β -Adrenergic Signaling Regulates Evolutionarily Derived Sleep Loss in the Mexican Cavefish

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Key Words

Astyanax · Mexican cavefish · Sleep · Propranolol · Catecholamine

Abstract

Sleep is a fundamental behavior exhibited almost universally throughout the animal kingdom. The required amount and circadian timing of sleep differs greatly between species in accordance with habitats and evolutionary history. The Mexican blind cavefish, *Astyanax mexicanus*, is a model organism for the study of adaptive morphological and behavioral traits. In addition to loss of eyes and pigmentation, cave populations of *A. mexicanus* exhibit evolutionarily derived sleep loss and increased vibration attraction behavior, presumably to cope with a nutrient-poor environment. Understanding the neural mechanisms of evolutionarily derived sleep loss in this system may reveal critical insights into the regulation of sleep in vertebrates. Here we report that blockade of β -adrenergic receptors with propranolol rescues the decreased-sleep phenotype of cavefish. This effect was not seen with α -adrenergic antagonists. Treatment with selective β_1 -, β_2 -, and β_3 -antagonists revealed that the increased sleep observed with propranolol could partially be explained via the β_1 -adrenergic system. Morphological analysis of catecholamine circuitry revealed conservation of gross catecholaminergic neuroanatomy between surface and

cave morphs. Taken together, these findings suggest that evolutionarily derived changes in adrenergic signaling underlie the reduced sleep of cave populations.

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Introduction

Astyanax mexicanus (*A. mexicanus*) is a powerful model for studying the genetic basis of evolutionary phenomena including developmental, morphological, and behavioral trait variation. *A. mexicanus* consists of numerous populations of eyed ‘surface’ morphs that live in the rivers and streams of Northeast Mexico and Texas, and 29 geographically isolated blind and depigmented cave morph populations inhabiting the region of the Sierra de El Abra in Northeast Mexico [Mitchell et al., 1977]. The invasion of caves by ancestral surface populations occurred in 2 distinct waves over periods ranging from 2 to 3 million years ago [Dowling et al., 2002; Ornelas-Garcia et al., 2008; Strecker et al., 2003, 2004]. In addition to morphological traits, a number of behaviors

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have evolved in response to a dramatically altered cave habitat. In contrast to rivers where photosynthesis-driven plant growth and constant water flow provide an abundance of food, the cave environment is characterized by a lack of photosynthesis, reduced food availability, and nearly constant water temperatures [Poulson, 1969]. *A. mexicanus* cave morphs have developed a number of metabolic and behavioral traits, presumably to cope with this environment, including increased mechanosensory capabilities, a keen ability to find mates, and a hypoactive metabolism [Burt de Perera, 2004; Filippi et al., 2010; Plath et al., 2006; Protas et al., 2008].

We previously reported that *A. mexicanus* cavefish have evolved a dramatically reduced sleep phenotype, presumably to increase the time available for foraging in a food-poor cave environment [Duboué et al., 2011]. Modulators of neurotransmission have a conserved role in the regulation of sleep [Cirelli, 2009]. For example, the neuropeptide orexin promotes sleep in fish and mammals [Lin et al., 1999; Prober et al., 2006; Yokogawa et al., 2007]. Elevated serotonin signaling in mice selectively increases REM sleep, suggesting a sleep-promoting role for serotonin [Wisor et al., 2003]. Conversely, mice lacking norepinephrine and epinephrine have an increase in total sleep and a decrease in sleep latency, and mice lacking histamine show an increase in sleep, indicating these neurotransmitters are wake promoting systems [Hunsley et al., 2006; Parmentier et al., 2002]. The amino acid neurotransmitters GABA and glutamate have also been implicated in the regulation of sleep. Injection of glutamate, or inhibition of GABA signaling in the dorsal subcoeruleus, a brain region involved in REM sleep, potently induces REM sleep [Pollock and Mistlberger, 2003; Simon et al., 2011]. Furthermore, pharmacological blockade of the glutamate N-methyl-D-aspartic acid (NMDA) receptor disrupts sleep [Stone et al., 1992]. Therefore, diverse neurotransmitter systems regulate sleep, and pharmacological manipulation of these systems may provide critical insight into the molecular basis of sleep-wake regulation. Given the dramatic differences in sleep between the surface and cave morphs as well as the conserved role for neurotransmitter systems in sleep, we sought to examine the role of neurotransmitter systems in evolutionarily derived sleep loss.

