QRFP and Its Receptors Regulate Locomotor Activity and Sleep in Zebrafish

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The hypothalamus plays an important role in regulating sleep, but few hypothalamic sleep-promoting signaling pathways have been identified. Here we demonstrate a role for the neuropeptide QRFP (also known as P518 and 26RFa) and its receptors in regulating sleep in zebrafish, a diurnal vertebrate. We show that QRFP is expressed in ~10 hypothalamic neurons in zebrafish larvae, which project to the hypothalamus, hindbrain, and spinal cord, including regions that express the two zebrafish QRFP receptor paralogs. We find that the overexpression of QRFP inhibits locomotor activity during the day, whereas mutation of qrfp or its receptors results in increased locomotor activity and decreased sleep during the day. Despite the restriction of these phenotypes to the day, the circadian clock does not regulate qrfp expression, and entrained circadian rhythms are not required for QRFP-induced rest. Instead, we find that QRFP overexpression decreases locomotor activity largely in a light-specific manner. Our results suggest that QRFP signaling plays an important role in promoting sleep and may underlie some aspects of hypothalamic sleep control.

Key words: 26RFa; Gpr103; P518; QRFP; sleep; zebrafish

Introduction

Sleep is an evolutionarily conserved behavior whose regulation is poorly understood. The hypothalamus is thought to play a key role in regulating sleep (for review, see Saper et al., 2010; Brown et al., 2012), in part due to the production of specific neuropeptides. For example, the neuropeptide hypocretin/orexin is produced in the hypothalamus of vertebrate animals, including zebrafish (de Lecea et al., 1998; Kaslin et al., 2004), and has a conserved role in promoting wakefulness (Chemelli et al., 1999; Lin et al., 1999; Peyron et al., 2000; Prober et al., 2006; Adamantidis et al., 2007; Yokogawa et al., 2007; Elbaz et al., 2012). Conversely, the hypothalamic neuropeptide melanin-concentrating hormone, and the neurons that produce it, has been shown to promote sleep in nocturnal rodents (Verret et al., 2003; Willie et al., 2008; Konahode et al., 2013; Tsunematsu et al., 2014). The hypothalamus produces many other neuropeptides (van den Pol, 2012), but their requirement for normal sleep/wake behaviors is largely unexplored, especially in diurnal animals.

A family of hypothalamic neuropeptides containing a C-terminal Arg-Phe-NH₂ motif has been described in several vertebrate and invertebrate animals, and these peptides are implicated in regulating a variety of physiological and behavioral processes (Sandvik et al., 2014). QRFP (also known as P518 and 26RFa), the most recently identified vertebrate member of this family, has been shown to affect feeding, locomotor activity, energy homeostasis, reproduction, bone formation, and nociception in mammals (Chartrel et al., 2011; Ukena et al., 2014). However, these findings are largely based on the injection of...
QRFP peptides into rodent brains and may not reflect the normal function of endogenous QRFP. One of the two identified murine QRFP receptors (gpr103a) has been knocked out in mice (Baribault et al., 2006). These mutants exhibit defects in bone formation but lack obvious additional phenotypes. This result suggests either that endogenous QRFP signaling is not required for other behavioral and physiological processes that are affected by QRFP peptide injection, or that other proteins function redundantly with Gpr103a. However, animals lacking qrfp or the second murine qrfp receptor paralog (gpr103b) have not been reported in any animal model. Zebrafish QRFP (Liu et al., 2009, 2015; Chen et al., 2013; Ukena et al., 2014) and Gpr103 (Chen et al., 2013; Larhammar et al., 2014; Ukena et al., 2014) orthologs have been identified, but have not been functionally characterized. Here we describe the zebrafish qrfp and gpr103 orthologs, characterize qrfp- and gpr103-expressing neurons, and show that QRFP and its receptors regulate locomotor activity and sleep in zebrafish larvae.

