Hypocretin underlies the evolution of sleep loss in the Mexican cavefish

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Abstract

The duration of sleep varies dramatically between species, yet little is known about genetic bases or evolutionary factors driving this variation in behavior. The Mexican cavefish, *Astyanax mexicanus*, exists as surface populations that inhabit rivers, and multiple independently derived cave populations with convergent evolution on sleep loss. The number of Hypocretin/Orexin (HCRT)-positive hypothalamic neurons is increased significantly in cavefish, and HCRT is upregulated at both the transcript and protein levels. Pharmacological inhibition of HCRT signaling increases sleep duration in cavefish without affecting sleep in surface fish, suggesting enhanced HCRT signaling underlies sleep loss in cavefish. Ablation of the lateral line or starvation, manipulations that selectively promote sleep in cavefish, inhibit *hcrt* expression in cavefish while having little effect in surface fish. These findings provide the first evidence of genetic and neuronal changes that contribute to the evolution of sleep loss, and support a conserved role for HCRT in sleep regulation.

Introduction

Sleep behavior is nearly ubiquitous throughout the animal kingdom and vital for many aspects of biological function (Campbell and Tobler, 1984; Hartmann, 1973; Musiek et al., 2015), While animals display remarkable diversity in sleep duration and architecture, little is known about the functional and evolutionary principles driving these differences (Allada and Siegel, 2008; Capellini et al., 2008; Siegel, 2005). We have previously identified the convergent evolution of sleep loss in the blind Mexican cavefish, *Astyanax mexicanus,* resulting in a striking 80% reduction in total sleep duration in independently derived cave-dwelling populations compared to extant surface fish without apparent adverse impacts on health or development (Duboué et al., 2011). The robust differences in sleep provide a unique model for investigating the genetic basis for sleep variation and identification of novel mechanisms underlying the evolution of sleep regulation.

The *Astyanax mexicanus* species consists of eyed 'surface' populations that inhabit open rivers in the Sierra del Abra region of Northeast Mexico, and at least 29 distinct populations of cavefish (Mitchell et al., 1977). Cavefish are derived from surface ancestors, which arose from colonization events within the past 2-5 million years (Gross, 2012; Jeffery, 2009; Keene et al., 2015). Independently-evolved cave populations of *A. mexicanus* share multiple morphological and developmental phenotypes including reduced size or complete absence of eyes and loss of pigmentation (Borowsky, 2008a; Gross and Wilkens, 2013; Protas et al., 2006). In addition, cavefish display an array of behavioral changes including reduced schooling, enhanced vibration attraction behavior, hyperphagia, and sleep loss (Duboué et al., 2011; Kowalko et al., 2013; Mcgaugh et al., 2014; Yoshizawa et al., 2010). Convergent evolution of shared traits in independent cavefish populations, combined with robust phenotypic differences with extant surface fish populations provides a system to examine how naturally occurring variation and

evolution shape complex biological traits.

While the ecological factors underlying phenotypic changes in cave populations are unclear, food availability and foraging strategy are hypothesized to be potent drivers of evolutionary change that contribute to the variation in sleep duration across animal species (Siegel, 2005). Many caves inhabited by *A. mexicanus* are nutrient poor compared to the above-ground river surrounding them (Mitchell et al., 1977), and previous field studies suggest cavefish subsist primarily off of bat guano, small insects, and organic matter washed into the cave by seasonal floods (Keene et al., 2015; Mitchell et al., 1977). Supporting this notion, we previously found that sleep is increased selectively in cavefish during periods of prolonged starvation, raising the possibility that cavefish suppress sleep to forage during the wet season when food is plentiful, and increase sleep to conserve energy during the dry season when food is less abundant (Jaggard et al., 2017). Therefore, sleep loss in cavefish appears to be an evolved feature as a consequence of food availability, providing a model to examine interactions between sleep and metabolism.

Despite the robust phenotypic differences in sleep between *A. mexicanus* surface and cave populations, little is known about the neural mechanisms underlying the evolution of sleep loss in cavefish. Here, we investigate the role of the wake-promoting hypothalamic neuropeptide, Hypocretin/Orexin (HCRT) is highly conserved across vertebrates, and deficiencies in HCRT signaling are associated with increased sleep duration and narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Prober et al., 2006). In zebrafish, HCRT is critical for normal sleep-wake regulation. Ectopic enhancement of *hcrt* expression increases locomotor activity, while deletion of *hcrt* or ablation of HCRT neurons increases daytime sleep= (Elbaz et al., 2012; Prober et al., 2006; Singh et al., 2015). Here, we report that HCRT is upregulated in cavefish, and pharmacological blockade of HCRT signaling selectively reduces sleep in cavefish. Further, HCRT expression is down-regulated in cavefish in response to sleep-promoting manipulations

including starvation and ablation of the lateral line. Together, these findings suggest plasticity of HCRT function underlies the evolved differences in sleep regulation in Mexican cavefish.

Results

Sleep is dramatically reduced in adult Pachón cavefish compared to surface fish counterparts (Fig. 1A, B) (Jaggard et al., 2017; Yoshizawa et al., 2015). To investigate the role of HCRT in cavefish, we compared sequence homology between surface fish and cavefish by a bioinformatic analysis of the sequences from the cavefish genome (Mcgaugh et al., 2014) and available full-length transcriptomic sequences (Gross et al., 2013). Alignment of the HCRT neuropeptide reveals that the *Astyanax* shares high sequence similarity to other fish species (35-48% percent identity) and mammals (35% percent identity), including conservation of domains that give rise to the HCRT neuropeptides (Fig. 1-S1; Wall and Volkoff, 2013). The HCRT peptide sequences of surface and Pachón cavefish are identical (100% percent identity). To determine if *hcrt* expression is altered in cavefish, we measured transcript levels in whole-brain extracts with quantitative real-time PCR (qPCR). Expression of *hcrt* was significantly elevated in Pachón cavefish to over three-fold the levels surface fish, raising the possibility that up-regulation of *hcrt* underlies sleep loss in Pachón cavefish (Fig. 1C). Neuropeptide levels were quantified by immunolabeling serial-sectioned brains, and examining the number of HCRT-positive cell bodies and the relative fluorescence of each cell under fed conditions (Fig 1-S2). The number of HCRT-positive cell bodies was significantly higher in Pachón cavefish compared to surface fish (Fig. 1D). Further, quantification of fluorescence intensity of individual cells revealed increased HCRT neuropeptide in cavefish (Fig. 1E-I). Enhanced levels of HCRT protein were also observed in 5 day post fertilization (dpf) larvae, suggesting the change in peptide levels were present at the time fish begin consuming food (Fig. 1-S3).

