

1 **Hypocretin underlies the evolution of sleep loss in the Mexican cavefish**

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1 **Abstract**

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

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2 **Introduction**

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

14 The *Astyanax mexicanus* species consists of eyed 'surface' populations that inhabit open rivers
15 in the Sierra del Abra region of Northeast Mexico, and at least 29 distinct populations of
16 cavefish (Mitchell et al., 1977). Cavefish are derived from surface ancestors, which arose from
17 colonization events within the past 2-5 million years (Gross, 2012; Jeffery, 2009; Keene et al.,
18 2015). Independently-evolved cave populations of *A. mexicanus* share multiple morphological
19 and developmental phenotypes including reduced size or complete absence of eyes and loss of
20 pigmentation (Borowsky, 2008a; Gross and Wilkens, 2013; Protas et al., 2006). In addition,
21 cavefish display an array of behavioral changes including reduced schooling, enhanced
22 vibration attraction behavior, hyperphagia, and sleep loss (Duboué et al., 2011; Kowalko et al.,
23 2013; Mcgaugh et al., 2014; Yoshizawa et al., 2010). Convergent evolution of shared traits in
24 independent cavefish populations, combined with robust phenotypic differences with extant
25 surface fish populations provides a system to examine how naturally occurring variation and

1 evolution shape complex biological traits.

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

15 Despite the robust phenotypic differences in sleep between *A. mexicanus* surface and cave
16 populations, little is known about the neural mechanisms underlying the evolution of sleep loss
17 in cavefish. Here, we investigate the role of the wake-promoting hypothalamic neuropeptide,
18 Hypocretin/Orexin (HCRT) is highly conserved across vertebrates, and deficiencies in HCRT
19 signaling are associated with increased sleep duration and narcolepsy (Chemelli et al., 1999;
20 Lin et al., 1999; Prober et al., 2006). In zebrafish, HCRT is critical for normal sleep-wake
21 regulation. Ectopic enhancement of *hcr*t expression increases locomotor activity, while deletion
22 of *hcr*t or ablation of HCRT neurons increases daytime sleep= (Elbaz et al., 2012; Prober et al.,
23 2006; Singh et al., 2015). Here, we report that HCRT is upregulated in cavefish, and
24 pharmacological blockade of HCRT signaling selectively reduces sleep in cavefish. Further,
25 HCRT expression is down-regulated in cavefish in response to sleep-promoting manipulations

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

3 **Results**

4 Sleep is dramatically reduced in adult Pachón cavefish compared to surface fish counterparts
5 (Fig. 1A, B) (Jaggard et al., 2017; Yoshizawa et al., 2015). To investigate the role of HCRT in
6 cavefish, we compared sequence homology between surface fish and cavefish by a
7 bioinformatic analysis of the sequences from the cavefish genome (Mcgaugh et al., 2014) and
8 available full-length transcriptomic sequences (Gross et al., 2013). Alignment of the HCRT
9 neuropeptide reveals that the *Astyanax* shares high sequence similarity to other fish species
10 (35-48% percent identity) and mammals (35% percent identity), including conservation of
11 domains that give rise to the HCRT neuropeptides (Fig. 1-S1; Wall and Volkoff, 2013). The
12 HCRT peptide sequences of surface and Pachón cavefish are identical (100% percent identity).
13 To determine if *hcr*t expression is altered in cavefish, we measured transcript levels in whole-
14 brain extracts with quantitative real-time PCR (qPCR). Expression of *hcr*t was significantly
15 elevated in Pachón cavefish to over three-fold the levels surface fish, raising the possibility that
16 up-regulation of *hcr*t underlies sleep loss in Pachón cavefish (Fig. 1C). Neuropeptide levels
17 were quantified by immunolabeling serial-sectioned brains, and examining the number of
18 HCRT-positive cell bodies and the relative fluorescence of each cell under fed conditions (Fig 1-
19 S2). The number of HCRT-positive cell bodies was significantly higher in Pachón cavefish
20 compared to surface fish (Fig. 1D). Further, quantification of fluorescence intensity of individual
21 cells revealed increased HCRT neuropeptide in cavefish (Fig. 1E-I). Enhanced levels of HCRT
22 protein were also observed in 5 day post fertilization (dpf) larvae, suggesting the change in
23 peptide levels were present at the time fish begin consuming food (Fig. 1-S3).