Materials and Methods

Zebrafish genetics

Zebrafish were raised on a 14 h/10 h light/dark cycle at 28.5°C, with lights on at 9:00 A.M. and off at 11:00 P.M. Wild-type (WT), transgenic, and mutant stocks come from a background of TL/H11003AB WT strains. All experiments were performed using standard protocols (Westerfield, 1993).
Figure 3. Hypothalamic qrfp expression does not colocalize with other hypothalamic markers. A–E, G–I. Images show immunohistochemistry using a GFP-specific antibody for Tg(qrfp:EGFP) larvae (green, A–E), or FISH using a qrfp-specific probe (green, G–I), and FISH using probes specific for other hypothalamic markers (red), including avp (A), ccka (B), cckb (C), oxt (D), vip (E), crhb (G), sst1.1 (H), and th1 (I). F, J, Native fluorescence in a Tg(hcrt:mRFP); Tg(qrfp:EGFP) larval and adult brain, respectively. Images show maximum intensity projections. A′–J′, Orthogonal views. Schematic diagrams indicate larval brain regions shown in panels A–I and A′–J′, and indicate rostral, caudal, lateral, and ventral axes. Brains from 120 hpf larvae (A–I) and a 22-month-old adult (J) are shown. Scale bars: A–I, 50 μm; J, 100 μm.
In accordance with the California Institute of Technology Institutional Animal Care and Use Committee guidelines. Zebrafish mutants were generated using zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs), as described previously (Chen et al., 2013).

**qrfp** mutant. ZFN binding sites were 5′/H11032-ACAGTCTTC-3′/H11032 and 5′/H11032-AGCACCAAC-3′/H11032.

**qrfp** mutant line i4 contains a 4b p insertion (TTCT, after nucleotide 51 of the open reading frame). The mutation results in a change in reading frame after amino acid (aa) 18 and a premature stop codon after aa 42, compared with 168 aa for the WT protein.

**qrfp** mutants were genotyped using the primers 5′/H11032-AATGGTCAGTTCAGGGTGATG-3′/H11032, 5′/H11032-CAGACCACAGTCTTCttctTTC-3′/H11032, and 5′/H11032-ATTGCTGCTTCCCATTCAG-3′/H11032. These primers produce a 212 bp band for homozygous WT; 216 and 123 bp bands for homozygous mutant; and 212, 216, and 123 bp bands for heterozygous mutant. These bands were resolved on a 4% agarose gel. For **qrfp** mutant behavioral experiments, we mated **qrfp**/**H11002**/**H11002** to **qrfp**/**H11001**/**H11002** fish and compared **qrfp**/**H11002** larvae to their **qrfp**/**H11001** siblings.

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**qrfp** (ZFIN gene name si:ch211-185o22.2) mutant. TALEN binding sites were 5′/H11032-TCCTGAAGTGCTGGAGCA-3′/H11032 and 5′/H11032-TCCTGACGGGTAAGATT-3′/H11032. **gpr103a** mutant d11 contains an 11 bp deletion (TGCAGTTTTAC, after nucleotide 1 of the open reading frame). The mutation results in a change in reading frame after amino acid (aa) 18 and a premature stop codon after aa 42, compared with 168 aa for the WT protein. **qrfp** mutants were genotyped using the primers 5′-AATGGTCAGTTCAGGGTGATG-3′, 5′-CAGACACAGCTTTCTcTcTT-3′, and 5′-ATTGCTGCTTCCCATTACAG-3′. These primers produce a 212 bp band for homozygous WT; 216 and 123 bp bands for homozygous mutant; and 212, 216, and 123 bp bands for heterozygous mutant. These bands were resolved on a 4% agarose gel. For **qrfp** mutant behavioral experiments, we mated **qrfp**−/− to **qrfp**−/− fish and compared **qrfp**−/− larvae to their **qrfp**−/− siblings.

**gpr103a** (ZFIN gene name qrfpra) mutant. TALEN binding sites were 5′-TCCTGAAGTGCTGGAGCA-3′ and 5′-TCCTGACGGGTAAGATT-3′. **gpr103a** mutant d11 contains an 11 bp deletion (TGCAgTcTcTT, after nucleo-
The image shown in Figure 2D is a Tg(qrfp:Brainbow) larva in which no recombination had been induced, and thus only tdTomato is expressed. This image stack was obtained using a two-photon microscope with avalanche photodiode detectors (710 LSM, Zeiss).