To directly assess the contributions of HCRT regulation to sleep loss, we measured the effect of HCRT receptor blockade on sleep in adult surface fish and Pachón cavefish. While mammals

possess two HCRT receptors (HCRTR1 and HCRTR2), zebrafish only possess HCRTR2 and our genome analysis revealed that both the cavefish and surface fish genomes encode only HCRTR2, which is proposed to be evolutionarily more ancient (Mcgaugh et al., 2014; Wong et al., 2011). Fish from both populations were bathed in the selective HCRTR2 pharmacological inhibitor, TCS0X229 (Plaza-Zabala et al., 2012). Surface fish sleep and maintenance of diurnal rhythms in light/dark conditions remained unchanged in the presence of 1μM or 10μM TCS0X229 (Fig. 2A, C). Conversely, treatment of TCS0X229 in Pachón cavefish increased sleep duration compared to solvent treated (DMSO) controls (Fig. 2B, C). While these results do not exclude the possibility that HCRT regulates sleep in surface fish, the sleep-promoting effect of TCS0X229 in Pachón cavefish suggests these fish are more sensitive to changes in HCRT signaling than surface fish. Treatment with TCS0X229 had no effect on waking velocity in surface fish or cavefish, suggesting that the increased quiescence observed in cavefish after drug treatment is not due to lethargy (Fig. 2D). Further analysis revealed that sleep-promoting effects of TCS0X229 in cavefish can be attributed to both an increase in bout number and bout duration, suggesting that HCRT blockade affects sleep onset and maintenance (Fig. 2E, F). Taken together, these findings support the notion that elevated HCRT signaling in cavefish underlies the evolution of sleep loss.

Sleep loss in *A. mexicanus* cavefish populations is conserved throughout development and previous studies have examined sleep in 25 dpf fry as well as in adults (Duboue et al, 2011; Yoshizawa et al, 2015). To determine whether blockade of HCRT signaling restores sleep in developing cavefish, we assessed the effect of TCS0X229 treatment on 25 dpf fry. Similar to findings in adults, TCS0X229 treatment did not affect sleep duration in surface fish (Fig. S4). Treatment significantly increased sleep in treated Pachón cavefish larvae by increasing bout length (Fig. 2-S1) indicating, the enhanced sensitivity to TCS0X229 during adulthood is conserved in early development.

Hypocretin neurons are modulated by sensory stimuli and feeding state, indicating that they are involved in the integration of environmental cues with sleep regulation. The number of mechanosenory neuromasts that comprise the lateral line, a neuromodulatory system used to detect waterflow, are increased in cavefish. This evolved trait is hypothesized to allow for an enhanced ability to forage object detection and social behaviors in the absence of eyes (Kowalko et al., 2013; Kulpa et al., 2015; Yoshizawa et al., 2010). We previously reported that ablation of the lateral line restores sleep to Pachón cavefish without affecting sleep in surface fish, raising the possibility that lateral input modulates HCRT signaling in cavefish to suppress sleep (Jaggard et al., 2017). To investigate the effects of lateral line input on HCRT, we pre-treated adult fish in the ototoxic antibiotic gentamicin, which effectively ablates the lateral line (Van Trump et al., 2010), and assayed sleep in adult cave and surface fish. In agreement with previous findings, gentamicin treatment fully ablated the lateral line (Fig. 3A-D) and restored sleep in cavefish without affecting sleep in surface fish (Jaggard et al., 2017; not shown). To determine the effect of lateral line ablation on HCRT regulation, we quantified *hcrt* expression or neuropeptide levels in adult cave and surface fish following gentamicin treatment. Quantitative PCR analysis of gentamicin treatment revealed that *hcrt* expression was significantly reduced in cavefish to levels equivalent to untreated surface fish (Fig. 3E). We observed a non-significant decrease in *hcrt* expression following gentamicin treatment in surface fish; however, the robust effect of lateral line ablation in cavefish indicates that the lateral line selectively enhances HCRT levels in cavefish. Comparison and detailed quantification of HCRT neuropeptide levels in the hypothalamus reveals that lateral line ablation does not impact the number of HCRT-positive hypothalamic neurons, but instead selectively reduces the level HCRT within each cell in cavefish (Fig. 3F-K), supporting the notion that the lateral line is required for enhances HCRT function in cavefish. Together, these findings reveal that sensory input from the lateral line promotes sleep and *hcrt* expression in Pachón cavefish, providing a link between sensory input and transcriptional regulation of a wake-promoting factor.

In addition to its potent role in sleep regulation, *hcrt* promotes food consumption in fish and mammals (Penney and Volkoff, 2014; Tsujino and Sakurai, 2013; Yokobori et al., 2011). We previously reported that prolonged starvation increases sleep in cavefish without affecting sleep in surface fish (Jaggard et al., 2017), but the role of HCRT in feeding-state dependent modulation of sleep-wake cycles has not been investigated. Quantitative PCR analysis from whole-brain extracts revealed that *hcrt* transcript is significantly reduced in cavefish following 30 days of starvation; however, the same treatment does not affect *hcrt* transcription in surface fish, indicating that cavefish are more sensitive to starvation-dependent changes in HCRT (Fig. 4A). To determine whether HCRT neuropeptide is produced in a greater number of cells during starvation, we quantified HCRT-positive neurons in fed and starved state (Fig. 4B-G). Similar to lateral line ablation, starvation reduced HCRT levels in each cell, without affecting the number of HCRT-positive neurons. Further, starvation did not affect the number of HCRT-positive cells or HCRT levels per cell in surface fish (Fig. 4B). These results indicate that the starvation modulates HCRT levels, rather than the number of cells that produce HCRT. The acute regulation of HCRT by feeding state and lateral-line dependent sensory input demonstrates a unique link between these neuronal systems and those mediating sleep/wake cycles.