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

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

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

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

1 In addition to its potent role in sleep regulation, *hcrt* promotes food consumption in fish and
2 mammals (Penney and Volkoff, 2014; Tsujino and Sakurai, 2013; Yokobori et al., 2011). We
3 previously reported that prolonged starvation increases sleep in cavefish without affecting sleep
4 in surface fish (Jaggard et al., 2017), but the role of HCRT in feeding-state dependent
5 modulation of sleep-wake cycles has not been investigated. Quantitative PCR analysis from
6 whole-brain extracts revealed that *hcrt* transcript is significantly reduced in cavefish following 30
7 days of starvation; however, the same treatment does not affect *hcrt* transcription in surface
8 fish, indicating that cavefish are more sensitive to starvation-dependent changes in HCRT (Fig.
9 4A). To determine whether HCRT neuropeptide is produced in a greater number of cells during
10 starvation, we quantified HCRT-positive neurons in fed and starved state (Fig. 4B-G). Similar to
11 lateral line ablation, starvation reduced HCRT levels in each cell, without affecting the number of
12 HCRT-positive neurons. Further, starvation did not affect the number of HCRT-positive cells or
13 HCRT levels per cell in surface fish (Fig. 4B). These results indicate that the starvation
14 modulates HCRT levels, rather than the number of cells that produce HCRT. The acute
15 regulation of HCRT by feeding state and lateral-line dependent sensory input demonstrates a
16 unique link between these neuronal systems and those mediating sleep/wake cycles.

1 **Discussion**

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

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

1 enhances locomotor response to sensory stimuli including light, a dark flash and mechanical
2 stimulation while ablation of HCRT neurons reduces response to sound stimulus (Elbaz et al.,
3 2012; Prober et al., 2006; Woods et al., 2014), suggesting that HCRT neurons mediate sensory
4 responsiveness and sleep-wake behavior. Our findings reveal that ablation of the lateral line in
5 cavefish reduces *hcrt* transcript and neuropeptide abundance to levels indistinguishable from
6 their surface fish conspecifics, indicating that lateral line input is a potent regulator of *hcrt*
7 production in cavefish. These discoveries postulate that evolution of sensory systems
8 dramatically affects central brain processes that regulate behavior, and provide further support
9 that HCRT neurons integrate sensory stimuli to modulate sleep and arousal.

10 Hypocretin is highly conserved and has been shown to promote wakefulness in species
11 including in animals ranging from zebrafish to humans (Appelbaum et al., 2009; Chemelli et al.,
12 1999; Mileykovskiy et al., 2005). Moreover, the Hypocretin system has been characterized in
13 detail in zebrafish and appears to be a key mediator of arousal (Appelbaum et al., 2009; Elbaz
14 et al., 2012; Kaslin et al., 2004; Prober et al., 2006). Our findings suggest that regulation of
15 HCRT signaling may be subject to evolutionary pressure, and implicate it as a ‘hot-spot’ for
16 variation in sleep throughout the animal kingdom.

17 While a full understanding of the neural circuitry regulating HCRT-positive neurons has not been
18 determined, HCRT neurons send projections to numerous areas implicated in behavioral
19 regulation including the periventricular hypothalamus, the raphe, and thalamic nuclei (Panula,
20 2010). Evidence suggests that wake-promoting role of HCRT neurons is dependent
21 norepinephrine signaling, and optogenetic activation of HCRT neurons activates the locus
22 coeruleus (Singh et al., 2015), raising the possibility that activation of this arousal pathway is
23 enhanced in Pachón cavefish. We previously demonstrated that treating cavefish with the β -
24 adrenergic inhibitor propranolol restores sleep in cavefish without affecting sleep in surface fish,
25 similar to findings observed in this study in fish pharmacologically treated with the HCRT

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

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

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

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

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4

1 **Materials and Methods**

2 **Fish maintenance and rearing**

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

13

14 **Sleep behavior**

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

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

13 **Vital dye labeling and lateral line ablation**

14 Fish were treated with 0.002% gentamicin sulfate as previously described (Sigma Aldrich 1405-
15 41-0) (Van Trump et al., 2010). Following baseline sleep recording and neuromast imaging, fish
16 were bathed in gentamicin for 24hrs. Following the treatment, a complete water change was
17 administered and behavior was again recorded for 24hrs. Fish treated with gentamicin were
18 housed in separate tanks for at least 1 month after treatment in order to avoid contamination.
19 Lateral line re-growth was measured with DASPEI staining two weeks following ablation to
20 confirm that there were no long-term effects from the ablation treatments.