In this study, we pharmacologically disrupted neurotransmitter signaling to gain insight into the molecular basis of evolutionarily derived sleep loss in *A. mexicanus*. We reasoned that a pharmacological manipulation of an evolved wake-promoting system in *A. mexicanus* cave morphs would be affected differentially between surface

and cave morphs. Here we identify a role for the noradrenergic system in the altered sleep/wake regulation exhibited by cave morphs. These findings are consistent with a conserved wake-promoting role for norepinephrine in flies, fish, and humans [Cirelli et al., 2005; Lebestky et al., 2009; Rihel et al., 2010a]. We find that propranolol, a general inhibitor of β -adrenergic signaling, significantly increases sleep in cavefish, but not surface fish. There were no significant effects with α -adrenergic antagonists suggesting the role is specific to the β -adrenergic system. Neuroanatomical analysis revealed striking conservation of catecholamine neuron morphology between surface and cavefish. These findings support the notion that evolutionarily derived sleep loss in cavefish populations may be due to hyperactive noradrenergic signaling and that the wake-promoting role of β -adrenergic signaling in cave populations is likely due to alterations in neural activity rather than changes in brain morphology. Our study is the first systematic study examining the contribution of distinct neurotransmitter classes to sleep regulation in *A. mexicanus*.

Materials and Methods

Animal Maintenance and Behavioral Analysis

Fish were housed in the core-fish facility at NYU with water temperature maintained at $21 \pm 1^\circ\text{C}$ and set to 12:12 light/dark cycles. Husbandry was carried out as previously described [Borowsky, 2008]. *A. mexicanus* breeders were fed a high-fat diet consisting of liver paste and live black worms (*Lumbriculus variegatus*) for 2 weeks prior to breeding. On the night before breeding, breeder fish were moved to individualized tanks and the temperature was raised to $25 \pm 1^\circ\text{C}$. *A. mexicanus* fry were fed a daily diet of *Artemia salina* (Jehmco Fish Supplies). Behavioral testing took place in 100-well plates, and sleep was assayed in 21–22 days post-fertilization (dpf) *A. mexicanus* surface and Pachón cave morphs as previously described [Duboué et al., 2011]. The time of behavioral testing (21–22 dpf) is pre-sexual differentiation and therefore fry are intersex. All recordings were carried out in a recording chamber with the temperature maintained at $21 \pm 1^\circ\text{C}$. The recording chamber was illuminated constantly with a custom designed IR LED source (940 nm) as well as a 175-lux LED white light, which illuminated for 12 h (10:00 a.m. to 10:00 p.m.). Following 18 h of acclimatization, either control dimethyl sulfoxide solvent (DMSO) or freshly prepared (prepared the morning of administration) drug dissolved in DMSO was added to each well and behavior was recorded for 24 h. Drugs were purchased from Sigma-Aldrich and tested at final water concentrations of 10–30 μM , as previously described in *Danio* [Rihel et al., 2010b]. Video records were gathered using a standard charge-coupled device camera and a Noldus MPEG recorder (v2.1; Noldus IT). Video was subsequently processed using Ethovision XT 7.1 (Noldus IT). Tracking parameters were consistent with those previously used [Duboué et al., 2011]. Parameters for detection were set as follows:

method set to dynamic subtraction; detection set to subject brighter than background and brightness contrast from 20 to 255; current frame weight set to 15; video sample rate set to 29.97, and pixel smoothing turned off. Data was subsequently processed using custom-written MATLAB scripts (Mathworks, Natick, Mass., USA). In accordance with previous experiments in *A. mexicanus* surface and cave fry, 1 min of complete immobility was defined as sleep [Duboue et al., 2011; Prober et al., 2006; Yokogawa et al., 2007]. For all experiments, $n \geq 10$ per group.

Brain Dissection and Analysis

Ten- or twenty-two-dpf *A. Mexicanus* surface and Pachón cave morphs were anesthetized in tricaine solution, and entire brains were removed in PBS and fixed in 4% formaldehyde, PBS, and 0.2% Triton-X 100 for 60 min. Brains were incubated overnight at 4°C in 1:1,000 polyclonal rabbit-anti-TH (AB152; Millipore). Brains were then incubated in 1:800 Alexa goat anti-rabbit (Invitrogen, Carlsbad, Calif., USA) for 90 min at room temperature and then mounted in Vectashield (VectorLabs) and imaged on a Zeiss SP5 confocal microscope (Manheim, Germany). Brains were imaged in 1.5- μ m sections and are presented as the Z-stack projection through the entire brain. The TH-positive cell numbers were counted manually by examining individual sections in Fiji imaging software. The number of cells per cluster represents the average of surface $n \geq 12$, Pachón cave $n \geq 10$ (10 dpf), and surface $n \geq 4$, Pachón $n \geq 8$ (22 dpf).

