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Morphological and Physiological Interactions between GnRH3 and Hypocretin/Orexin Neuronal Systems in Zebrafish (*Danio rerio*)

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Abstract

GnRH neurons integrate internal and external cues to control sexual maturation and fertility. Homeostasis of energy balance and food intake correlates strongly with the status of reproduction. Neuropeptides secreted by the hypothalamus involved in modulating energy balance and feeding may play additional roles in the regulation of reproduction. Hypocretin (also known as orexin) is one such peptide, primarily controlling sleep/wakefulness, food intake, and reward processing. There is a growing body of evidence indicating that hypocretin/orexin (Hcrt) modulates reproduction through interacting with the hypothalamo-pituitary gonadal axis in mammals. To explore potential morphological and functional interactions between the GnRH and Hcrt neuronal systems we employed a variety of experimental approaches including confocal imaging, immunohistochemistry, and electrophysiology in transgenic zebrafish in which fluorescent proteins are genetically expressed in GnRH3 and Hcrt neurons. Our imaging data revealed close apposition and direct connection between GnRH3 and Hcrt neuronal systems in the hypothalamus during larval development through adulthood. Furthermore, the Hcrt receptor (HcrtR) is expressed in GnRH3 neurons. Electrophysiological data revealed a reversible inhibitory effect of Hcrt on GnRH3 neuron electrical activity, which was blocked by the HcrtR antagonist almorexant. In addition, Hcrt had no effect on the electrical activity of GnRH3 neurons in the HcrtR null mutant zebrafish ($HcrtR^{-/-}$). Our findings demonstrate a close anatomical and functional relationship between Hcrt and GnRH neuronal systems in zebrafish. It is the first demonstration of a link between neuronal circuits controlling sleeping/arousal/feeding and reproduction in zebrafish – an important animal model for investigating the molecular genetics of development.

Introduction

Hypocretin (Hcrt; also known as orexin) is the highly conserved peptide product of preprohypocretin, with two enzymatically cleaved hypocretin peptides: hypocretin 1/orexin A (33 aa) and hypocretin 2/orexin B (28 aa). These peptides are synthesized in a cluster of neurons in the lateral hypothalamus, and their neuronal processes extend widely in the brain (1-5). Mammals have two Hcrt receptors (HcrtR 1/OrxR A and HcrtR 2/OrxR B) that are distributed throughout the central nervous system and in peripheral organs (5-8). To date, a large body of evidence suggests that Hcrt is involved in multiple physiological processes, such as sleep/wakefulness, food intake, and energy homeostasis (9-13). The Hcrt system has also been reported to regulate reproduction (14-20). However, the pathway and mechanism involved in this regulation are still not clear. In the hypothalamus and other areas of the forebrain, GnRH neurons integrate multiple internal and external factors (neuropeptides, neurotransmitters, hormones, metabolic cues, social cues, photoperiod) as the final common pathway for central control of reproduction (21-33). Given the impact of energy homeostasis on reproduction, studying interactions between Hcrt and GnRH neurons is crucial to understanding the integration of multiple neuronal circuits that impact reproduction.

Currently, zebrafish is one of the favorable animal models widely used in studies of development, molecular genetics, toxicology, pharmacology, pathophysiology, and neuroscience, including neuroendocrinology (34-38). Two forms of GnRH peptides are expressed in zebrafish, GnRH2 and GnRH3, which are encoded by distinct genes with different expression patterns and functions. GnRH2 neurons are located in the midbrain tegmentum, while there are multiple populations of GnRH3 neurons, including in the terminal nerve (TN), trigeminal ganglia (TG), ventral telencephalon (TEL), preoptic area (POA), and hypothalamus (Hypo) (37, 39). GnRH3 neurons located in the TEL, POA and Hypo are considered hypophysiotropic, similar to GnRH1 neurons that control reproduction in other species (37,

40, 41). On the other hand, the organization and functions of the Hcrt system in zebrafish are similar to that of the Hcrt system in mammals (5, 42-44), although the zebrafish genome only contains a single HcrtR ortholog (45).

In the present study, we take advantage of established transgenic zebrafish model systems of *gnrh3:EMD* and *hcrt:RFP* in which the *gnrh3* promoter and *hcrt* promoter drive the expression of a bright variant of green fluorescent protein (Emerald-EMD) and red fluorescent protein (RFP), respectively (46, 47). Using confocal microscopy, we investigated anatomical interactions between these two neuronal systems from the larval stage to adulthood. Using immunohistochemistry, we studied overlapping expression patterns of HcrtR and GnRH3:EMD neurons in the hypothalamus. In addition, we investigated the role of Hcrt in physiological properties of POA-hypothalamic GnRH3 neurons in adults using a combination of electrophysiology, pharmacology, and genetic manipulations. Our findings provide a unique perspective on the anatomical and functional interactions of two hypothalamic neuronal circuits that ultimately impact reproduction.

Materials and methods

Animals

Tg(gnrh3:EMD), *Tg(gnrh3:EMD);(hcrt:RFP)*, *Tg(gnrh3:EMD);hcrtr*$^{-/-}$ (HcrtR null mutant) and *Tg(gnrh3:EMD);hcrtr*$^{+/-}$ (HcrtR heterozygous control) were maintained in a zebrafish aquarium system on a 14L:10D photoperiod at 28°C, and fed with flake food and live brine shrimp twice daily. The double transgenic zebrafish in this study were obtained by crossing the pair of breeders from each line (46, 47). Both male and female sexually mature zebrafish were maintained in separate tanks until the day before breeding. The divider separating males and females was removed shortly after the lights were turned on for timed breeding, and fertilized eggs were collected. Embryos and larvae were maintained in a 28°C

incubator. All procedures were carried out in accordance with the Institutional Animal Care and Use Committees of UCLA and California Institute of Technology.