21 **Sequence analysis**

22 To compare Hypocretin/Orexin (HCRT), we aligned the accessioned protein sequences of *A.*
23 *mexicanus* surface fish ([SRR639083.116136.2](https://www.ncbi.nlm.nih.gov/nuclot/SRR639083.116136.2), SRA) and Pachón cavefish
24 (ENSAMXP00000000478) to orthologous HCRT in zebrafish (ENSARP000000095322),
25 Medaka (ENSORLP00000004866), Tetraodon (ENSTNIP00000014660), mouse

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

6 7 **Quantitative PCR (qPCR)**

8 To measure levels of *hcrt* mRNA, whole brains of one-two year old fish were extracted
9 immediately after behavior was recorded (ZT2). After extraction, individual brains were frozen
10 and homogenized in trizol (QIAGEN, Valencia,CA). RNA was extracted with an RNeasy Mini Kit
11 (QIAGEN, Valencia,CA). All RNA samples were standardized to 10ng/μL concentrations and
12 cDNA synthesis was carried out using iScript (BioRad, Redmond, WA). RT-qPCR was carried
13 out using SsoAdvanced Universal SYBR Green Supermix (BioRad, Redmond, WA). qPCR
14 primers were used at an annealing temperature of 53.3°C; their sequences follow: *hcrt* forward
15 5'-CAT-CTC-CTC-AGC-CAA-GGT-TT-3', *hcrt* reverse 5'-TAG-AGT-CCG-TGC-TGT-TAC-ACC-
16 3'. Two housekeeping genes were amplified with the following primers: *rpl13a* forward 5'-TCT-
17 GAA-GGA-CTG-TAA-GAG-GTA-TGC-3', *rpl13a* reverse 5'-AGA-CGC-ACA-ATC-TTG-AGA-
18 GCA-G-3'; *gapdh* forward 5'-GTG-TCC-ACA-GAC-TTC-AAC-GG-3', *gapdh* reverse 5'-CAT-TGT-
19 CAT-ACC-ATG-TCA-CCA-G-3'. The following qPCR protocol was run on a Bio Rad CFX96 with
20 a C1000 thermal cycler: 95.0°C for 3min followed by a plate read at 95.0°C for 10sec to 53.3°C
21 for 30sec followed by a plate read repeated 39 times. All samples were compiled into Bio Rad
22 CFX manager gene study (version 3.1) to account for inter-run calibration. All samples were
23 normalized to one (relative to surface fish controls).

24 25 **Immunohistochemistry**

1 Following euthanasia in MS-222 (Sigma) and ice-water, brains were immediately dissected from
2 adults in ice-cold PBS and fixed overnight in 4% Paraformaldehyde/1x PBS (PFA). Adult brains
3 were then placed in 20% sucrose for cryoprotection overnight or until the brains sunk to the
4 bottom of the well (Kaslin et al, 2004). Whole brains were then flash frozen and mounted in OCT
5 compound (23-730-571 Fisher scientific) for sectioning. Whole brains were serial sectioned in
6 50 μ m slices, all slices were floated in PBS to rinse out embedding solution. Slice sections were
7 then washed in 0.5% Triton-X 100/PBS (PBT) for 3X 15 minutes and co-incubated in 0.5% PBT
8 and 2% Bovine serum albumin (BSA) (Sigma) with primary antibody anti-ORX-A 1:2000
9 (AB3704 EMD Millipore) overnight at 4°C in . The slices were rinsed again in 0.5% PBT, 3X for
10 15 minutes and placed in secondary antibody 1:600 (Goat anti-rabbit 488; Life Technologies) for
11 90 minutes at room temperature. Slices were mounted on slides in Vectashield with DAPI
12 (VectorLabs) and imaged on a Nikon A1 confocal microscope. Whole-mount larvae were fixed
13 overnight in 4% PFA, rinsed 3X 15 min in 0.5% PBT, then placed in 0.5% PBT with 2% BSA
14 and primary antibody anti-ORX-A 1:2000 overnight. Following 3X 15 min 0.5% PBT rinse, larvae
15 were placed in secondary antibody 1:600. Larvae were then placed in Vectashield with DAPI
16 until mounted in 2% low melt temp agarose (Sigma) for imaging. All samples were imaged in
17 2 μ m sections and are presented as the Z-stack projection through the entire brain. For
18 quantification of HCRT levels, all hypothalamic slices were imaged in 2 μ m sections, merged into
19 a single Z-stack as maximum fluorescence, and the total brain fluorescence was determined by
20 creating individual ROIs for each cell expressing HCRT.