Statistics

For drug screens, multivariate ANOVA tests were carried out to test the effects of drug, genotype, and the drug*genotype interaction. Significant drug*genotype interaction ($p < 0.05$) revealed drugs that differentially affected sleep between surface and cave forms. The F statistic and p values for these tests are given in figures 1 and 3. For detailed analysis of propranolol (fig. 2b–e), we implemented a Kruskal-Wallis one-way ANOVA. When significance was detected, we followed with a multiple comparisons post-test correcting for the number of comparisons made. For cell counting, pairwise comparisons were carried out using a non-parametric t test (fig. 4j, k). All statistical analyses were implemented in the R Language for Statistical Computing [Team RDC, 2011].

Results

To identify the biological basis for reduced sleep in cavefish populations, we screened putative arousal system antagonists for drugs that selectively promote sleep in cavefish. We reasoned that blockade of a signaling pathway involved in evolutionarily derived sleep loss would have little effect on sleep in surface populations while increasing sleep in cavefish. We tested antagonists directed toward glutamatergic, serotonergic, histaminergic, cholinergic, dopaminergic, and noradrenergic systems that have previously been implicated in sleep [Cirrelli, 2009; Hunsley et al., 2006; Parmentier et al., 2002;

Pollock and Mistlberger, 2003; Simon et al., 2011; Stone et al., 1992; Wisor et al., 2003] (fig. 1a). We found that the NMDA receptor antagonist MK-801 as well as the β -adrenergic antagonist propranolol (P0884; Sigma Chemical Co., St. Louis, Mo., USA) potentially increased sleep in Pachón cavefish while having little effect on surface fish (fig. 1a). The tyrosine hydroxylase (TH) inhibitor 3-iodo-L-tyrosine disrupts synthesis of dopamine and norepinephrine approached significance ($p = 0.06$). Because of a known role for noradrenergic neurons of the ascending reticular activating system (ARAS) in sleep-wake regulation, we focused on the role of propranolol on sleep [Berridge and Foote, 1991]. Propranolol is a general inhibitor of β -adrenergic receptors that has been previously reported to modulate sleep in *Danio* and mammals [Mendelson et al., 1980; Rihel et al., 2010a; Whitehurst et al., 1999]. Furthermore, norepinephrine is an important component of the wake-promoting ascending reticular activating system [Berridge and Foote, 1991]. A dose-response curve for propranolol in concentrations ranging from 10^{-9} M through 10^{-4} M resulted in a concentration-dependent increase in sleep in the cavefish population (fig. 1b). There was no significant effect at any dose in surface populations. Because cavefish sleep loss could result from inadequate sleep-promoting effects, we wanted to explore the role of gamma-aminobutyric acid (GABA) signaling, a primary sleep-promoting system in vertebrates. We tested the GABA-A receptor antagonist bicuculine and found that it did not affect sleep in cave or surface populations at any dose tested (fig. 1c), indicating that the diminished sleep in cave populations is due to an increase in wake-promoting signaling rather than altered sleep-inducing signaling.

Quantification of total sleep reveals that surface fish sleep significantly more than cave populations, as has previously been reported [Duboue et al., 2011]. Propranolol has no effect on sleep in surface fish, while it significantly increases sleep in cavefish (fig. 1a, b, 2a, b). These findings suggest that increased β -adrenergic signaling confers sleep loss in cave populations. We next quantified activity per waking minute to determine whether propranolol induces consolidated periods of sleep. In agreement with previous findings, surface and cavefish show similar activity per waking minute. Treatment with propranolol results in no change in waking activity for both cave and surface fish. Total quantification showed no significant differences for both daytime ($p = 0.50$) and nighttime ($p = 0.82$) waking activity (fig. 2c). Thus, it is likely that propranolol treatment leads to increased sleep rather than affecting motor function.