Tg(hsp:QRFP). Full-length zebrafish gpr103 cDNA was isolated using 5' and 3' rapid amplification of cDNA ends (RACE; FirstChoice RLM-RACE, Ambion), and the qrfp open reading frame was cloned down-stream of the zebrafish hsp70c promoter (Halloran et al., 2000) using the primers 5'-ATGAAATATCTAGCTCAGGCACTGCTTATTCACTG-3' and 5'-TATCTAGCTCAGGAGTACGCGCCTGATTATGTCG-3' (452 bp); cbb, 5'-CTCTCTCTGCGTCTCTGCAA-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'. Probes specific for arginine vasopressin (avp; Eaton et al., 2008); oxytocin (ox; Unger and Glasgow, 2003); tyrosine hydroxylase 1 (th1; Guo et al., 1999); vasoactive intestinal polypeptide (vip; Wolf and Ryu, 2013); and glutamate decarboxylase 65 (gad65), gad67, vesicular glutamate transporter 1 (vglut1), vglut2a, and vglut2b (Hagishima et al., 2004) have been described. Plasmids containing gpr103 (GenBank clone CR297915, 531 bp) and corticotropin releasing hormone b (crh) (GenBank clone CK352624, 849 bp) were expressed sequence tags were used for riboprobe synthesis. PCR products generated from larval zebrafish cDNA were used as templates for riboprobe synthesis using the following primers: cholecystokinin a (cca), 5'-TTCCTTCCTGTCTTCTGACA-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'; 5'-TAATAGAGCTCCTATAGGTTAGTACGCGCACCGCTG-3'. FISH of Tg(qrfp:EGFP) larvae used DIG-labeled riboprobes followed by immunohistochemistry with a rabbit anti-GFP primary antibody (MBL International) and an Alexa Fluor 488 anti-rabbit secondary antibody (Life Technologies). Samples were mounted in 50% glycerol/PBS and imaged using a compound microscope (Axioimager, Zeiss) or confocal microscope (780 LSM, Zeiss).

Passive clarity technique

Passive clarity technique (PACT) clearing was performed as described previously (Yang et al., 2014). Adult (22-month-old) Tg(qrfp:EGFP); Tg(hcrnrnRFP) zebrafish (Liu et al., 2015) were anesthetized in 0.2% tricaine, killed by incubation in ice water for 15 min, and decapitated. Intact heads were fixed in 4% PFA/PBS overnight at 4°C, then whole brains were dissected out from their skulls and placed in A4P0 hydrogel monomer solution at 4°C overnight with agitation. Samples were then degassed with nitrogen for 5 min and incubated for 2–3 h at 37°C to allow for polymerization. Samples were rinsed in 0.1 M PBS and excess polymerized gel was removed with a Kimsipe. Samples were then rinsed in 0.1 M PBS and placed in 8% SDS at 37°C with agitation for 48 h or until clearing was complete. Samples were then washed with 0.1 M PBS and placed in Refractive Index Matching Solution (RIMS) with a refractive index of 1.42. Samples were mounted in 1.42 RIMS, and native fluorescence was imaged using a confocal microscope (780 LSM, Plan-Apochromat 10×/0.45 M27 objective, Zeiss). Image reconstructions were performed using Imaris (Bitplane).
Behavioral analysis
Larval zebrafish were placed into each well of a 96-well plate (7701–1651, Whatman) containing E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4; Prober et al., 2006). Plates were sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. The sealing process introduces air bubbles in some wells, which are discarded from analysis. Locomotor activity was monitored using an automated videotracking system (Viewpoint Life Sciences) with a Dimion one-third inch Monochrome camera (Dragonfly 2, Point Grey) fitted with a fixed-angle megapixel lens (M50 18-MP, Computar) and infrared filter. The movement of each larva was captured at 15 Hz and recorded using the quantization mode with 1 min integration time bins. The 96-well plate and camera were housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared red lights and with white lights from 9:00 A.M. to 11:00 P.M., unless noted otherwise. The 96-well plate was housed in a chamber filled with recirculating water to maintain a constant temperature of 28.5°C. Heat shocks (HSs) were performed by placing 96-well plates in a 37°C water bath for 1 h. The parameters used for detection were as follows: detection threshold, 15; burst, 29; freeze, 3. For experiments that did not involve a heat shock and in which larvae were tested in 14 h/10 h light/dark cycles, daytime averages were calculated from Days 5, 6, and 7, and nighttime averages were calculated from Nights 5 and 6. For larvae raised and tested in constant dark or constant light, data were averaged for 30 h before HS (pre-HS) and 30 h after HS (post-HS). Data were processed using custom PERL and Matlab (The MathWorks) scripts, and statistical tests were performed using Matlab and Prism (GraphPad).

Statistical analysis
In all statistical tests, the significance threshold was set to \( p < 0.05 \). Parametric analyses were applied because data follow an approximately normal distribution. Asterisks in figures denote statistics for pairwise comparisons performed using an unpaired Student’s t test or Tukey’s HSD test to correct for multiple comparisons where appropriate. Error bars indicate the SEM. To determine whether the observed changes in Tgg(hsp:QRFP) larval behavior are due to heat shock, WT and Tgg(hsp:QRFP) locomotor activity pre-HS and post-HS was compared using two-way ANOVA.