21

22 **Statistics**

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

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

5

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10 **Competing Interests**

11 There are no competing interests associated with this work.

12

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1 **Figure Legends**

2 **Figure 1: Hypocretin transcript and peptide levels are elevated in Pachón cavefish.**

3 **A.** Representative images of surface fish and Pachón cavefish **B.** Sleep duration is significantly
4 reduced in Pachón cavefish compared to surface morph (Unpaired t-test, $t=5.56$, $n=26$,
5 $P<0.0001$). **C.** Expression of *hcrt* normalized by GAPDH and *rp13 α* in adult whole-brain
6 extracts is significantly enhanced in Pachón cavefish compared to surface fish (Unpaired t-test,
7 $t=11.15$, $n=8$, $P<0.0001$). **D.** HCRT neuropeptide signal is significantly increased in Pachón
8 cavefish compared to surface fish (Unpaired t-test, $t=5.94$, $n=8$, $P<0.001$). **E.** The number of
9 HCRT-positive cells in the hypothalamus is significantly increased in cavefish compared to
10 surface fish (Unpaired t-test, $t=9.984$, $n=8$, $P<0.0001$). **F-I.** Representative 2 μm confocal
11 images from coronal slices of surface fish or Pachón brains immunostained with anti-HCRT
12 (green) and DAPI (white) **F.** Surface whole brain coronal slice. **G.** Pachón whole brain coronal
13 slice. **H.** Surface fish dorsal hypothalamus containing HCRT positive cells **I.** Pachón cavefish
14 dorsal hypothalamus containing HCRT neurons in view. Scale bar denotes 500 μm (F,G); 50 μm
15 (H,I).

16 **Figure 2. Pharmacological inhibition of HCRT Receptor 2 promotes sleep in Pachón**

17 **cavefish. A,B.** Twenty-four hour sleep profile in surface fish (A) Pachón cavefish (B) treated
18 with DMSO (black), 1 μM TCS (light blue) or 10 μM TCS (dark blue). **C.** TCS treatment does not
19 affect total sleep duration in surface fish (1 μM $p>0.999$, $n=12$, 10 μM $P>0.941$, $n=12$). Pachón
20 cavefish treated with 1 μM TCS trended towards increased sleep ($P>0.178$, $n=12$) while
21 treatment with 10 μM significantly increased sleep ($P<0.01$, $n=13$; $F(1, 73) = 25.00$,) compared
22 to control treated fish. **D.** Waking activity was not significantly altered in surface fish or cavefish
23 or in response to drug treatment, 2-way ANOVA, ($F(1, 73) = 2.73$, $P>0.103$, $n=79$) **E.** Treatment
24 with TCS did not affect average sleep bout duration in surface fish (1 μM TCS, $P>0.430$, $n=12$;
25 10 μM TCS, $P>0.518$, $n=12$) Treatment of Pachón cavefish with 1 μM TCS trended towards

1 increased bout duration, $P > 0.051$, $n = 12$, while $10 \mu\text{M}$ TCS treatment significantly increased bout
2 duration in Pachón cavefish, ($P < 0.01$, $n = 13$; $F(1, 73) = 47.42$). **F.** TCS treatment did not affect
3 total sleep bout number in surface fish $1 \mu\text{M}$ TCS, $P > 0.976$, $n = 12$; $10 \mu\text{M}$ TCS, $P > 0.998$, $n = 12$.
4 In Pachón cavefish, treatment with $1 \mu\text{M}$ TCS did not affect sleep bout number ($P > 0.828$, $n = 12$).
5 Treatment with $10 \mu\text{M}$ TCS significantly increased bout duration in Pachón cavefish, ($P < 0.001$,
6 $n = 13$; 2-way ANOVA, $F(1, 68) = 3.309$).