Two-way ANOVA of drug screen						
Control						
MK-801	Glutamate	Potent nonselective NMDA glutamate receptor antagonist that acts at the NMDA receptor-operated ion channel as an open channel blocker	Form: $F_{(1,11)} = 8.38$ Drug: $F_{(1,11)} = 0.39$ Form*drug: $F_{(1,11)} = 7.09$	$p = 0.02$ $p = 0.54$ $p = 0.02$		
Propranolol	Noradrenergic	Potent β -adrenergic receptor antagonist that acts at all β -class adrenergic receptors	Form: $F_{(1,11)} = 0.73$ Drug: $F_{(1,11)} = 9.92$ Form*drug: $F_{(1,11)} = 17.59$	$p = 0.41$ $p = 0.009$ $p = 0.001$		
Atropine	Cholinergic	Competitive nonselective antagonist at central and peripheral muscarinic acetylcholine receptors	Form: $F_{(1,11)} = 4.54$ Drug: $F_{(1,11)} = 1.76$ Form*drug: $F_{(1,11)} = 1.29$	$p = 0.05$ $p = 0.21$ $p = 0.27$		
Diphenhydramine	Histaminergic	Potent competitive inverse agonist of the histamine H_1 receptor and hypnotic	Form: $F_{(1,12)} = 28.28$ Drug: $F_{(1,12)} = 0.12$ Form*drug: $F_{(1,12)} = 0.26$	$p = 0.0002$ $p = 0.73$ $p = 0.62$		
3-Iodo-L-tyrosine	Dopaminergic	Reversible inhibitor of the enzyme tyrosine hydroxylase and general inhibitor of catecholamine synthesis	Form: $F_{(1,10)} = 4.96$ Drug: $F_{(1,10)} = 0.17$ Form*drug: $F_{(1,10)} = 4.65$	$p = 0.05$ $p = 0.68$ $p = 0.06$		
Amitriptyline	Serotonergic	Tricyclic antidepressant; serotonergic reuptake inhibitor	Form: $F_{(1,9)} = 7.72$ Drug: $F_{(1,9)} = 0.02$ Form*drug: $F_{(1,9)} = 0.09$	$p = 0.02$ $p = 0.09$ $p = 0.76$		

a

Fig. 1. Propranolol and MK-801 increase sleep in Pachón cavefish. **a** The effects of six drugs targeting neurotransmitter systems were assayed at a 30- μ M concentration for 24 h. The total minutes of sleep per 24-hour period reveal no effect of any drug on surface populations while propranolol and MK-801 significantly increased sleep in Pachón cavefish. The mean total sleep times for control surface and Pachón were 846 ± 37.2 min and 318 ± 62.57 min, respectively, and they were significantly different as previously reported (two-tailed Mann-Whitney U test, $p < 0.01$). Val-

ues for drug-treated groups were as follows: MK801: surface = 662.2 ± 55.46 min, Pachón = 620.9 ± 83.19 min; propranolol: surface = 697.9 ± 84.65 min, Pachón = $1,061.0 \pm 33.41$ min; atropine: surface = 857.0 ± 62.88 min, Pachón = 589.1 ± 115.5 min; diphenhydramine: surface = 863.5 ± 42.3 min, Pachón = 398.1 ± 82.9 min; 3-iodo-L-tyrosine: surface = 613.9 ± 70.8 min, Pachón = 589.4 ± 73.0 min; amitriptyline: surface = 942.5 ± 120.2 min, Pachón = 273.6 ± 49.5 min. Two-way ANOVA F and p values are shown.

Sleep consists of individual sleep bouts. We previously reported that reduced sleep in cave populations was due to shortened lengths of individual sleep bouts rather than changes in bout number [Duboue et al., 2011]. We measured the effect of propranolol on the number and length of individual sleep bouts. Propranolol dramatically in-

creased sleep bout length while decreasing sleep bout number (fig. 2d, e). Therefore, propranolol both increases the total sleep time and promotes the consolidation of sleep bouts in cave populations.

We next assessed the effects of subtype-specific adrenergic antagonists on sleep in surface and cave popula-

Fig. 1. Propranolol and MK-801 increase sleep in Pachón cavefish. **b** A dose-response curve for total sleep time in propranolol-treated fish revealed a dose-dependent increase in sleep in Pachón cave populations and no effect at any dose in surface fish. The total sleep (mean $\pm$ SEM) times for the dose-response curve and statistical analysis (two-tailed nonparametric t test) relative to the control (0 μ M) were as follows: surface (control = 589.4 $\pm$ 45.6 min; 10^{-9} μ M = 588.0 $\pm$ 35.8 min, $p = 0.36$; 10^{-8} μ M = 520.0 $\pm$ 46.9 min, $p = 0.12$; 10^{-7} μ M = 589.0 $\pm$ 101.5 min, $p = 0.13$; 10^{-6} μ M = 800.0 $\pm$ 156.4 min, $p = 0.24$; 10^{-5} μ M = 650.0 $\pm$ 86.9 min, $p = 0.57$; 10^{-4} μ M = 701.0 $\pm$ 26.4 min, $p = 0.21$, 10^{-3} μ M = lethal) and Pachón (control = 365.7 $\pm$ 14.6 min; 10^{-9} μ M = 850.0 $\pm$ 43.2 min, $p < 0.001$; 10^{-8} μ M = 974.2 $\pm$ 45.7 min, $p < 0.001$; 10^{-7} μ M = 1,031.7 $\pm$ 113.4 min, $p < 0.001$; 10^{-6} μ M = 1,061.0 $\pm$ 84.89 min, $p < 0.001$; 10^{-5} μ M = 1,082.3 $\pm$ 112.4 min, $p < 0.001$; 10^{-4} μ M = 1,359.0 $\pm$ 40.2 min, $p < 0.001$; 10^{-3} μ M = lethal). **c** At all doses