Immunohistochemistry

Adult wildtype (WT), HcrtR mutants (*hcrtr*$^{+/-}$ and *hcrtr*$^{-/-}$*),* and *Tg(gnrh3:EMD)* zebrafish (both male and female) were used in this study. Animals were anesthetized by immersion in MS-222 solution (150 mg/L). The intact brains were fixed with 4% paraformaldehyde for at least overnight at 4°C. Fixed specimens were washed with PBS, and permeabilized in ice-cold acetone for 30 min, rehydrated in a graded series of solutions containing 75%, 50%, 25% methanol, then blocked in a 10% goat serum in PBST solution for 1 hour at room temperature. Following blocking, samples were washed and incubated for 48 hrs at 4°C on a rotator with the primary antibody solutions (anti-HcrtR2, mouse, R&D Systems, Inc., 1:200) in phosphate buffered saline tween-20 (PBST). After washing with PBST, samples were incubated in the secondary antibody solution (1:1000) for 48 hrs at 4°C. Following a series of rinses in PBST (10 min, 20 min, 30 min, 45 min), the samples were mounted in 0.8% agarose for confocal microscopy imaging. The HcrtR antibody was validated by immunohistochemistry using HcrtR null mutant (*hcrtr*$^{-/-}$*)* and HcrtR heterozygous control *(hcrtr*$^{+/-}$) adult zebrafish. No HcrtR antibody staining was observed in *hcrtr*$^{-/-}$ animals (Fig. 2, right panel), unlike WT and *hcrtr*$^{+/-}$ zebrafish (Figure 2, left and middle panels).

Confocal microscopy

Whole zebrafish larvae (8 dpf) or intact adult brains were mounted in 0.8% agarose (Sigma glass bottomed culture dish (MetTek) (35 mm diameter with 14 mm glass, Mat Tek Corp., Ashland, MA, USA) in a ventral side up position. Samples were covered with PBS to keep them moist and then imaged with a confocal microscope system (upright, Olympus America Inc., Center Valley, PA, USA) (46). Using Fluoview software, samples were then viewed and imaged using 5X, 20X and 40X objectives. EMD

fluorescence was observed using an Argon laser (488nm) with an emission barrier filter of 510 nm. RFP was visualized using a HeNe laser (543nm) with an emission filter of 560–600nm. All images were captured using the sequence mode to avoid bleaching of the two fluorescent signals. Images were taken at 0.5-1.0 µm steps. Optical sections were made along the *z*-axis from the dorsal to the ventral side of the samples. Images were achieved through projections of the *z*-stack. To control the quality of the images, the parameters of the Fluoview program and the microscope were adapted but kept constant for each set of experiments (31).

Electrophysiology

Both loose-patch and whole-cell electrophysiology in current-clamp mode were performed to record the spontaneous electrical activity from the POA-hypothamic GnRH3:EMD neurons located in both preoptic area (POA) and hypothalamus (Hypo) as described previously (39, 48). Briefly, adult zebrafish (3-6 months of age, male and female) were anesthetized by immersion in MS-222 (150 mg/L) and decapitated. The entire brain was carefully removed from the skull and then glued ventral-side up to a glass coverslip at the bottom of a flow-through recording chamber (P1; Warner Instrument Corp., Hamden, CT). The meninges were gently peeled away to expose the POA and Hypo. EMD labeled GnRH3 neurons in POA and Hypo were visualized under an upright microscope (BX50W, Olympus, Melville, NY, USA). Data was acquired by PowerLab instrumentation and software (ADInstruments Inc., Colorado Springs, CO, USA), and analyzed by AxoGraph software (Axon Instruments, Foster City, CA, USA). Following a stable baseline-recording period in fish saline, test solutions (Hcrt and the HcrtR antagonist almorexant) were bath applied for 5 min, followed by a washout period. Aerated solutions were perfused continuously through the recording chamber. It takes about 4 min to reach the final test solution concentration or complete washout in the recording chamber with a perfusion rate at 200 µl/min. One neuron was recorded per animal. To study the effects of Hcrt and almorexant on GnRH3 neuron electrical activity, experiments were performed using *gnrh3:EMD* transgenic zebrafish in order to

positively identify GnRH neurons in live brain. To further study the requirement of functional HcrtR in mediating the effect of Hcrt on GnRH3 neuron electrical activity, experiments were performed using heterozygous control (*Tg(gnrh3:EMD);hcrtr*$^{+/-}$) and homozygous mutant (*Tg(gnrh3:EMD);hcrtr*$^{-/-}$) zebrafish. The last minute of recordings for each treatment period was analyzed for frequency of action potential firing (both whole cell and loose patch recordings) and membrane potential (whole cell recordings only).

Solutions and pharmacological agents

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fish saline contained 134 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, and 1.2 mM $MgCl_2$ in 10 mM Hepes. Osmolarity was adjusted to 290 mOsm with glucose, and pH was adjusted to 7.8 with NaOH. The recording electrode was filled with the solution containing 112.5 mM potassium gluconate, 4 mM NaCl, 17.5 mM KCl, 0.5 mM CaCl2, 1 mM MgCl2, 5 mM MgATP, 1 mM EGTA, 10 mM Hepes, 1 mM GTP, 0.1 mM leupeptin, and 10 mM phosphocreatine. Osmolarity was adjusted to 290 mOsm by titrating the final volume of water, and pH was adjusted to 7.2 with KOH (48). 10 nM Hcrt (Orexin A; Bachem, Torrance, CA) was used in this study following pilot dose-response testing (1 nM, 10 nM, 20 nM, 1 μM; data not shown). Similar dose response was observed as Gaskins (19), not shown. Almorexant (Actelion Pharmaceuticals, Allschwil, Switzerland) was used in 100 nM solution based on the binding kinetic analyses by Malherbe et al. (49).