Discussion

Cavefish are a unique model for investigating neural and genetic regulation of sleep, particularly from an evolutionary vantage point. Robust phenotypic differences have been observed in multiple populations of cavefish, but our findings provide the first evidence of altered regulation at transcript and protein levels of a neuromodulatory peptide associated with the evolution of sleep loss. Alignment of *hcrt* sequences derived from surface and Pachón cavefish indicate that there are no differences in the genomic sequences between the two morphs. Our findings do, however, reveal dramatic differences in *hcrt* expression and neuron number between surface fish and cavefish, raising the possibility that regulation of *hcrt* is under evolutionary pressure. These findings suggest that functional differences between surface fish and cavefish likely occur at the level of differences in genomic enhancers or neuronal connectivity that affect *hcrt* expression. Because our findings also reveal an increased number of HCRT-positive neurons from an early in development, it is also likely that developmental differences between the brains of surface and cavefish underlie differences in *hcrt* function. Examination of cell body number in six-day-old fry reveals increased HCRT-positive neurons in cavefish, indicating HCRT differences are present during early development. In agreement with these findings, broad anatomical differences in forebrain structure have previously been documented between surface fish and cavefish including an expanded hypothalamus (Menuet et al., 2007). Therefore, it is likely that developmentally-derived differences in the number of HCRT-positive neurons and modified hypothalamic neural circuitry contribute sleep loss in cavefish.

While the neural processes regulating HCRT activity are not fully understood, growing evidence suggests these neurons integrate sleep-wake regulation with responses to sensory stimuli (Mileykovskiy et al., 2005; Woods et al., 2014). In mice, HCRT neurons are transiently activated by sound, feeding, and cage exploration, suggesting that HCRT neurons are generally regulated by external stimuli (Mileykovskiy et al., 2005). Further in zebrafish, overexpression of HCRT

enhances locomotor response to sensory stimuli including light, a dark flash and mechanical stimulation while ablation of HCRT neurons reduces response to sound stimulus (Elbaz et al., 2012; Prober et al., 2006; Woods et al., 2014), suggesting that HCRT neurons mediate sensory responsiveness and sleep-wake behavior. Our findings reveal that ablation of the lateral line in cavefish reduces *hcrt* transcript and neuropeptide abundance to levels indistinguishable from their surface fish conspecifics, indicating that lateral line input is a potent regulator of *hcrt* production in cavefish. These discoveries postulate that evolution of sensory systems dramatically affects central brain processes that regulate behavior, and provide further support that HCRT neurons integrate sensory stimuli to modulate sleep and arousal.

Hypocretin is highly conserved and has been shown to promote wakefulness in species including in animals ranging from zebrafish to humans (Appelbaum et al., 2009; Chemelli et al., 1999; Mileykovskiy et al., 2005). Moreover, the Hypocretin system has been characterized in detail in zebrafish and appears to be a key mediator of arousal (Appelbaum et al., 2009; Elbaz et al., 2012; Kaslin et al., 2004; Prober et al., 2006). Our findings suggest that regulation of HCRT signaling may be subject to evolutionary pressure, and implicate it as a 'hot-spot' for variation in sleep throughout the animal kingdom.

While a full understanding of the neural circuitry regulating HCRT-positive neurons has not been determined, HCRT neurons send projections to numerous areas implicated in behavioral regulation including the periventricular hypothalamus, the raphe, and thalamic nuclei (Panula, 2010). Evidence suggests that wake-promoting role of HCRT neurons is dependent norepinephrine signaling, and optogenetic activation of HCRT neurons activates the locus coeruleus (Singh et al., 2015), raising the possibility that activation of this arousal pathway is enhanced in Pachón cavefish. We previously demonstrated that treating cavefish with the β-adrenergic inhibitor propranolol restores sleep in cavefish without affecting sleep in surface fish, similar to findings observed in this study in fish pharmacologically treated with the HCRT

receptor inhibitor TCS0X229. (Duboué et al., 2012). Therefore, it is possible that differences in norepinephrine signaling contribute to sleep loss in cavefish. Further investigation of the synergistic effects of norepinephrine and *hcrt* in surface and cavefish, and the effects of their pre-supposed interaction on feeding- and sensory-mediated *hcrt* production, will be critical in our understanding of how sleep changes can be driven by alterations in the environment.

In addition to its role in sleep-wake regulation, *hcrt* neurons regulate feeding and metabolic function, raising the possibility that HCRT neurons are integrators of sleep and metabolic state. Previous findings reveal that injection of HCRT peptide increases food consumption in cavefish, suggesting the consummatory behavior induced by HCRT in mammals is conserved in *A. mexicanus* (Penney and Volkoff, 2014; Wall and Volkoff, 2013) In addition, studies in mammals and zebrafish indicate the *hcrt* neurons are regulated by the adipose peptide hormone, Leptin (Levitas-Djerbi et al, 2015). Adipose levels – white? -in cavefish are elevated compared to surface fish (Aspiras et al., 2015), and it is possible that prolonged starvation reduces leptin levels, thereby inhibiting *hcrt* expression to promote sleep in cavefish. These findings indicate that cavefish provide a model for examining the leptin-hcrt axis and, more generally, interactions between sleep and metabolic function.