tested the GABA-A receptor antagonist bicuculine failed to effect sleep in cave and surface populations. Bicuculine induced lethality in cave populations at concentrations that did not affect surface fish. Total sleep (mean $\pm$ SEM) times for the dose-response curve and statistical analysis (two-tailed nonparametric t test) relative to the control (0 μ M) were as follows: surface (control = 790.2 $\pm$ 51.9 min; 10^{-10} μ M = 837.2 $\pm$ 53.6 min, $p = 0.61$; 10^{-9} μ M = 862.8 $\pm$ 65.6 min, $p = 0.45$; 10^{-8} μ M = 811.5 $\pm$ 73.6 min, $p = 0.83$; 10^{-7} μ M = 778.0 $\pm$ 107.7 min, $p = 0.92$; 10^{-6} μ M = 863.1 $\pm$ 59.7 min, $p = 0.041$; 10^{-5} μ M = 740.2 $\pm$ 90.3 min, $p = 0.66$; 10^{-4} μ M = 808.7 $\pm$ 140.5 min, $p = 0.91$) and Pachón (control = 351.4 $\pm$ 83.7 min; 10^{-10} μ M = 325.5 $\pm$ 91.2 min, $p = 0.83$; 10^{-9} μ M = 170.5 $\pm$ 43.5 min, $p = 0.08$; 10^{-8} μ M = 170.0 $\pm$ 44.7 min, $p = 0.08$; 10^{-7} μ M = 271.8 $\pm$ 80.4 min, $p = 0.50$; 10^{-6} μ M = 308.3 $\pm$ 77.2 min, $p = 0.71$; 10^{-5} μ M = lethal; 10^{-4} μ M = lethal). All graphs represent means $\pm$ SEM. * $p < 0.05$; *** $p = 0.001$.

Fig. 2. Propranolol selectively increases sleep bout duration in Pachón cavefish. **a** After an 18-hour acclimation period, both surface and Pachón cavefish treated with either DMSO or 30 μ M propranolol were assayed for sleep over a 24-hour period.

Fig. 2. Propranolol selectively increases sleep bout duration in Pachón cavefish. **b** Propranolol significantly increased the total sleep over a 24-hour day in Pachón cavefish compared to controls (DMSO: $1,041 \pm 38.21$ min; propranolol: 318 ± 40.8 min; $p < 0.0001$) while having little effect on surface fish (DMSO: 582 ± 48.64 min; propranolol: 646.1 ± 85.75 min; $p > 0.05$). **c** Quantification of activity per waking minute (scored as average mm/s velocity) suggests no significant difference among groups for either daytime [ANOVA, $H_{(30, 4)} = 2.345$, $p = 0.50$] or nighttime [ANOVA, $H_{(30, 4)} = 0.9424$, $p = 0.81$]. Waking activities were as follows: daytime: surface DMSO = 2.44 ± 0.08 mm/s; surface propranolol = 3.12 ± 0.35 mm/s; cave DMSO = 2.91 ± 0.29 mm/s; cave propranolol = 3.99 ± 0.59 mm/s; nighttime: surface DMSO = 2.69 ± 0.08 mm/s; surface propranolol = 3.20 ± 0.33 mm/s; cave DMSO = 2.94 ± 0.30 mm/s; cave propranolol = 3.76 ± 0.71 mm/s. **d** Quantification of the number of bouts revealed a significant difference among groups for daytime [Kruskal-Wallis ANOVA, $H_{(30, 4)} = 7.712$, $p < 0.05$] and nighttime bout numbers [Kruskal-Wallis ANOVA $H_{(30, 4)} = 16.99$, $p > 0.001$]. **a, b** Black and dark grey bars denote surface, white and light grey bars denote Pachón. Dark grey and light grey bars denote drug-