Data analysis

Data are shown as mean ± SEM. For analysis of electrophysiology data (membrane potential and action potential firing frequency) from experiments involving more than two treatment periods, statistical significance between treatments was determined by one-way ANOVA followed by the Tukey's multiple-comparison test. For analysis of electrophysiology data using the HcrtR mutant, paired t-test was used.

Statistical analysis used GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Differences were considered significant if $P < 0.05$.

Results

Close apposition and interaction between GnRH and Hcrt neuronal systems in the hypothalamus

In this study, we first generated a double transgenic *Tg(gnrh3:EMD);(hcrt:RFP)* zebrafish by crossing the stable transgenic *gnrh3:EMD* and *hcrt:RFP* zebrafish lines in which the GnRH3 and Hcrt neuronal systems are genetically labeled with EMD and RFP, respectively (46, 47). This new transgenic line allowed us to visualize morphological interactions between the two hypothalamic neuropeptidergic systems at different life stages. In this study, we focused on the larval stage (8 dpf) and adulthood (>3 months). Fixed larvae (n=4) were positioned ventral-side up, and low power confocal images of the forebrain revealed localization of GnRH3:EMD neurons and their processes in green and Hcrt:RFP neurons and their processes in red in whole brain (Fig.1: A1-A3). Previous studies in zebrafish embryos showed that GnRH3:EMD neurons form a network of multiple populations including the terminal nerve associated with the olfactory bulb (TN), trigeminal ganglion (TG), pre-optic area (POA), and hypothalamus (Hypo) (39， 47). Hcrt neurons are localized as only two bilateral clusters in the hypothalamus (5, 42). In the present study, we focused only on the hypothalamus to explore the potential interactions between these two neuronal networks. Higher power images of the hypothalamus (Fig.1 A4-A6) reveal that in the larval stage (8 dpf), GnRH3:EMD neurons form two bilateral clusters immediately caudal to the Hcrt neurons, but are adjacent to one another at their rostral border. The Hcrt:RFP neurons are located laterally in the rostral hypothalamus in two clusters as previously reported (42). Both sets of neuronal projections are broadly distributed throughout the hypothalamus, with close apposition between GnRH3 and Hcrt cell bodies and neuronal projections (Fig. 1 A4-A6). The sites of close apposition are shown in single plane images (0.5 μm; Fig. 1 A5 and A6). Further, we examined the interactions between

these two neuronal circuits in the hypothalamus of adult brain (3 months). Whole prefixed brains of the double transgenic *Tg(gnrh3:EMD);(hcrt:RFP)* adult zebrafish (n=4) were examined in the ventral side up position. Merged confocal image (Fig. 1 B1) illustrates that GnRH3 cell bodies are scattered in the hypothalamus, with abundant neuronal projections emerging bilaterally from ventral periventricular hypothalamic areas. Hcrt projections were intertwined with GnRH3 neuronal projections, with additional dense fibers located anterior to GnRH3 projections. Single optical section (0.5 μm) illustrates the anatomical relationship of these two sets of neuronal circuits (Fig. 1 B2 and B3). The close apposition (Fig. 1 B3) suggests direct contact between GnRH3 and Hcrt neurons in the adult hypothalamus, similar to what we observed in the larvae.

Expression of HcrtR in GnRH3:EMD-expressing neurons in the hypothalamus

Wildtype (WT) and *hcrtr*$^{+/-}$ adult zebrafish showed similar patterns of Hcrt receptor expression (Fig. 2 A, left and middle panels). However, HcrtR immunoreactivity was absent in the *hcrtr*$^{-/-}$ mutant (Fig. 2 A, right panel). Using this antibody, we examined HcrtR protein distribution in the *gnrh3:EMD* adult brain (Fig. 2 B; whole mount). HcrtR positive staining neurons (red) were detected in the hypothalamus, especially around the periventricular region. Merged images revealed the HcrtR-ir co-localized in GnRH3:EMD cell bodies (Fig. 2 B4a) and neuronal processes (Fig. 2 B2 and B4b), indicating HcrtR expression in GnRH3 neurons in the hypothalamus in zebrafish. Analysis of four brains showed about 20% of GnRH3:EMD cell bodies around the periventricular region of the hypothalamus co-localized with HcrtR.

Inhibitory effect of Hcrt on the electrical activity of POA-hypothamic GnRH3 neurons

To determine the effect of Hcrt on the electrophysiology of POA-hypothamic GnRH3 neurons in adult zebrafish, extracellular loose patch and whole cell recordings were performed. Figure 3A shows 17 minutes of continuous whole-cell recording from a GnRH3:EMD neuron in the hypothalamus, illustrating

the electrical response of a representative GnRH3 neuron to Hcrt treatment and washout. A low dose of 1 nM Hcrt slightly hyperpolarized the cell membrane potential (from -40 mV to -43 mV) and decreased firing rate (from 1.2 to 0.8 Hz). Subsequent treatment with 10 nM Hcrt dramatically hyperpolarized the cell (from -40 mV to -53 mV) and decreased firing rate (from 1.2 to 0 Hz) in the representative GnRH3 neuron. The average changes of membrane potential and action potential firing frequency (n=5 neurons) in response to 10 nM Hcrt treatments and washout are summarized in Fig. 3B and 3C. 10 nM Hcrt significantly hyperpolarized membrane potential (-47.00 ± 1.95 to -51.40 ± 2.31 mV; *: $P<0.05$) and deceased firing frequency (0.99 ± 0.18 to 0.31 ± 0.22 Hz; *: $P<0.05$). Loose patch recording confirmed the findings from whole cell electrophysiology: 10 nM Hcrt significantly inhibited the electrical activity of GnRH3:EMD neurons, (0.93 ± 0.14 to 0.44 ± 0.11 Hz; n=11, $P<0.05$) (Fig. 3D and 3E). This inhibitory effect was reversible following washout, as shown by both whole cell and loose patch recordings. This loose patch experiment was performed in both males (n=5) and females (n=6), one neuron from each animal. There were no significant sex differences in electrical activity of the baseline and the inhibitory response to Hcrt treatment.