Our findings specifically examine neural mechanism underlying sleep loss in the Pachón cave populations. Both morphological and genomic data suggest Pachón cavefish are one of the oldest, and most troglomorphic of the 29 *A. mexicanus* cavefish populations (Bradic et al., 2012; Dowling et al., 2002; Ornelas-García et al., 2008; Strecker et al., 2003).. We have also demonstrated evolutionary convergence on sleep loss in other populations of cavefish, including Molino, Tinaja and Chica cave populations (Jaggard et al., 2017). However, ablation of the lateral line has no effect on sleep in Molino, Tinaja and Chica populations, suggesting distinct neural mechanism underlie sleep loss between Pachón cavefish, and other cavefish populations assayed (Jaggard et al., 2017). Future studies will reveal if enhanced HCRT function represents

a conserved mechanism for sleep loss, or that sleep loss in other fish populations is HCRT independent.

Materials and Methods

Fish maintenance and rearing

Animal husbandry was carried out as previously described (Borowsky, 2008b) and all protocols were approved by the IACUC Florida Atlantic University. Fish were housed in the Florida Atlantic University core facilities at 21°C±1°C constant water temperature throughout rearing for behavior experiments (Borowsky, 2008b). Lights were kept on a 14:10 hr light-dark cycle that remained constant throughout the animal's lifetime. Light intensity was kept between 25-40 Lux for both rearing and behavior experiments. All fish used for experiments were raised to adulthood and housed in standard 18-37L tanks. Adult fish were fed a mixture diet of black worms to satiation twice daily at zeitgeber time (ZT) 2 and ZT12, (Aquatic Foods, Fresno, CA,) and standard flake fish food during periods when fish were not being used for behavior experiments or breeding (Tetramine Pro).

Sleep behavior

Adult fish were recorded in standard conditions in 10L tanks with custom-designed partitions that allowed for five fish (2L/fish) to be individually housed in each tank as previously described (Yoshizawa et al., 2015). Recording chambers were illuminated with custom-designed IR LED source (Infrared 850 nm 5050 LED Strip Light, Environmental Lights). After a 4-5 day acclimation period, behavior was recorded for 24hrs beginning ZT0-ZT2. Videos were recorded at 15 frames/sec using a USB webcam (LifeCam Studio 1080p HD Webcam, Microsoft) fitted with a zoom lens (Zoom 7000, Navitar). An IR high-pass filter (Edmund Optics Worldwide) was placed between the camera and the lens to block visible light. For larval fish recordings, individual fish were placed in 12 well tissue culture plates (BD Biosciences). Recording chambers were lit with a custom-designed IR LED light strip and placed beneath the recording platform. Fish were allowed to acclimate for 24 hours before starting behavioral recordings.

Videos were recorded using Virtualdub, a video-capturing software (Version 1.10.4) and were subsequently processed using Ethovision XT 9.0 (Noldus, IT). Water temperature and chemistry were monitored throughout recordings, and maintained at standard conditions in all cases. Ethovision tracking was setup as previously described (Yoshizawa et al., 2015). Data was then processed using Perl scripts (v5.22.0, developed on-site) and Excel macro (Microsoft) (Yoshizawa et al., 2015). These data were used to calculate sleep information by finding bouts of immobility of 60sec and greater, which are highly correlated with increased arousal threshold, one of the hallmarks of sleep (Yoshizawa et al., 2015). For drug treatment studies, fish were allowed normal acclimation periods, followed by 24hrs of baseline recording. At ZT0 fish were treated with either control dimethyl sulfoxide solvent (0.1% DMSO) or freshly prepared TCS0X229 (Tocris) (Plaza-Zabala et al., 2012) dissolved into each 2-gallon tank system and behavior was recorded for 24hrs.

Vital dye labeling and lateral line ablation

Fish were treated with 0.002% gentamicin sulfate as previously described (Sigma Aldrich 1405-41-0) (Van Trump et al., 2010). Following baseline sleep recording and neuromast imaging, fish were bathed in gentamicin for 24hrs. Following the treatment, a complete water change was administered and behavior was again recorded for 24hrs. Fish treated with gentamicin were housed in separate tanks for at least 1 month after treatment in order to avoid contamination. Lateral line re-growth was measured with DASPEI staining two weeks following ablation to confirm that there were no long-term effects from the ablation treatments.

Sequence analysis

To compare Hypocretin/Orexin (HCRT), we aligned the accessioned protein sequences of *A. mexicanus* surface fish (SRR639083.116136.2, SRA) and Pachón cavefish (ENSAMXP00000000478) to orthologous HCRT in zebrafish (ENSDARP00000095322), Medaka (ENSORLP00000004866), Tetraodon (ENSTNIP00000014660), mouse

(ENSMUSP00000057578) and human (ENSP00000293330). Protein alignment, neighbor joining tree (cladogram) and sequences analyses were performed with Clustal Omega (v.1.2.1, EMBL-EBI, (Sievers et al., 2011)). HCRT domains (PF02072/IPR001704) were determined using Ensembl genome browser (v.83, EMBL-EBI/Sanger) and PFam/Interpro (v.28.0, EMBL-EBI).

Quantitative PCR (qPCR)

To measure levels of *hcrt* mRNA, whole brains of one-two year old fish were extracted immediately after behavior was recorded (ZT2). After extraction, individual brains were frozen and homogenized in trizol (QIAGEN, Valencia,CA). RNA was extracted with an RNeasy Mini Kit (QIAGEN, Valencia,CA). All RNA samples were standardized to 10ng/µL concentrations and cDNA synthesis was carried out using iScript (BioRad, Redmond, WA). RT-qPCR was carried out using SsoAdvanced Universal SYBR Green Supermix (BioRad, Redmond, WA). qPCR primers were used at an annealing temperature of 53.3ºC; their sequences follow: *hcrt* forward 5'-CAT-CTC-CTC-AGC-CAA-GGT-TT-3', *hcrt* reverse 5'-TAG-AGT-CCG-TGC-TGT-TAC-ACC-3'. Two housekeeping genes were amplified with the following primers: *rpl13α* forward 5'-TCT-GAA-GGA-CTG-TAA-GAG-GTA-TGC-3', *rpl13α* reverse 5'-AGA-CGC-ACA-ATC-TTG-AGA-GCA-G-3'; *gapdh* forward 5'-GTG-TCC-ACA-GAC-TTC-AAC-GG-3', *gapdh* reverse 5'CAT-TGT-CAT-ACC-ATG-TCA-CCA-G-3'. The following qPCR protocol was run on a Bio Rad CFX96 with a C1000 thermal cycler: 95.0ºC for 3min followed by a plate read at 95.0ºC for 10sec to 53.3ºC for 30sec followed by a plate read repeated 39 times. All samples were compiled into Bio Rad CFX manager gene study (version 3.1) to account for inter-run calibration. All samples were normalized to one (relative to surface fish controls).