treated groups. A post hoc Dunn's multiple comparisons test revealed that propranolol reduces daytime (DMSO: 36.2 ± 6.8 bouts; propranolol: 85.0 ± 15.2 bouts, $p < 0.001$) and nighttime (DMSO: 100 ± 12.2 bouts; propranolol: 16.7 ± 4.5 bouts, $p < 0.001$) bout numbers in cave populations while having no significant effect on bout number in surface fish (daytime – DMSO: 57.5 ± 9.2 bouts; propranolol: 57.4 ± 10.9 bouts; nighttime – DMSO: 61.0 ± 14.0 bouts; propranolol: 51.1 ± 8.9 bouts; $p > 0.05$ for both). **e** Quantification of the average bout duration revealed a significant difference among groups for daytime [Kruskal-Wallis ANOVA, $H_{(30, 4)} = 20.02$, $p < 0.001$] and nighttime bout numbers [Kruskal-Wallis ANOVA $H_{(30, 4)} = 17.87$, $p > 0.001$]. A post hoc Dunn's multiple comparisons test revealed that propranolol increased the average daytime (DMSO: 2.12 ± 0.27 min; propranolol: 18.21 ± 3.1 min, $p < 0.001$) and nighttime (DMSO: 1.92 ± 0.2 min; propranolol: 70.1 ± 50.1 min, $p < 0.001$) bout duration in cave populations while having no significant effect on bout number in surface fish (daytime – DMSO: 2.73 ± 0.5 min; propranolol: 4.03 ± 1.1 min; nighttime – DMSO: 10.92 ± 6.3 min; propranolol: 18.07 ± 6.8 bouts; $p > 0.05$ for both).

Fig. 3. Propranolol selectively increases sleep bout duration in Pachón cavefish. The three β -adrenergics (β 1-atenolol, β 2-butaxamine, and β 3-SR59203A) did not increase sleep in surface populations, consistent with the lack of effect observed for propranolol. A significant increase in cavefish sleep was observed with the β 1-antagonist atenolol, suggesting that β 1-adrenergic receptors regulate evolutionarily derived sleep loss. No effect of the α -adrenergic antagonists phenoxybenzamine and idazoxan was observed in cave or surface populations. The mean total sleep times for control surface and Pachón fish were 842.3 ± 63.4 min and $319.0 \pm$

62.6 min, respectively, and they were significantly different as previously reported (two-tailed Mann-Whitney U test, $p < 0.01$). Values for drug-treated groups were as follows: phenoxybenzamine: surface = 877.3 ± 80.02 min, Pachón = 301.2 ± 94.78 min; idazoxan: surface = 728.4 ± 86.3 min, Pachón = 413.2 ± 61.82 min; atenolol: surface = 493.2 ± 76.08 min, Pachón = 555.5 ± 52.01 min; butaxamine: surface = 644.9 ± 88.49 min, Pachón = 274.8 ± 47.29 min; SR59230A: surface = 808.0 ± 82.92 min, Pachón = 515.4 ± 98.58 min. Two-way ANOVA F and p values are shown.

tions. In vertebrates, norepinephrine signals through α -adrenergic receptors and β -adrenergic receptors. We assayed drugs that specifically targeted α -adrenergic and β -adrenergic subtypes. There was no effect with the α -adrenergic antagonist phenoxybenzamine or the α 2-adrenergic antagonist idazoxan suggesting the rescue effect of propranolol was specific to β -adrenergic receptors (fig. 3). While the *Danio* genome encodes for four β -ad-

renergic receptors [Ruuskanen et al., 2004], the number of β -adrenergic receptor genes in the *A. mexicanus* genome remains unknown. To gain insight into the subclass of β -adrenergic receptors modulating sleep, we assayed the effects of subtype-specific β -adrenergic antagonists on sleep. The three β -adrenergic antagonists (β 1-atenolol, β 2-butaxamine, and β 3-SR59203A) did not affect sleep in surface populations, consistent with the

Fig. 4. Propranolol reduces the sleep bout number and increases the average sleep bout length in Pachón cavefish. **a, b** Whole mount projection of 10-dpf surface (**a**) and cavefish (**b**) brains stained with anti-TH alongside. Scale bars represent 200 μ m. **c** Diagram depicting cell bodies of catecholamine regions quantified. Orange: hypothalamus (Hypo), green: right and left locus coeruleus (rLC and lLC), red: reticular formation (RF), blue: cau-

dal nuclei (CN). **d–i** Single 1.5- μ m sections were used to quantify the number of TH-positive cells within surface (**d, f, h**) and cavefish brains (**e, g, i**). Scale bars represent 50 μ m. **d, e** Hypothalamus. **f, g** Locus coeruleus. **h, i** Reticular formation and caudal nuclei. **j, k** Total quantification of TH-positive cells revealed no significant difference among groups ($p > 0.05$ for all groups) for 10 dpf (**j**) and 22 dpf (**k**). All bars represent means $\pm$ SEM.

