscillator, and melatonin production are located in the photoreceptor cells of the pineal gland. Thus, the fish pineal gland is considered as an autonomous organ able to rhythmically produce melatonin (19, 20). There are only a few described examples of pineal innervations in teleosts (20, 31). In this study, we show HCRT innervation of the pineal gland and that HCRT signal modulates pineal melatonin production. This is an example of peptidergic innervation and functional control of the fish pineal gland. Thus, the dogma that pineal melatonin production in teleost is totally independent of CNS regulation may need to be revisited.

This study not only describes a neuroanatomical connection between two major sleep/circadian systems, it also demonstrates functional relevance of this sleep regulatory circuit at the molecular and behavioral levels. Application of zebrafish HCRT on wild-type pineal glands consistently stimulates the release of melatonin. This promoting effect was confirmed in vivo by the down-regulation of *aanat2* in *hcrtr*−/− fish pineal glands, suggesting that the HCRT signaling pathway likely modulates melatonin production by acting upstream of the transcriptional cascade controlling *aanat2* expression (32). Furthermore, *hcrtr*−/− fish are strikingly hypersensitive to the hypnotic effects of melatonin. This effect is specific, as four other classes of hypnotics (Fig. S3) show no differences between *hcrtr*−/− fish and wild-type siblings. This hypersensitivity may be interpreted in different ways. A first possibility could be that *hcrtr*−/− fish are sleepier, and thus more sensitive to sleep induction by melatonin. In this case, however, we would have expected similar results with other hypnotics. An attractive alternative hypothesis supported by our results, could be that HCRT promotes, at least partially, sleep consolidation in the dark through the stimulation of the melatonin sleep-promoting system. This would explain sleep disruption in the *hcrtr*−/− fish when in the dark, but not the light that is a suppressor of melatonin release of its own. Furthermore, in the absence of HCRT signaling, the downstream melatonin pathway and/or circuit could be up-regulated to compensate for the decrease of melatonin production, thus explaining the hypersensitivity to melatonin of *hcrtr*−/− fish. Such a regulatory loop has been previously reported in mammals and birds where melatonin receptor numbers increased after pinealectomy (24). Moreover, interestingly, HCRT fiber and *hcrtr* mRNA distributions (Fig. 3) strongly resemble melatonin receptors' expression pattern (Fig. S4) especially in the periventricular gray zone of the optic tectum, and the periventricular thalamus and hypothalamus. Thus, HCRT and melatonin pathways could also interact at downstream levels in brain regions where both HCRT and melatonin receptors are coexpressed. These potential downstream interconnections might also be responsible for the *hcrtr*−/− melatonin hypersensi-

tivity phenotype. Overall, future studies will be required to investigate the full extent of melatonin and HCRT pathway collaboration (synergy or compensation) and to further test the wake-promoting and sleep-consolidating dual function of HCRT.

Whether or not HCRT promotion of melatonin release and sleep is relevant to humans is unknown at this stage. Studies in narcoleptics have not shown dramatic differences in melatonin release, although light regime was not controlled (33). In addition, humans display variable sensitivity to exogenous melatonin's hypnotic effects. Melatonin acts as a mild hypnotic when administered during the daytime and can cause small shifts in the timing of the circadian clock (34). Hypnotic effects of melatonin at night are more debated, although notably in Smith Magenis syndrome strong beneficial effects on disturbed nocturnal sleep have been documented (35). The use of melatonin for the treatment of insomnia in narcolepsy has only been reported in a single case, with a dramatic REM sleep promoting effect (36). As this is not in line with clinical impression, a more systematic evaluation is needed.

In summary, because HCRT is not present in nonvertebrate lineages, zebrafish provide an excellent simplified system in which to dissect how HCRT neurons and their projections may affect the sleep/wake cycle and other behaviors. This work reveals a sleep consolidating circuit and shows that melatonin can act as one of the mediators of the HCRT system in regulating sleep.

Materials and Methods

The methods are described in detail in *SI Materials and Methods*. Imaging was performed using a custom-made two-photon laser-scanning microscope and Zeiss LSM 510 META confocal microscope. *hcrt:EGFP* stable transgenic lines were established with the tol2 technique. Cultured pineal assay was done in Dr. Gothilf laboratory. Behavior was monitored with either Adult Fish Sleep Recording System or ViewPoint system.