HcrtR activation is required for the inhibitory effect of Hcrt on GnRH3 neuron electrical activity

To explore the mechanism of the Hcrt inhibitory effect on GnRH3 neuron electrical activity (monitored by loose-patch recording), we used both a pharmacological approach (the competitive HcrtR antagonist, almorexant) and a genetic manipulation approach (homozygous null mutant $hcrt^{-/-}$). Figure 4A and 4B shows that 10 nM Hcrt alone inhibits action potential firing of POA-hypothamic GnRH3 neurons, and that the inhibitory effect is blocked by the HcrtR antagonist almorexant (100 nM). Fig. 4B shows analysis of firing rate from six replicate experiments: baseline: 2.30 ± 0.65 Hz; Hcrt treatment: 0.77 ± 0.34 Hz; Hcrt with almorexant treatment: 2.28 ± 0.61 Hz; *: $P<0.05$). Figure 4C and 4D show that 10 nM Hcrt treatment fails to suppress the firing frequency of GnRH3 neurons in $hcrt^{-/-}$ zebrafish (baseline: 1.71. ±0.75; Hcrt: 2.21± 0.83; n=5), while 10 nM Hcrt inhibited electrical activity in $hcrtr^{+/-}$ zebrafish (baseline:

1.62±0.40; Hcrt: 0.39±0.15; n=5; *: P<0.05). The pharmacological and genetic manipulations demonstrate that the inhibitory action of Hcrt on GnRH3 neuron activity requires the HcrtR. In total we recorded from 22 active neurons treated with 100 nM Hcrt ('active' defined as having a firing frequency greater than 0.05 Hz); 21 of them showed a decrease in firing frequency – a 95% response rate (1.32 ± 0.22 to 0.50 ± 0.12 Hz, n= 22; P<0.0001).

Discussion

While the hypothalamus is only a small portion of the brain, as a center of neuronal and hormonal integration it controls essential physiological functions including energy balance, temperature regulation, body growth, stress response, and reproduction (50). Multiple nuclei and cell types within the hypothalamus send projections to each other, as well as to other brain regions. This neuronal communication within a complex functional network is achieved through synaptic and paracrine secretory means (51). The biology of GnRH neurons in the hypothalamus is modulated through integration of multiple inputs, ultimately regulating the pituitary-gonadal axis (52). Nearby hypothalamic Hcrt neurons synthesize and secrete hypocretins/orexins in the hypothalamus, and are recognized primarily as an important regulator of sleep/wakefulness, energy homeostasis, and appetite (2, 5, 42, 44, 53, 54). Recent studies in mammals suggest that hypocretin/orexin play an important role in reproduction by modulating the hypothalamo-pituitary-gonadal axis at different levels (55-60). A few studies provided evidence for interaction between the Hcrt and GnRH neuronal systems and the role of Hcrt in the modulation of reproduction. They showed that Hcrt fibers project to brain areas involved in the control of the hypothalamo-gonadotropic axis, and make anatomical contacts with GnRH cells in different species (61-64). A study by Campbell and colleagues (61) first reported that Hcrt receptors were expressed on GnRH neurons in rat (61). Analysis of the electrophysiological response of GnRH neurons to hypocretin/orexin treatment in mouse revealed that orexin 1 suppressed GnRH neuron activity via the OX-R1 receptor (19). More recently, in goldfish, intracerebroventricular administration of Hcrt inhibited spawning behavior

302 and lowered GnRH2 mRNA levels, while treatment with GnRH decreased HcrtR1 mRNA levels
303 suggesting reciprocal feedback between the two neuronal systems (65).
304
305 Zebrafish is a highly favorable animal model to study the biology of reproduction for its remarkable
306 advantages compared to vertebrates, such as: hundreds of eggs produced from a single female with each
307 mating, rapid embryonic development, transparency during embryogenesis, genetic similarity to
308 mammals, and feasibility for cellular, molecular and genetic manipulation (39-40, 43). Previously, using
309 transgenic *gnrh3:EMD* zebrafish we described the development and biology of GnRH3 neurons within
310 its intact neural circuitry (39). In the present study, we generated a double transgenic zebrafish line
311 *Tg(gnrh3:EMD);(hcrt:RFP)* by crossing established stable transgenic lines *Tg (gnrh3:EMD)* (30) and *Tg*
312 *(hcrt:RFP)* (46), which allowed us to explore interactions between these two neuronal systems. We found
313 that both GnRH3 and Hcrt neurons are densely packed in the hypothalamus, with close apposition of
314 neurons with occasional direct contact that was evident by 8 dpf and continued into adulthood. The
315 majority of Hcrt positive neurons and processes were localized bilaterally in the anterior hypothalamus,
316 while GnRH3:EMD fibers spread widely in the hypothalamus. Close apposition of these two neuronal
317 systems provides the anatomical potential for physiological interactions. This is the first study in any
318 animal to show the anatomical relationship between GnRH and Hcrt neurons from an early developmental
319 stage to adulthood.
320
321 Zebrafish have only one HcrtR, which is similar to HCRTR2 in mammals (5, 45). To elucidate if the
322 GnRH3 neurons in the hypothalamus express HcrtR as shown in rat (61), we conducted whole mount
323 immunohistochemistry with anti-HCRTR2 antibody in adult brains from *Tg(gnrh3:EMD)* zebrafish. As
324 previously reported with *in situ* hybridization (45), HcrtR in the present work was found to be expressed
325 broadly in multiple regions of the brain, including hypothalamus, especially around the periventricular
326 area. Co-localization of HcrtR-ir and GnRH3:EMD (both cell body and processes) in the hypothalamus

indicate the presence of HcrtR in GnRH3 neurons. Our results showed that about 20% of GnRH3:EMD neurons in the ventral periventricular hypothalamic region were co-localized with HcrtR in the cell body. This rate is much lower than 85% reported by Campbell et al. in female rat (61). Abundant GnRH3:EMD neuronal processes were observed with HcrtR co-localization. Therefore, Hcrt regulation of GnRH3 biological functions could be occurring at both the cell bodies and neural processes.