lack of effect observed for propranolol. A significant increase in cavefish sleep was observed with the β_1 -antagonist atenolol, suggesting that β_1 -adrenergic receptors regulate evolutionarily derived sleep loss. Cavefish treated with the β_3 -antagonist SR59203A approached a significant increase in sleep, raising the possibility that a combination of β_1 and β_3 signaling results in reduced sleep in Pachón populations (fig. 3).

We next asked how β -adrenergic signaling affected sleep. It is possible that reduced sleep in cave populations results from neuroanatomical changes such as an increased number of noradrenergic neurons or increased innervations of targets. This notion is supported by the differential distribution of catecholaminergic cell bodies among various fish species [Brinon et al., 1998]. Alternatively, subtle changes in neural function may underlie the

dramatic reduction in sleep observed in cave populations. The enzyme TH is an obligate precursor for the synthesis of all catecholamine. In *Danio*, two *TH* genes (*Th1* and *Th2*) are expressed in neural clusters of the olfactory bulb, forebrain, hypothalamus, and hindbrain [Jang et al., 2011; Prober et al., 2006; Yamamoto et al., 2011; Yamamoto and Vernier, 2011]. We found that anti-mouse TH labels *A. mexicanus* brains in an expression pattern similar to the known pattern observed in *Danio*, suggesting conservation between the two systems.

For neuroanatomical analysis we imaged 10-dpf fry because brains at this stage are small enough for whole-mount confocal microscopy. Additionally, we used 22-dpf fry that match the age of fish used for behavioral analysis. Ten-dpf juvenile surface and cave morphs were dissected and stained with anti-TH. Immunostaining of the

surface and cavefish brain revealed that catecholamine cell bodies and projections are highly conserved with those previously reported in *Danio rerio* in both 10-dpf (fig. 4a–c) and 22-dpf fry (online suppl. fig. 1, see www.karger.com/doi/10.1159/000341403 for all online suppl. material). The number of catecholamine-positive cell bodies were quantified in all clusters including those of the hypothalamus [Prober et al., 2006], locus coeruleus [Filippi et al., 2010], reticular formation, and caudal nuclei [Berger, 1969; Moruzzi and Magoun, 1949] in order to test whether an altered number of catecholamine neurons are present in cave populations (fig. 4d–k). No significant differences were observed in any clusters of 10- and 22-dpf fry, suggesting that the gross morphology of catecholamine neurons is conserved between cave and surface populations (fig. 4j, k). To our knowledge, these findings represent the first analysis of central brain circuitry in cavefish and reveal an anatomical conservation of the catecholamine systems between distinct *A. mexicanus* morphs. These results suggest that catecholamine function, rather than neuroanatomical changes, underlie evolutionarily derived sleep loss.

Discussion

A. mexicanus cave populations appear to be uniquely adapted to life in a nutrient-poor environment. In addition to reduced sleep, cavefish display a number of traits that are adaptive to nutrient-poor environments including lower basal metabolic rates and a heightened attraction to vibration [Ercolini et al., 1987; Salin et al., 2010; Yoshizawa et al., 2010]. We previously reported that the reduced sleep in cave populations is due to shortened sleep bout duration. Here we extend these observations through pharmacological interrogation of sleep-wake regulation in *A. mexicanus*. We identified the NMDA receptor antagonist MK-801 and the β -adrenergic antagonist propranolol as increasing sleep selectively in cave populations, suggesting that blockade of β -adrenergic receptors in cave populations induces surface fish-like sleep. Further characterization of β -adrenergic signaling reveals a role for β_1 -adrenergic receptors, but not β_2 - or α -adrenergic receptors, in evolutionarily derived sleep loss. This finding suggests that the noradrenergic wake-promoting system is finely tuned in both cave and surface *A. mexicanus* in accordance with their natural ecology, and that noradrenergic antagonists in cavefish may act to mimic a neural system of sleep consolidation seen in *A. mexicanus* surface conspecifics. It is possible that β -ad-

renergic signaling generally modulates metabolism or hunger-induced behaviors. Alternatively, differences in β -adrenergic signaling may be specific to sleep. Future examination of the effects of propranolol on cavefish metabolism and foraging behaviors will allow differentiation between these two possibilities.