Immunohistochemistry

Following euthanasia in MS-222 (Sigma) and ice-water, brains were immediately dissected from adults in ice-cold PBS and fixed overnight in 4% Paraformaldehyde/1x PBS (PFA). Adult brains were then placed in 20% sucrose for cryoprotection overnight or until the brains sunk to the bottom of the well (Kaslin et al, 2004). Whole brains were then flash frozen and mounted in OCT compound (23-730-571 Fisher scientific) for sectioning. Whole brains were serial sectioned in 50 µm slices, all slices were floated in PBS to rinse out embedding solution. Slice sections were then washed in 0.5% Triton-X 100/PBS (PBT) for 3X 15 minutes and co-incubated in 0.5% PBT and 2% Bovine serum albumin (BSA) (Sigma) with primary antibody anti-ORX-A 1:2000 (AB3704 EMD Millipore) overnight at 4°C in . The slices were rinsed again in 0.5% PBT, 3X for 15 minutes and placed in secondary antibody 1:600 (Goat anti-rabbit 488; Life Technologies) for 90 minutes at room temperature. Slices were mounted on slides in Vectashield with DAPI (VectorLabs) and imaged on a Nikon A1 confocal microscope. Whole-mount larvae were fixed overnight in 4% PFA, rinsed 3X 15 min in 0.5% PBT, then placed in 0.5% PBT with 2% BSA and primary antibody anti-ORX-A 1:2000 overnight. Following 3X 15 min 0.5% PBT rinse, larvae were placed in secondary antibody 1:600. Larvae were then placed in Vectashield with DAPI until mounted in 2% low melt temp agarose (Sigma) for imaging. All samples were imaged in 2µm sections and are presented as the Z-stack projection through the entire brain. For quantification of HCRT levels, all hypothalamic slices were imaged in 2µm sections, merged into a single Z-stack as maximum fluorescence, and the total brain fluorescence was determined by creating individual ROIs for each cell expressing HCRT.

Statistics

Two-way ANOVA tests were carried out to test the effects of pharmacological and starvation paradigms among different groups and populations on behavior. Each was modeled as a function of genotype (Surface and Pachón) and genotype by treatment interaction (TCS, gentamicin, or starvation, respectively). Significance for all tests was set at $p<0.05$. When the

ANOVA test detected significance, the Holm-Sidak multiple comparison post-test was carried out to correct for the number of comparisons. For comparison of two baseline groups, non-parametric t-tests were carried out to test for significance. All statistical analysis were carried out using SPSS (IBM, 22.0) or InStat software (GraphPad 6.0).

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Competing Interests

There are no competing interests associated with this work.

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Figure Legends

Figure 1: Hypocretin transcript and peptide levels are elevated in Pachón cavefish. **A.** Representative images of surface fish and Pachón cavefish **B.** Sleep duration is significantly reduced in Pachón cavefish compared to surface morph (Unpaired t-test, t=5.56, n=26, P<0.0001). **C.** Expression of *hcrt* normalized by GAPDH and *rpl13α* in adult whole-brain extracts is significantly enhanced in Pachón cavefish compared to surface fish (Unpaired t-test, t=11.15, n=8, P<0.0001). **D.** HCRT neuropeptide signal is significantly increased in Pachón cavefish compared to surface fish (Unpaired t-test, t=5.94, n=8, P<0.001). E. The number of HCRT-positive cells in the hypothalamus is significantly increased in cavefish compared to surface fish (Unpaired t-test, t=9.984, n=8, P<0.0001). **F-I.** Representative 2 μm confocal images from coronal slices of surface fish or Pachón brains immunostained with anti-HCRT (green) and DAPI (white) **F.** Surface whole brain coronal slice. **G.** Pachón whole brain coronal slice. **H.** Surface fish dorsal hypothalamus containing HCRT positive cells **I.** Pachón cavefish dorsal hypothalamus containing HCRT neurons in view. Scale bar denotes 500μm (F,G); 50μm (H,I).

Figure 2. Pharmacological inhibition of HCRT Receptor 2 promotes sleep in Pachón cavefish. A,B. Twenty-four hour sleep profile in surface fish (A) Pachón cavefish (B) treated with DMSO (black), 1μm TCS (light blue) or 10μm TCS (dark blue). **C.** TCS treatment does not affect total sleep duration in surface fish (1μM p>0.999, n=12, 10 uM P>0.941, n=12). Pachón cavefish teated with 1μM TCS trended towards increased sleep (P>0.178, n=12) while treatment with 10μM significantly increased sleep (P<0.01, n=13; $F_{(1, 73)}$ = 25.00,) compared to control treated fish. **D.** Waking activity was not significantly altered in surface fish or cavefish or in response to drug treatment, 2-way ANOVA, ($F_{(1, 73)}$ = 2.73, P>0.103, n=79) **E.** Treatment with TCS did not affect average sleep bout duration in surface fish (1 μM TCS, P>0.430, n=12; 10μM TCS, P>0.518, n=12) Treatment of Pachón cavefish with 1μM TCS trended towards

increased bout duration, P>0.051, n=12, while 10μM TCS treatment significantly increased bout duration in Pachón cavefish, (P<0.01, n=13; $F_{(1, 73)}$ = 47.42). **F.** TCS treatment did not affect total sleep bout number in surface fish 1μM TCS, P>0.976, n=12; 10μM TCS, P>0.998, n=12). In Pachón cavefish, treatment with 1μM TCS did not affect sleep bout number (P>0.828, n=12). Treatment with 10μM TCS significantly increased bout duration in Pachón cavefish,(P<0.001, n=13; 2-way ANOVA, $F_{(1, 68)}$ = 3.309).