A unique feature of our transgenic *gnrh3:EMD* zebrafish model is that it allowed us to monitor the electrical activity of GnRH3:EMD neurons within the whole adult brain with intact neuronal circuits (31, 39, 48,). We utilized whole cell and loose patch electrophysiological recordings, and found that Hcrt significantly decreased the firing frequency of GnRH3 neurons in the POA and hypothalamus. This inhibitory effect was reversible. Whole cell recording revealed that this inhibitory effect on firing frequency was induced by hyperpolarizing the membrane potential.

Based on our morphological evidence showing HcrtR expression on GnRH3:EMD neurons in the hypothalamus, we hypothesized that Hcrt inhibits GnRH3 neuron electrical activity via HcrtR activation. To test this hypothesis, we first blocked HcrtR activation with the specific competitive antagonist, almorexant. Consistent with our hypothesis, Hcrt failed to inhibit GnRH3:EMD neuron firing frequency in the presence of almorexant. Second, we employed the HcrtR heterozygous control transgenic *gnrh3:EMD (Tg(gnrh3:EMD);hcrtr$^{+/-}$)* and HcrtR null mutant transgenic *gnrh3:EMD (Tg(gnrh3:EMD);hcrtr$^{-/-}$)* adult zebrafish, and found that Hcrt only decreased the firing frequency of GnRH3:EMD neurons in *Tg(gnrh3:EMD);hcrtr$^{+/-}$*, but not in *Tg(gnrh3:EMD);hcrtr$^{-/-}$* animals. Together, the results indicate that Hcrt inhibits GnRH3 neuronal activities via HcrtR activation. Notably, 95% of neurons recorded in this study showed a decrease in firing frequency, but anatomical analysis indicated that only about 20% of GnRH3 cell bodies are co-localized with Hcrtr (while expression in the complex web of neuronal processes are not feasible to count). The inhibitory effect of Hcrt on GnRH3 neurons

through activation of Hcrtr may be occurring directly on both GnRH3 cell bodies and the neuronal processes. Since there is Hcrtr expression on unidentified neurons in the hypothalamus, the inhibitory response could also be indirect through interneurons.

Our study is the first to investigate actions of Hcrt on the physiology of GnRH neurons in any fish species. Earlier work showed that overexpression of Hcrt caused an insomnia-like phenotype in zebrafish (42) and Hcrt treatment stimulated feeding behavior in zebrafish (65). These effects of Hcrt on sleep and feeding are very similar to what has been reported in mammals. In goldfish, Hcrt treatment was shown to decrease expression of *gnrh2* (66). That the work in goldfish and the present study in zebrafish show a suppressive effect of Hcrt on GnRH neuronal biology is also similar to what was reported in mice (19). All these findings suggest that the functional link between the Hcrt and GnRH systems is conserved across species.

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Figure Legends

Figure 1. Representative confocal images of GnRH3 and Hcrt neurons in hypothalamus of *Tg (gnrh3:EMD);(hcrt:RFP)* **zebrafish larvae (8 dpf) and adult.** A1-A3) Ventral-side up view of z-stack images through whole larvae head, 20x magnification; A1: GnRH3:EMD image, A2: Hcrt:RFP image, A3: merge of A1 and A2 images. A4-A5) 40x magnification of the white box in A3; A4: z-stack and A5: single 0.5 µm thick optical section of A4. A6) Magnification of white box in A5. B1) Ventral-side up view of z-stack image of hypothalamus from adult brain, 20x magnification; B2) magnification of white box in B1, single 1 µm thick optical section; B3) magnification of white box in B2. Green: GnRH3:EMD; Red: Hcrt:RFP. TN: terminal nerve; Hypo: hypothalamus. Scale bars: A1-A3 and B1: 200 µm; A4, A5: 50 µm; A6 and B2: 20 µm; B3: 10 µm.

Figure 2. HcrtR expression in hypothalamus of transgenic *gnrh3:EMD* adult zebrafish. A) HcrtR immunoreactivity in hypothalamus in WT (left panel), $HcrtR^{+/-}$ (middle panel) and $HcrtR^{-/-}$ (right panel). B1 and B3) Z-stack confocal images of ventral view of GnRH3:EMD (green) and HcrtR-ir (red) in hypothalamus from two excised intact adult brains. B2) Magnification and single 0.5 µm thick optical section of the box in B1. B4a and B4b) Magnification and single 0.5 µm thick optical sections of the boxes in B3. Co-localizations (yellow) of GnRH3:EMD and HcrtR-ir are shown with arrows on the neuronal processes (B2 and B4b) and cell body (B4a). Scale bars: A and B3: 50 µm; B1: 30 µm; B2, B4a and B4b: 10 µm.