We performed a detailed neuroanatomical analysis of TH-immunoreactive cells in both morphs. The finding of conserved catecholamine neuroanatomy in surface and cave morphs suggests that the differential effects of propranolol are likely due to altered β -adrenergic signaling. While there are no obvious differences in projections of catecholamine neurons, it is possible that subtle changes in synaptic connectivity are responsible for the increased wakefulness in cave populations. Alternatively, increased epinephrine/norepinephrine release or increased signaling may be responsible for the effects of propranolol. Future experiments quantifying epinephrine/norepinephrine levels and release in surface populations may be informative.

Our findings that blockade of β -adrenergic receptors selectively rescues sleep in cave populations suggest an evolutionarily derived increase in arousal systems in cave populations. It is also possible that decreased sensitivity of sleep-promoting systems modulates reduced sleep in cave populations. Activation of GABA-A receptors and adenosine have been shown to modulate sleep in other systems [Basheer et al., 2004; Tobler et al., 2001]. Future work examining the effects of benzodiazepines and adenosine on sleep in cave populations will help to identify the role of sleep-promoting systems in evolutionarily derived sleep loss.

Both the functions and the evolutionary basis of sleep have been the subject of great debate [Lesku et al., 2006]. Sleep has been suggested to be a state of adaptive inactivity derived from a more general rest-like state, and it has been suggested that the ecological factors in which species live serve as a driving force impacting sleep need [Allada and Siegel, 2008]. The differences in habitat between surface and cave populations present a unique opportunity to examine the effects of ecology and evolutionary history on sleep. Our findings provide the first insight into the biological basis of reduced sleep in cave populations.

It is likely that the mechanisms underlying sleep-wake regulation in cavefish extend to affect a number of processes beyond sleep. Studies in the Somalian blind cavefish (*P. Andruzzii*) reveal that these fish have retained a circadian clock but lost the ability to respond to light and temperature, which is likely an adaptation to perpetual darkness and near constant temperature in the cave en-

vironment. The fish are capable of generating free-running 47-hour rhythms in response to feeding [Cavallari et al., 2011]. Feeding entrainment has been observed in *Danio*, suggesting that this process is conserved in terrestrial and subterranean fish populations [Lopez-Olmeda et al., 2010]. It will be of great interest to determine whether *A. mexicanus*, like *P. andruzzii*, can generate food-entrained circadian rhythms.

Foraging requires alterations in a number of behaviors including decreased sleep and increased activity. There are a number of feeding-related behaviors that differ between cave and surface *A. mexicanus*. It will be important to determine whether β -adrenergic signaling selectively regulates sleep or generally regulates feeding-associated behaviors. *A. mexicanus* cave morphs differ in feeding behavior from surface morphs. Cavefish approach food at a 45° angle, while surface morphs approach food vertically [Schemmel, 1980]. It is suggested that the diminished angle of food approach is an adaptation for a slower food approach in cavefish. The altered approach was found to be recessive and segregation analysis in F2 surface-cave hybrids suggests surface-cave differences in approach are mono-allelic; however, the gene involved has not been identified [Schemmel et al., 1980]. Reduced sleep in cave populations is dominant [Duboue et al., 2011], supporting the notion that changes in sleep and feeding angle are controlled by distinct genetic mechanisms.

Fish detect food sources through vibration in the water, and one manifestation of foraging in *A. mexicanus* is vibration attraction behavior (VAB). Cavefish appear to be specifically tuned to detect water droplets at 35 Hz, the approximate frequency generated from a water droplet

falling from a cave ceiling [Yoshizawa et al., 2010]. The increased sensitivity to vibrations in the water may allow animals to locate prey in the cave environment. Evidence suggests that VAB is directly related to foraging capabilities. Prey capture experiments have found that cave populations with increased VAB outcompete surface fish in the dark [Yoshizawa et al., 2010]. Testing the effects of propranolol on VAB will provide insight into whether similar molecular mechanisms underlie the evolutionarily derived changes in sleep and feeding behavior.

Taken together, we have provided insight into the molecular basis for evolutionarily derived sleep loss in the Mexican cavefish. We show that that sleep loss in the Pachón cavefish can be rescued by blockade of β -adrenergic or NMDA receptors. Future studies examining additional feeding-related behaviors will examine whether β -adrenergic signaling is a general regulator of feeding-associated behaviors. These studies will provide critical insight into the evolutionary basis for behavioral changes of subterranean fish.

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