Figure 3: Chemical ablation of mechanosensory lateral line reduces HCRT levels in Pachón cavefish. A-D. Photomicrographs of surface fish cranial regions stained with DASPEI to reveal lateral line mechanosensory neuromasts. Treatment with gentamicin ablates lateral line neuromasts in surface fish (B) and Pachón cavefish (D) **E.** Gentamicin treatment has no significant effect on *hcrt* expression in surface fish (P>0.635, n=8) while in Pachón cavefish gentamicin treatment significantly reduces *hcrt* expression, restoring surface-like levels. (Pachón treated vs. untreated, P<0.0001; Pachón treated vs. surface untreated, P>0.635, n=8, $F_{(1,28)}$=21.28). **F.** Fluorescent intensity per hypothalamic HCRT-cell was not altered with gentamicin treatment in surface fish, P=0.590, n=4. In Pachón cavefish, HCRT neuropeptide levels are significantly lower following gentamicin treatment (P<0.0001, n=4; 2-way ANOVA, F (1, 13) =0.0001 **G.** Gentamicin treatment has no effect on total number of HCRT cell number in either surface or Pachón cavefish (P>0.494, n=8, F (1, 13C) = 0.4967). **H-K** . Representative 2 μm confocal images of the dorsal hypothalamic region in surface fish and Pachón cavefish immunostained with HCRT (green) and DAPI (white) **H.** Surface control **I.** Pachón control **J.** Surface gentamicin K. Pachón gentamicin. Scale bar= 50 μm

Figure 4. Starvation selectively inhbits HCRT levels in cavefish A. Starvation does not affect *hcrt* expression in surface fish (P>0.832, n=4) while *hcrt* expression is significantly reduced in Pachón cavefish (P>0.001, n=4, 2-way ANOVA, F(1,13)=13.54)) **B.** Fluorescent

intensity in HCRT cells was not affected by 30 days starvation in surface fish (P>0.788, n=4). In Pachón cavefish, HCRT neuropeptide was significantly reduced following starvation (P<0.004, n=4, 2-way ANOVA, F(1,12)=10.17)) **C.** Starvation has no significant effect on total number of HCRT-positive cells in either surface or Pachón cavefish (Surface, P=0.452, n=4; Pachón, P>0.979, n=4, 2-way ANOVA, F(1,11)=3.65)) **D.** Surface control **E.** Pachón control **F.** Surface starved **G.** Pachón Starved. Scale bar =50 μm

Supplementary Figures

Figure 1, Figure Supplement 1. HCRT sequence is identical between surface fish and Pachón cavefish. A. Sequence alignment for HCRT in *A. mexicanus* reveals a homology of 35-48% percent identity compared to other fish species including zebrafish and medaka, and a 35% percent identity conserved compared to mammalian HCRT. **B.** 5dpf Surface fish **C.** five dpf Pachón cavefish **D.** HCRT neuropeptide levels are significantly increased in five dpf Pachón larvae compared to surface (Unpaired t-test, t= 3.17, df=10, P=0.01).

Figure 1, Figure Supplement 2. HCRT peptide levels are increased in Pachón cavefish. A. Surface fish whole brain from sagittal 2 μm confocal slice **B.** Pachón cavefish whole brain sagittal 2 μm confocal slice. **C.** Surface fish hypothamus sagittal 2 μm confocal slice. **D.** Pachón cavefish hypothalmus 2 μm confocal slice

Figure 1, Figure Supplement 3. Hypocretin levels are increased in early development in Pachón cavefish. A. Five dpf whole-mount surface fish immunostained with anti-HCRT (green) and DAPI (white). **B.** Whole mount five dpf Pachón cavefish immunostained with anti-HCRT (Green) and DAPI (white). **C.** HCRT neuropeptide levels are significantly increased in five dpf Pachón larvae compared to surface (Unpaired t-test, t= 3.17, df=10, P<0.01).

Figure 2, Figure Supplement 1 HCRTR2 blockade selectively increases sleep in Pachón

cavefish. A,B. Twenty-four hour sleep profiles of surface fish (A) and Pachón cavefish (B) **C.** TCS treatment did not affect sleep duration in surface fish (1 uM, p=0.782, n=18, 10 uM P>0.891, n=18) while Pachón cavefish increased total sleep duration in response to 10uM TCS treatment (1uM, P>0.116, n=17; 10uM, P<0.031, n=18, 2-way ANOVA, F(2,88)=12.178). **D.** Waking activity was not altered in either surface or cavefish, or with any drug treatment (2-way ANOVA, F(2,88)=0.297; SF 1uM P>0.725, n=18; SF 10 uM P>0.341, n=18; Pa 1 uM P>0.997, n=17; Pa 10 uM P>0.9350, n=18). **E.** Sleep bout duration was not significantly changed in response to TCS treatment in either surface or Pachón (Surface 1 μM TCS, P>0.231, n=18; Surface 10 μM, P=0.921, n=18; Pachón 1 μM TCS, P>0.085, n=17; Pachón 10 μM TCS, P>0.232, n=18; 2-way ANOVA, F(2,88) =0.426)) F. Total sleep bout number was not significantly changed in surface fish (Surface 1 μM TCS, P>0.295, n=18; surface 10 μM TCS, P>0.999, n=18) while Pachón cavefish significantly increase total bout number in response to TCS treatment (Pachon 1 μM TCS, P=0.177, n=17; Pachon 10 μM TCS, P>0.450, n=18; 2-way ANOVA, F(2, 88)=3.175))