Figure 3. Inhibitory effects of Hcrt on electrical activity of POA-hypothalamic GnRH3:EMD neurons from intact adult zebrafish brain. A) Representative continuous whole cell electrophysiology recording from a GnRH3:EMD neuron through applications of 1 and 10 nM Hcrt treatment and followed by washout. Summary of membrane potential (mean ± SEM) (B) and firing frequency (mean ± SEM) (C)

in response to the treatments with 10 nM Hcrt treatment followed by washout (n=5, *: P<0.05). D) Loose patch electrophysiology recordings from **POA-hypothalamic** GnRH3:EMD neurons. Sample traces showing the pattern of action potential firing during baseline, Hcrt treatment and washout. E) Summary of the firing frequency (mean ± SEM) with 10 nM Hcrt treatment for 5 min followed by the washout (n=11, *: P<0.05).

Figure 4. HcrtR is required for the inhibitory action of Hcrt on GnRH3:EMD neurons in the adult zebrafish brain. A) Representative loose patch electrophysiology recording from a neuron during baseline, followed by hypocretin (10 nM) treatment, then hypocretin (10 nM) + almorexant (100 nM) treatment. B) Data (mean ± SEM) from n=6 replicate experiments show that almorexant completely blocks the inhibitory effect of hypocretin on GnRH3 firing rate. *: P<0.05. C) Representative loose patch electrophysiology recordings from *hcrtr*$^{+/-}$ heterozygote mutant control (n=5) and *hcrtr*$^{-/-}$ null mutant (n=5) during baseline and Hcrt treatment. D) Summary data (mean ± SEM) from n=5 replicate experiments showing that the null receptor mutation completely blocks the inhibitory effect of Hcrt on GnRH3 firing rate.

ANTIBODY TABLE

Peptide/protein target	**Antigen sequence (if known)**	**Name of Antibody**	**Manufacturer, catalog #, and/or name of individual providing the antibody**	**Species raised in; monoclonal or polyclonal**	**Dilution used**
Hypocretin Receptor Type 2 (HCRTR2)		anti-HCRTR2	R&D Systems, Inc.	mouse, monoclonal	1:200