7 **Figure 3: Chemical ablation of mechanosensory lateral line reduces HCRT levels in**
8 **Pachón cavefish. A-D.** Photomicrographs of surface fish cranial regions stained with DASPEI
9 to reveal lateral line mechanosensory neuromasts. Treatment with gentamicin ablates lateral
10 line neuromasts in surface fish (B) and Pachón cavefish (D) **E.** Gentamicin treatment has no
11 significant effect on *hcrt* expression in surface fish ($P > 0.635$, $n = 8$) while in Pachón cavefish
12 gentamicin treatment significantly reduces *hcrt* expression, restoring surface-like levels.
13 (Pachón treated vs. untreated, $P < 0.0001$; Pachón treated vs. surface untreated, $P > 0.635$, $n = 8$,
14 $F(1, 28) = 21.28$). **F.** Fluorescent intensity per hypothalamic HCRT-cell was not altered with
15 gentamicin treatment in surface fish, $P = 0.590$, $n = 4$. In Pachón cavefish, HCRT neuropeptide
16 levels are significantly lower following gentamicin treatment ($P < 0.0001$, $n = 4$; 2-way ANOVA, F
17 $(1, 13) = 0.0001$ **G.** Gentamicin treatment has no effect on total number of HCRT cell number in
18 either surface or Pachón cavefish ($P > 0.494$, $n = 8$, $F(1, 13) = 0.4967$). **H-K.** Representative 2
19 μm confocal images of the dorsal hypothalamic region in surface fish and Pachón cavefish
20 immunostained with HCRT (green) and DAPI (white) **H.** Surface control **I.** Pachón control **J.**
21 Surface gentamicin **K.** Pachón gentamicin. Scale bar = $50 \mu\text{m}$

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

1 intensity in HCRT cells was not affected by 30 days starvation in surface fish ($P>0.788$, $n=4$). In
2 Pachón cavefish, HCRT neuropeptide was significantly reduced following starvation ($P<0.004$,
3 $n=4$, 2-way ANOVA, $F(1,12)=10.17$) **C.** Starvation has no significant effect on total number of
4 HCRT-positive cells in either surface or Pachón cavefish (Surface, $P=0.452$, $n=4$; Pachón,
5 $P>0.979$, $n=4$, 2-way ANOVA, $F(1,11)=3.65$) **D.** Surface control **E.** Pachón control **F.** Surface
6 starved **G.** Pachón Starved. Scale bar =50 μm

7 **Supplementary Figures**

8 **Figure 1, Figure Supplement 1. HCRT sequence is identical between surface fish and**
9 **Pachón cavefish. A.** Sequence alignment for HCRT in *A. mexicanus* reveals a homology of 35-
10 48% percent identity compared to other fish species including zebrafish and medaka, and a
11 35% percent identity conserved compared to mammalian HCRT. **B.** 5dpf Surface fish **C.** five dpf
12 Pachón cavefish **D.** HCRT neuropeptide levels are significantly increased in five dpf Pachón
13 larvae compared to surface (Unpaired t-test, $t= 3.17$, $df=10$, $P=0.01$).