Figure 1

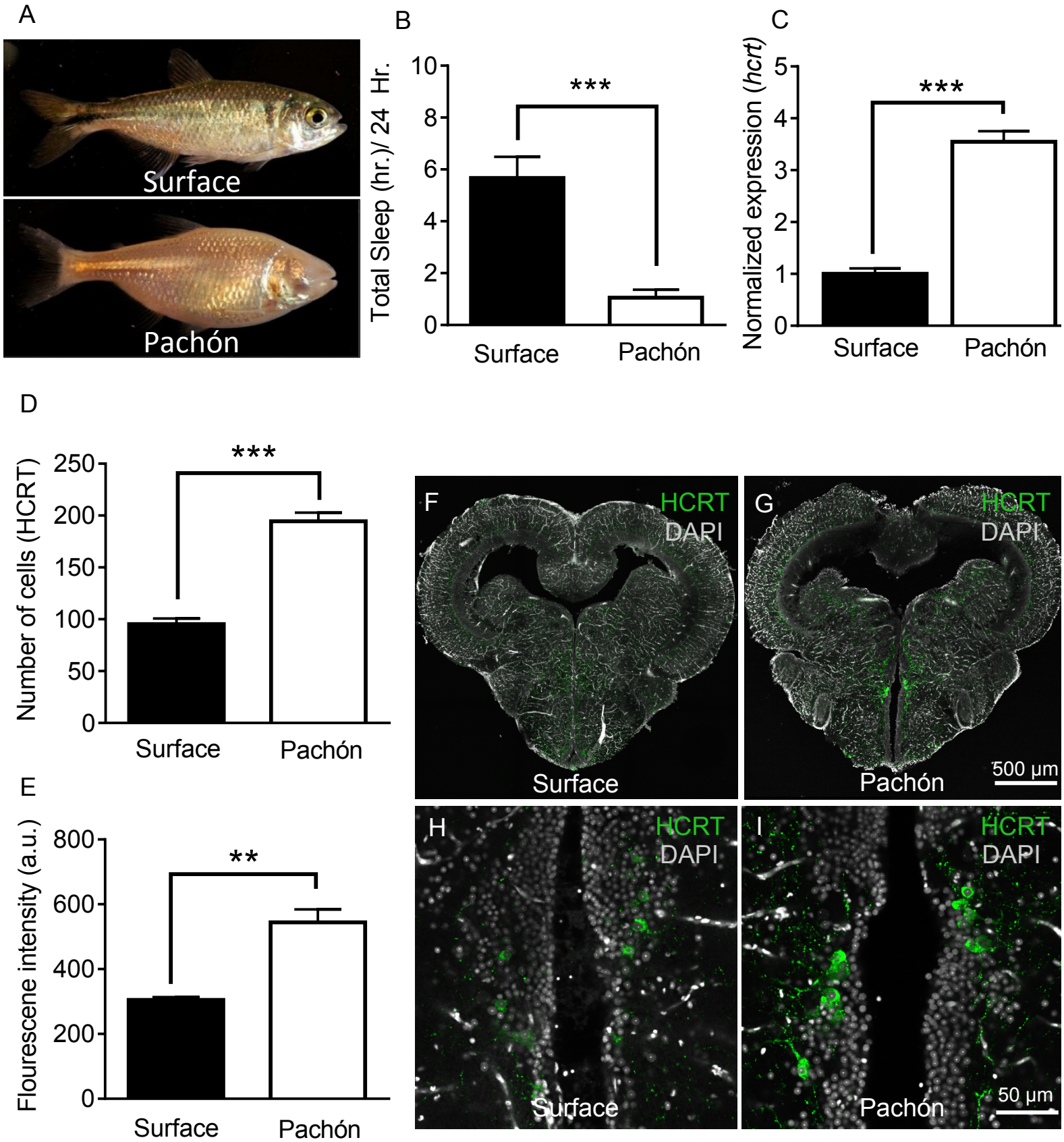

Figure 2