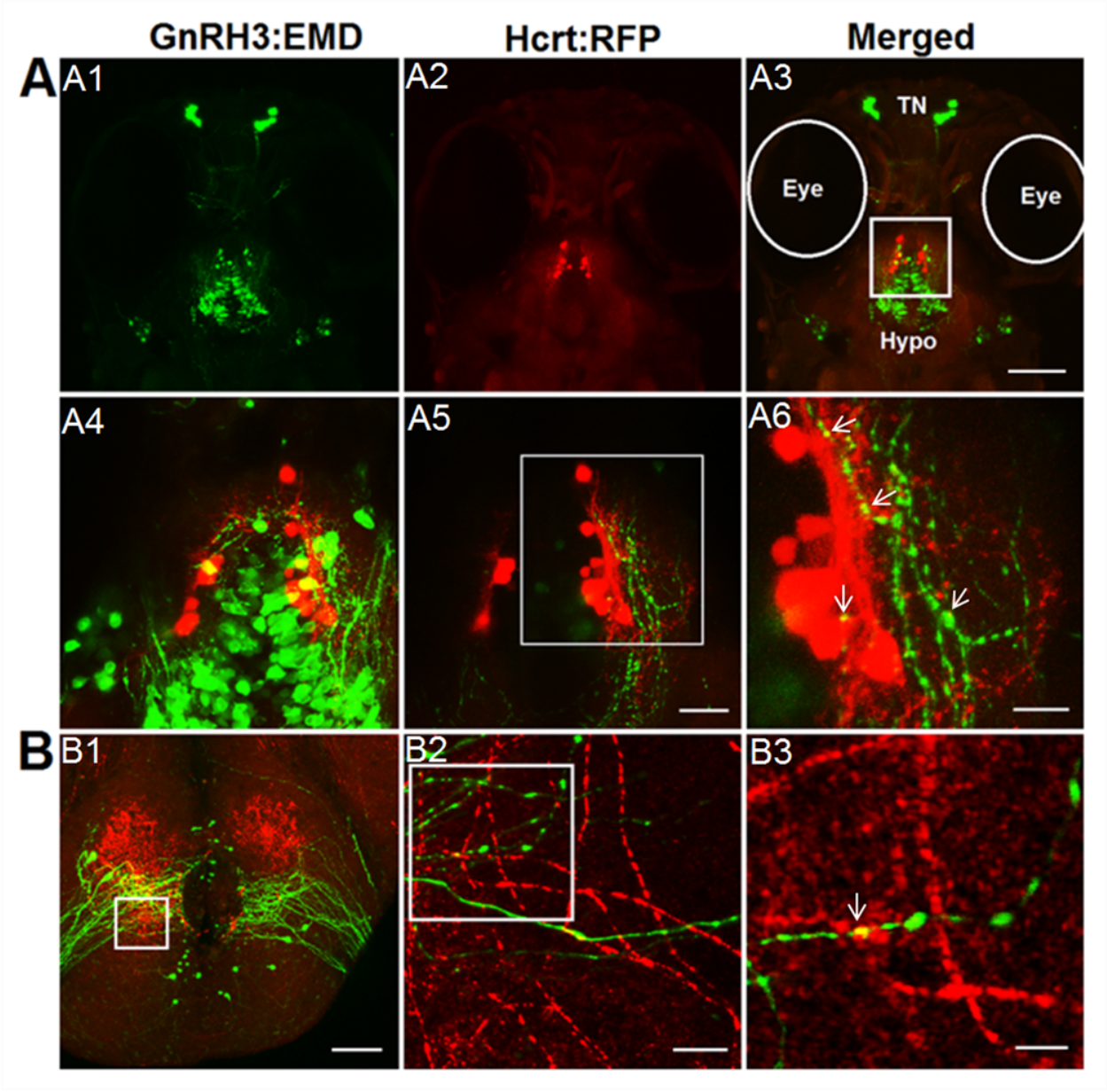
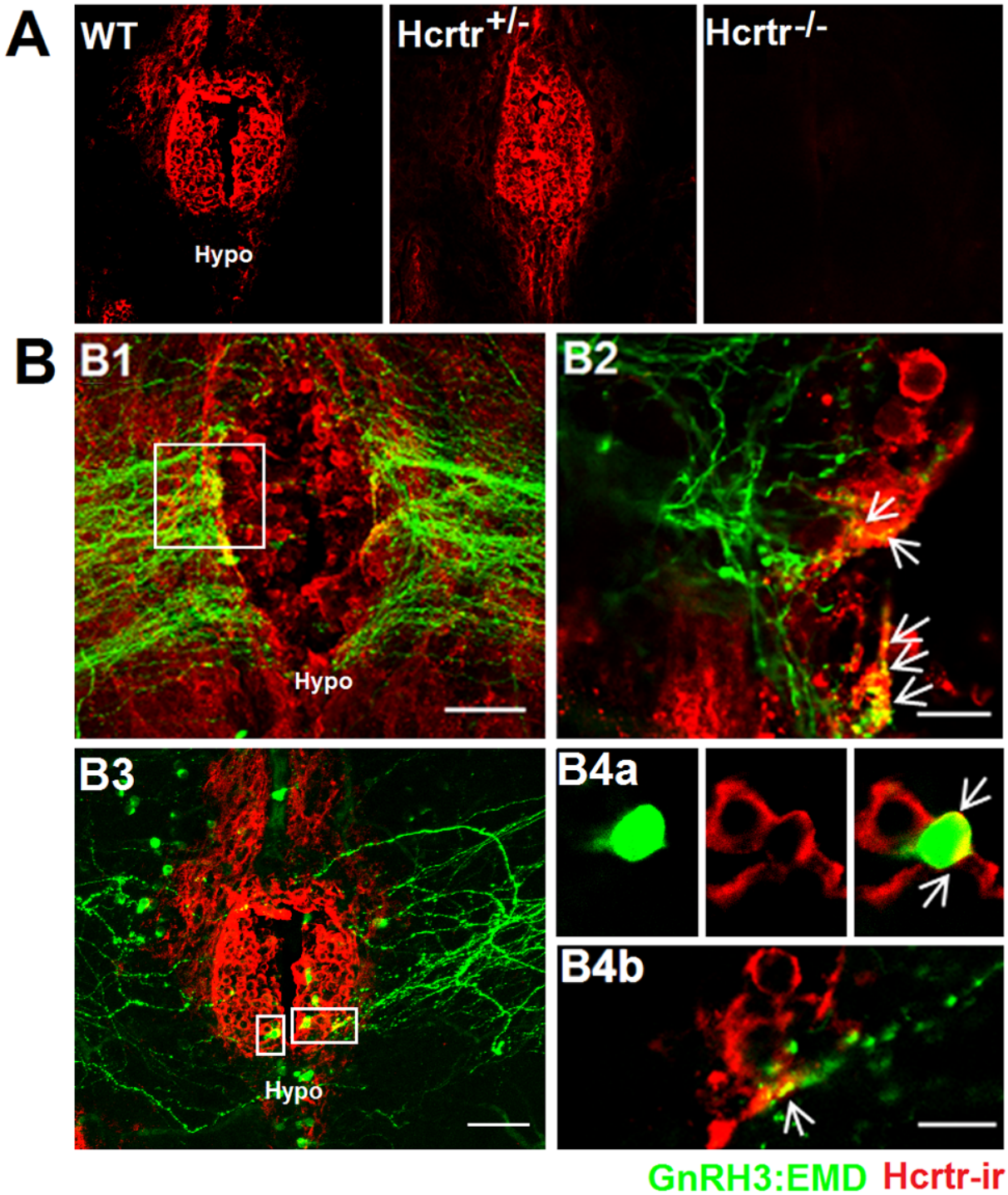