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

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

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

1 **cavefish. A,B.** Twenty-four hour sleep profiles of surface fish (A) and Pachón cavefish (B) **C.**
2 TCS treatment did not affect sleep duration in surface fish (1 uM, $p=0.782$, $n=18$, 10 uM
3 $P>0.891$, $n=18$) while Pachón cavefish increased total sleep duration in response to 10uM TCS
4 treatment (1uM, $P>0.116$, $n=17$; 10uM, $P<0.031$, $n=18$, 2-way ANOVA, $F(2,88)=12.178$). **D.**
5 Waking activity was not altered in either surface or cavefish, or with any drug treatment (2-way
6 ANOVA, $F(2,88)=0.297$; SF 1uM $P>0.725$, $n=18$; SF 10 uM $P>0.341$, $n=18$; Pa 1 uM $P>0.997$,
7 $n=17$; Pa 10 uM $P>0.9350$, $n=18$). **E.** Sleep bout duration was not significantly changed in
8 response to TCS treatment in either surface or Pachón (Surface 1 μ M TCS, $P>0.231$, $n=18$;
9 Surface 10 μ M, $P=0.921$, $n=18$; Pachón 1 μ M TCS, $P>0.085$, $n=17$; Pachón 10 μ M TCS,
10 $P>0.232$, $n=18$; 2-way ANOVA, $F(2,88) =0.426$) **F.** Total sleep bout number was not
11 significantly changed in surface fish (Surface 1 μ M TCS, $P>0.295$, $n=18$; surface 10 μ M TCS,
12 $P>0.999$, $n=18$) while Pachón cavefish significantly increase total bout number in response to
13 TCS treatment (Pachon 1 μ M TCS, $P=0.177$, $n=17$; Pachon 10 μ M TCS, $P>0.450$, $n=18$; 2-way
14 ANOVA, $F(2, 88)=3.175$)