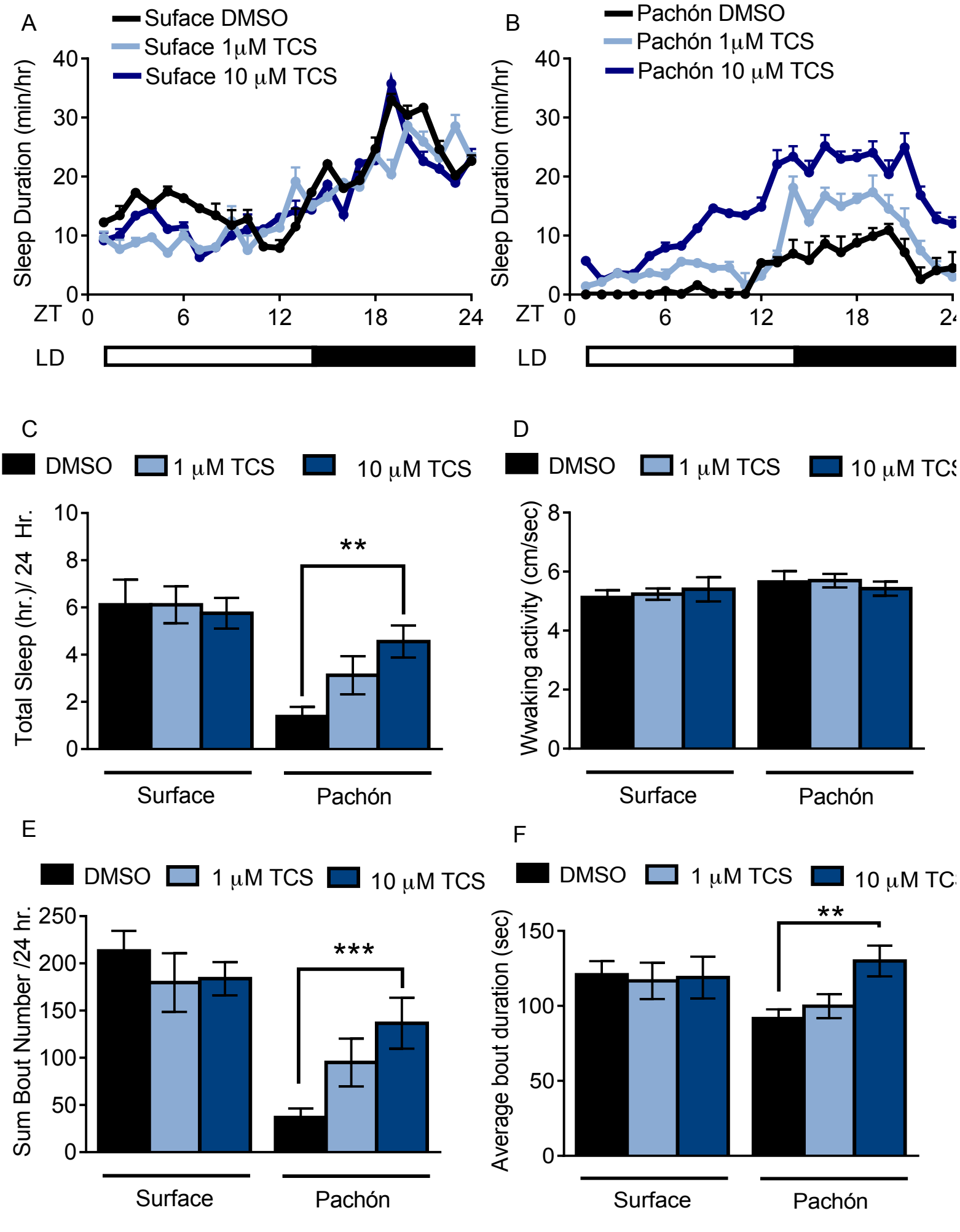

Figure 3

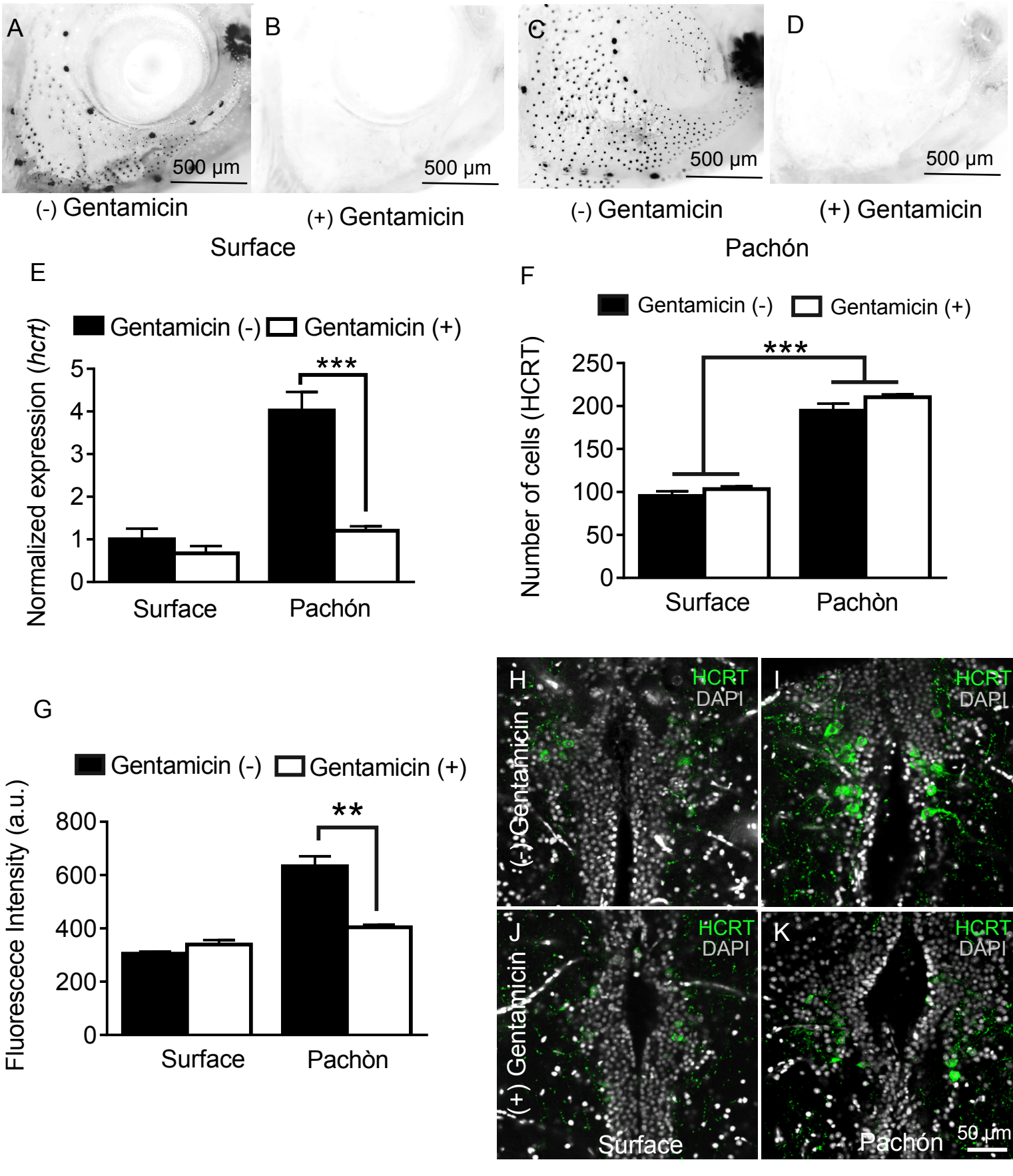

Figure 4