GnRH3:EMD
Hcrt:RFP
Merged
A
A1
A2
A3
TN
Eye
Eye
Hypo
A4
A5
A6
B
B1
B2
B3

Figure 1



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601 Figure 2

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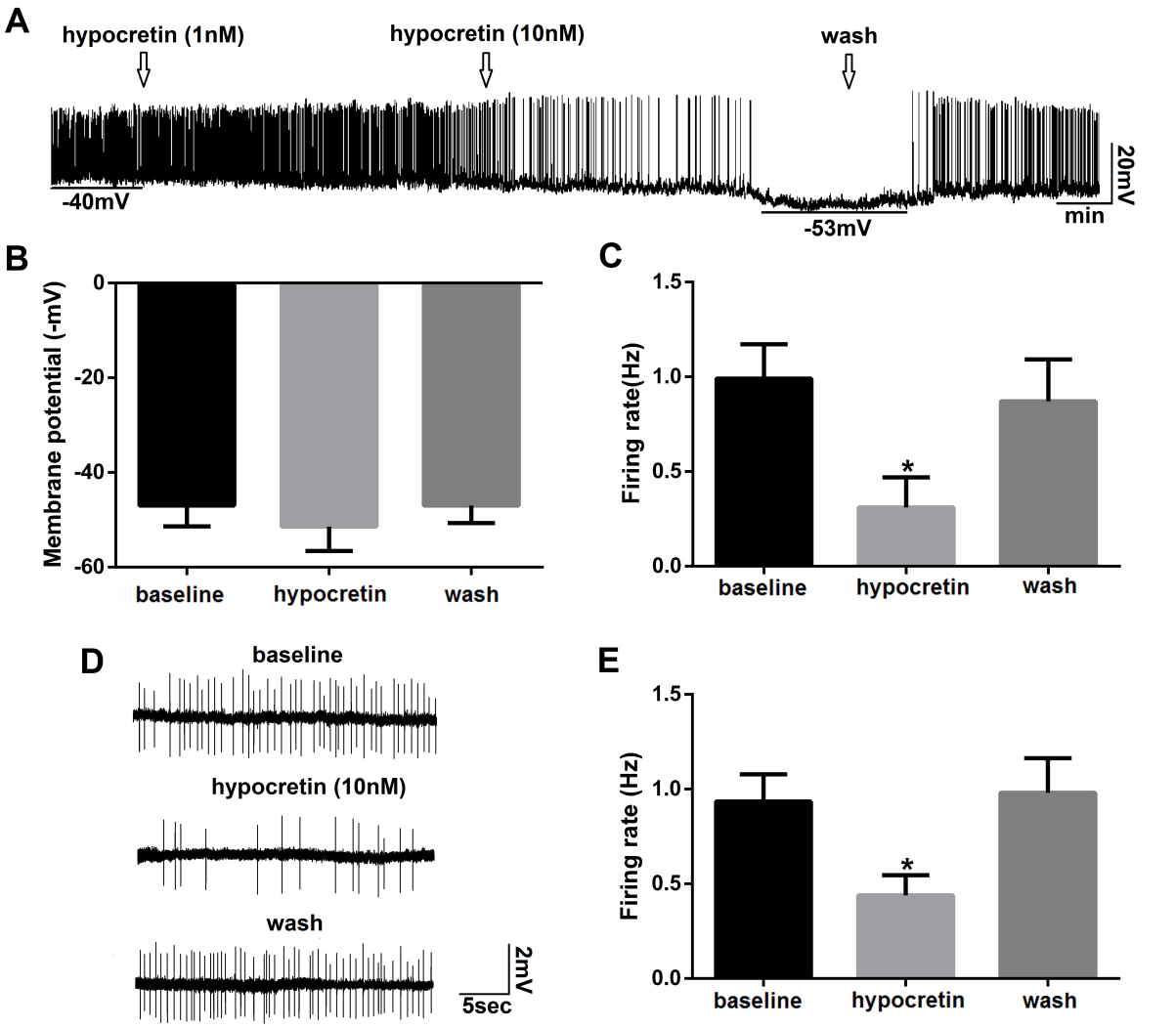

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604 Figure 3

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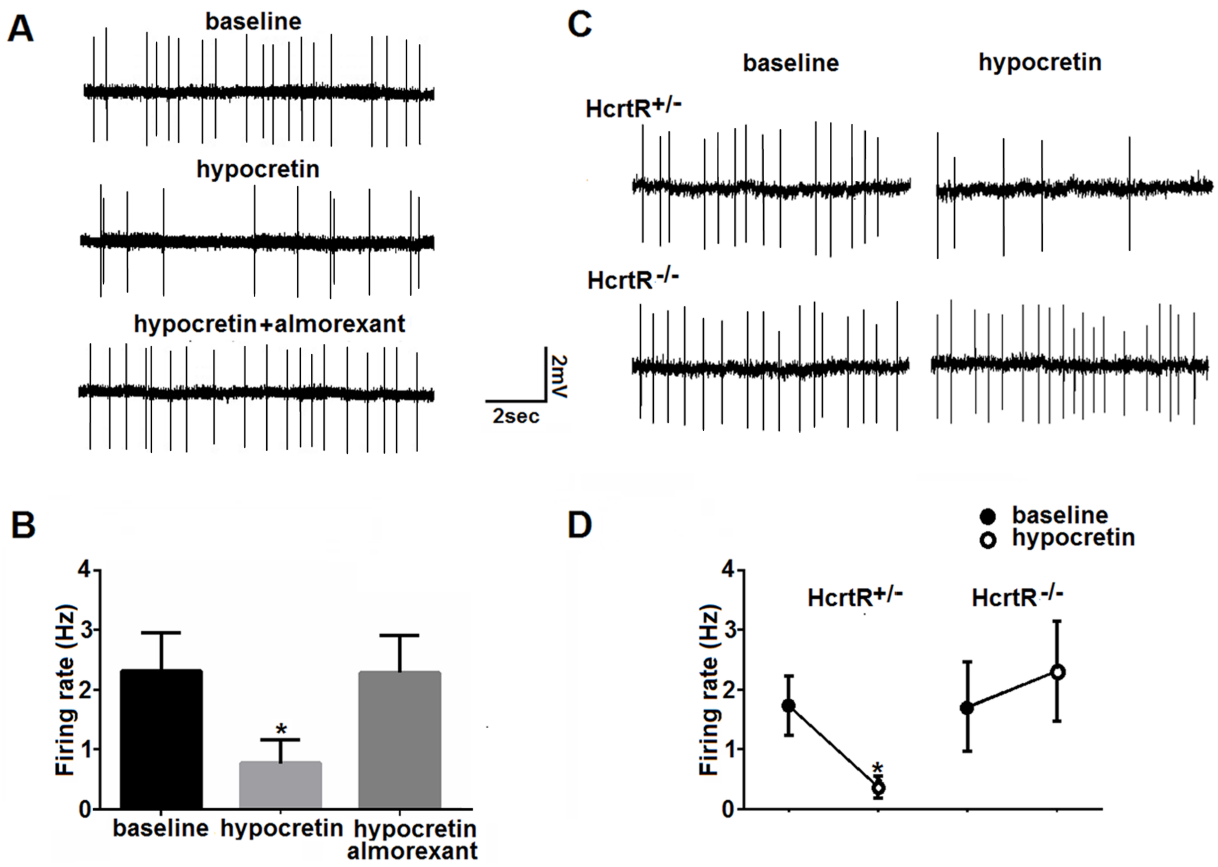

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613 Figure 4