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Figure 1

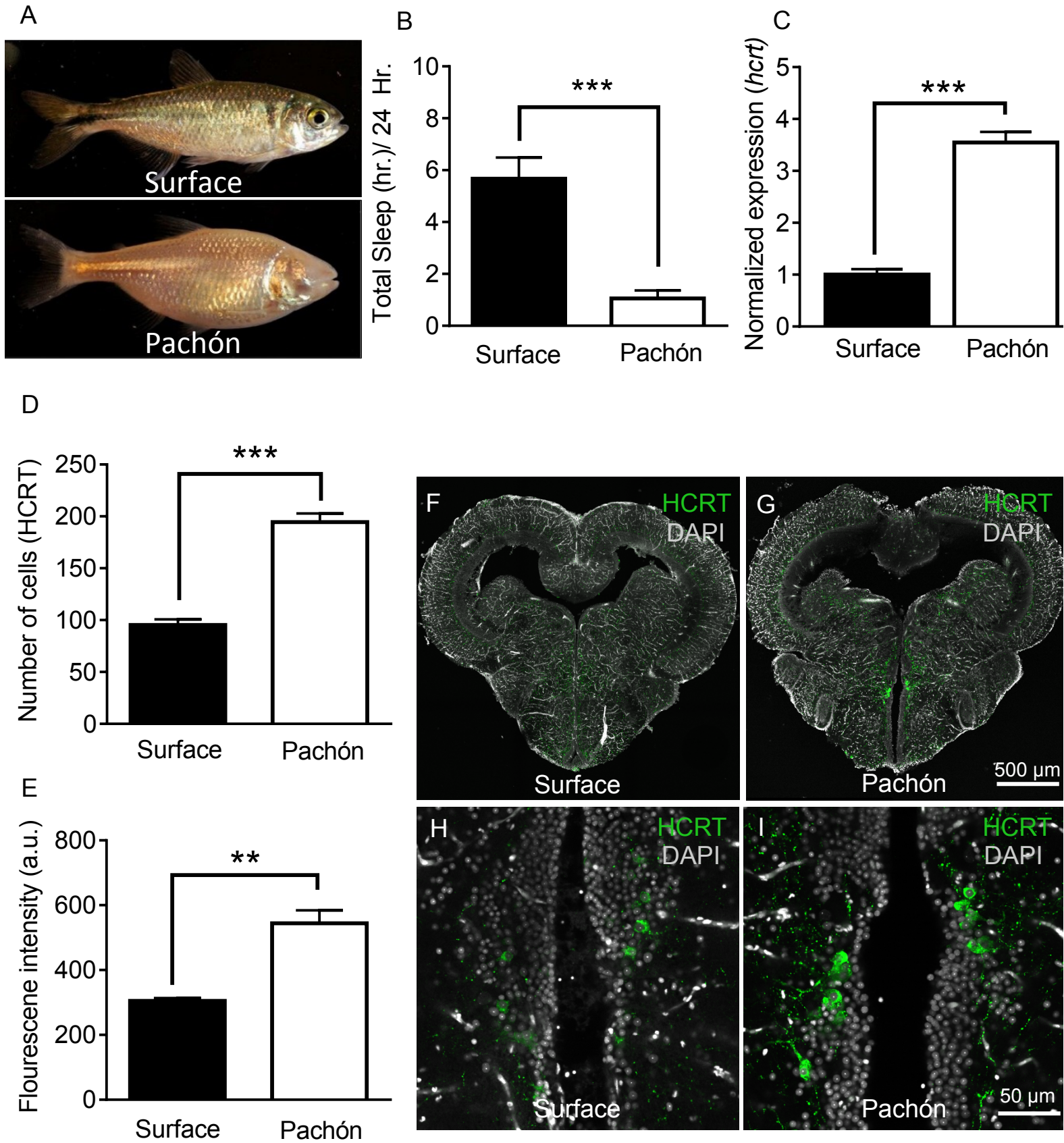