1. de Lecea L, et al. (1998) The hypocretins: Hypothalamus-specific peptides with neuro-excitatory activity. *Proc Natl Acad Sci USA* 95:322–327.
2. Sakurai T, et al. (1998) Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573–585.
3. Peyron C, et al. (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18:9996–10015.
4. de Lecea L, Sutcliffe JG (2005) The hypocretins and sleep. *FEBS J* 272:5675–5688.
5. van den Pol AN (1999) Hypothalamic hypocretin (orexin): Robust innervation of the spinal cord. *J Neurosci* 19:3171–3182.
6. Marcus JN, et al. (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 435:6–25.
7. Zeitzer JM, Nishino S, Mignot E (2006) The neurobiology of hypocretins (orexins), narcolepsy and related therapeutic interventions. *Trends Pharmacol Sci* 27:368–374.
8. Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. *J Neurosci* 25:6716–6720.
9. Piper DC, Upton N, Smith MI, Hunter AJ (2000) The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. *Eur J Neurosci* 12:726–730.
10. Nishino S, et al. (2000) Is narcolepsy a REM sleep disorder? Analysis of sleep abnormalities in narcoleptic Dobermans. *Neurosci Res* 38:437–446.
11. Willie JT, et al. (2003) Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: Molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* 38:715–730.
12. Arnulf I, Mignot E (2004) Sodium oxybate for excessive daytime sleepiness in narcolepsy-cataplexy. *Sleep* 27:1242–1243.
13. Kaslin J, Nystedt JM, Ostergard M, Peitsaro N, Panula P (2004) The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. *J Neurosci* 24:2678–2689.
14. Faraco JH, et al. (2006) Regulation of hypocretin (orexin) expression in embryonic zebrafish. *J Biol Chem* 281:29753–29761.
15. Prober DA, Rihel J, Onah AA, Sung RJ, Schier AF (2006) Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. *J Neurosci* 26:13400–13410.
16. Yokogawa T, et al. (2007) Characterization of sleep in zebrafish and insomnia in hypocretin receptor mutants. *PLoS Biol* 5:2379–2397.
17. Rosin DL, Weston MC, Sevigny CP, Stornetta RL, Guyenet PG (2003) Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. *J Comp Neurol* 465:593–603.
18. Arendt J, Deacon S, English J, Hampton S, Morgan L (1995) Melatonin and adjustment to phase shift. *J Sleep Res* 4:74–79.
19. Klein DC, et al. The melatonin rhythm-generating enzyme: Molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent Prog Horm Res* 52:307–357, 1997; discussion 357–308.

20. Falcon J (1999) Cellular circadian clocks in the pineal. *Prog Neurobiol* 58:121–162.
21. Zhdanova IV, Wang SY, Leclair OU, Danilova NP (2001) Melatonin promotes sleep-like state in zebrafish. *Brain Res* 903:263–268.
22. Gothilf Y, et al. (2002) Pineal-specific expression of green fluorescent protein under the control of the serotonin-N-acetyltransferase gene regulatory regions in transgenic zebrafish. *Dev Dyn* 225:241–249.
23. Gothilf Y, et al. (1999) Zebrafish serotonin N-acetyltransferase-2: Marker for development of pineal photoreceptors and circadian clock function. *Endocrinology* 140:4895–4903.
24. Lu J, Cassone VM (1993) Pineal regulation of circadian rhythms of 2-deoxy[^{14}C]glucose uptake and 2[^{125}I]iodomelatonin binding in the visual system of the house sparrow, Passer domesticus. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 173:765–774.
25. Matsuki T, et al. (2009) Selective loss of GABA(B) receptors in orexin-producing neurons results in disrupted sleep/wakefulness architecture. *Proc Natl Acad Sci USA* 106:4459–4464 .
26. Zeitzer JM, et al. (2003) Circadian and homeostatic regulation of hypocretin in a primate model: Implications for the consolidation of wakefulness. *J Neurosci* 23:3555–3560.
27. Xi MC, Chase MH (2006) Neuronal mechanisms of active (rapid eye movement) sleep induced by microinjections of hypocretin into the nucleus pontis oralis of the cat. *Neuroscience* 140:335–342.
28. Zhang S, et al. (2005) Expression of orexin receptors in the brain and peripheral tissues of the male sheep. *Regul Pept* 124:81–87.
29. Mikkelsen JD, et al. (2001) Hypocretin (orexin) in the rat pineal gland: A central transmitter with effects on noradrenaline-induced release of melatonin. *Eur J Neurosci* 14:419–425.
30. Fabris C, Cozzi B, Hay-Schmidt A, Naver B, Moller M (2004) Demonstration of an orexinergic central innervation of the pineal gland of the pig. *J Comp Neurol* 471:113–127.
31. Falcon J, Besseau L, Sauzet S, Boeuf G (2007) Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends Endocrinol Metab* 18:81–88.
32. Appelbaum L, Anzulovich A, Baler R, Gothilf Y (2005) Homeobox-clock protein interaction in zebrafish. A shared mechanism for pineal-specific and circadian gene expression. *J Biol Chem* 280:11544–11551.
33. Blazejova K, Illnerova H, Hajek I, Nevsimalova S (2008) Circadian rhythm in salivary melatonin in narcoleptic patients. *Neurosci Lett* 437:162–164.
34. Srinivasan V, et al. (2009) Melatonin and melatonergic drugs on sleep: Possible mechanisms of action. *Int J Neurosci* 119:821–846.
35. De Leersnyder H, et al. (2003) Beta 1-adrenergic antagonists and melatonin reset the clock and restore sleep in a circadian disorder, Smith-Magenis syndrome. *J Med Genet* 40:74–78.
36. Pavel S, Goldstein R, Petrescu M (1980) Vasotocin, melatonin, and narcolepsy: Possible involvement of the pineal gland in its patho-physiological mechanism. *Peptides* 1:281–284.

NEUROSCIENCE