Figure 2

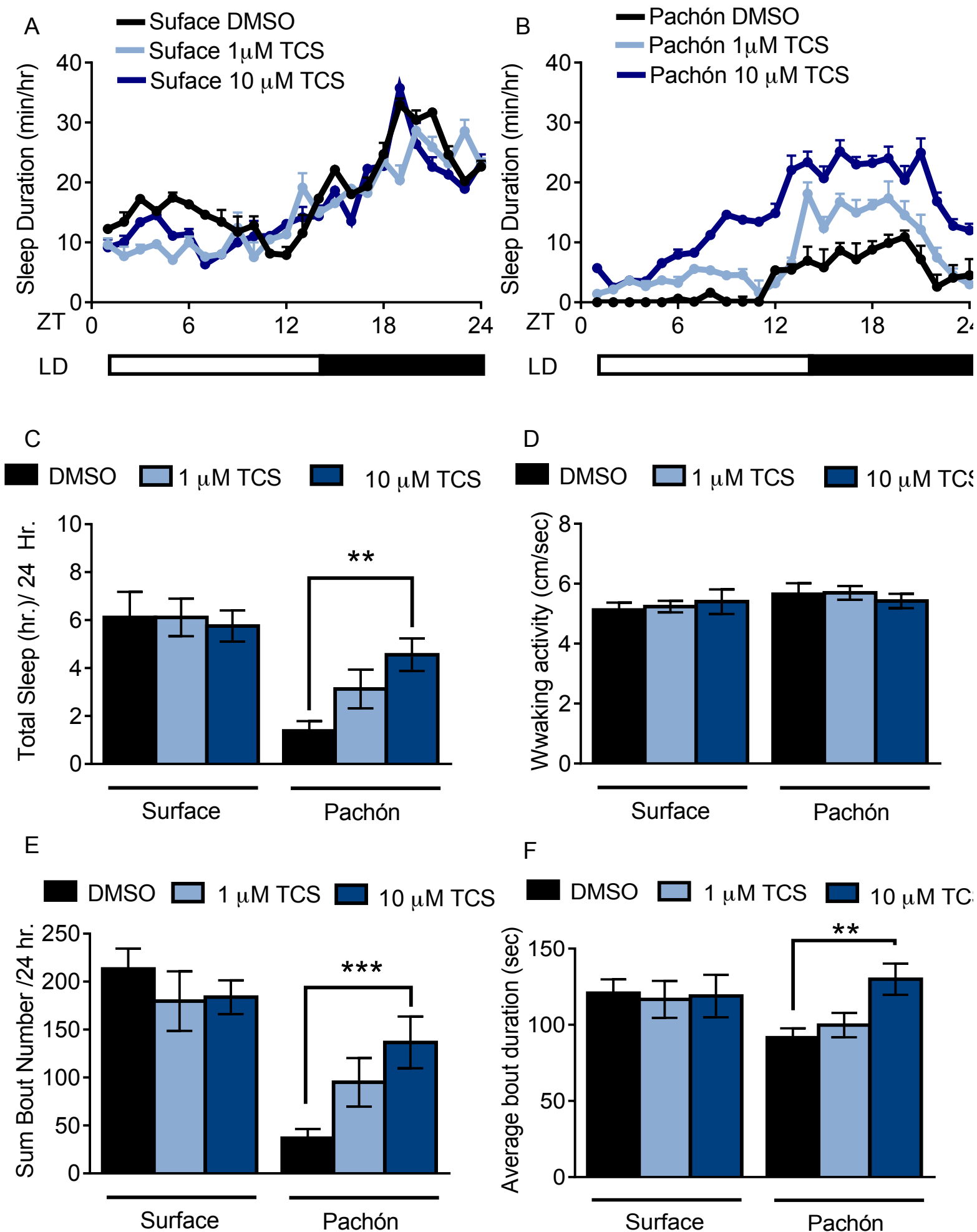


Figure 3

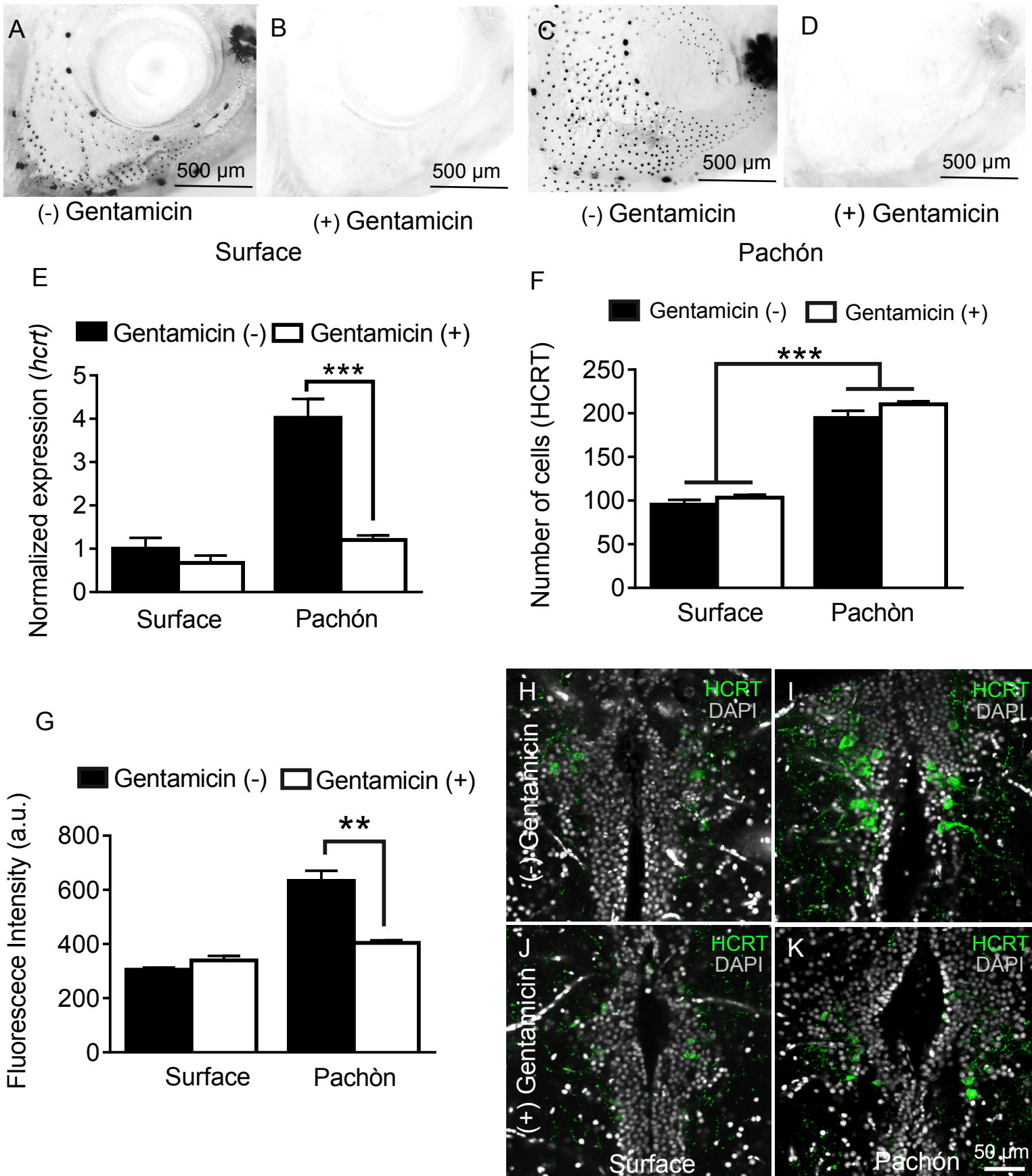


Figure 4