Quantitative RT-PCR
Larval zebrafish were raised on a 14 h/10 h light/dark cycle at 22°C with lights on at 9:00 A.M. and off at 11:00 P.M. At 6 d post-fertilization (dpf), individual larvae were placed into each well of 96-well plates (675075, Greiner) and placed into constant darkness starting at 6:00 A.M. After 24 h in the dark, total RNA was isolated using Trizol (15596–026, Life Technologies) from 24 pooled larvae every 6 h for 24 h. cDNA was synthesized from 5 μg total RNA using Superscript III Reverse Transcriptase (18080–051, Invitrogen), and quantitative PCR was performed using SYBR green master mix (4364346, Life Technologies) in an ABI PRISM 7900HT (Life Technologies) instrument using the following primers: Perib-F: 5’-ATCCGACACCCAATAACAC-3’; Perib-R: 5’-GGGAGACTCTGCTCCTTCT-3’; QRFP-F: 5’-TGGATGTAGATCGCTGTA-3’; and QRFP-R: 5’-GGGAGGATGAACCAGTAGCA-3’. \( \Delta Ct \) was calculated using ribosomal protein L13a (rpl13a) as a reference gene, using the primers: Rpl13a F: 5’-TCTGGAGGACTGTAAGAGGTATGC-3’ and Rpl13a R: 5’-AGACCCACATTTGAGGACGAGCAGC-3’. Relative expression levels were determined by using the \( \Delta \)ΔCt method (Livak and Schmittgen, 2001), normalized to the highest Ct value for each gene.

Results
The QRFP neuropeptide is conserved in zebrafish
The QRFP prepropeptide is known or predicted to be cleaved into a 26 aa peptide (26RFa) in humans (Chartrel et al., 2003; Jiang et al., 2003), rodents (Chartrel et al., 2003; Jiang et al., 2003), frogs (Chartrel et al., 2003), and goldfish (Liu et al., 2009), and a 25 or 27 aa peptide in birds (Ukena et al., 2010; Tobari et al., 2011). The prepropeptide is also cleaved into an N-terminal elon-
The genome contains a single C-terminal 7 aa motif (GGFXFRF-NH₂) that is critical for biological activity. We first observed qrfp expression at 24 h post-fertilization (hpf) in a bilateral hypothalamic population of two to four neurons (Fig. 2A). At 120 hpf, at which time zebrafish larvae exhibit sleep/wake behaviors (Zhdanova et al., 2001; Prober et al., 2006), we observed qrfp expression in a bilateral population of 10–15 neurons in the hypothalamus (Fig. 2B). This expression pattern persists in adult zebrafish, which contain ~120 qrfp-expressing neurons (Fig. 3D). At all developmental stages analyzed, qrfp expression in the brain was restricted to the hypothalamus. These observations indicate that the mammalian qrfp expression pattern is conserved in zebrafish, and the small number of qrfp-expressing neurons suggests that zebrafish may provide a simpler system to study the development and function of these neurons.

We next attempted to identify enhancer elements in the qrfp promoter that are sufficient to regulate gene expression in qrfp-expressing neurons. qrfp is predicted to contain two exons separated by a short intron. We confirmed this gene structure using 5′ RACE and found that the second exon contains the entire QRFP coding sequence. We cloned 1 kb of genomic DNA immediately upstream of the start codon, including the first noncoding exon and the intron, and found that this sequence was sufficient to drive transgene expression in qrfp-expressing neurons in transient injection experiments. As little as 475 bp of genomic sequence immediately 5′ to the start codon was sufficient to drive EGFP expression in qrfp-expressing neurons, but was somewhat less specific than the 1 kb sequence (data not shown). Transient injections using the 1 kb promoter often produced EGFP expression in body wall muscle, but little muscle

**ISH experiments have shown that qrfp expression is largely restricted to the hypothalamus in all species studied, including human (Bruzzone et al., 2006), mouse (Takayasu et al., 2006), rat (Chartrel et al., 2003; Fukusumi et al., 2003; Kampe et al., 2006), quail (Ukena et al., 2010), and zebra finch (Tobari et al., 2011); although expression in other tissues, such as the human spinal cord, has been observed (Bruzzone et al., 2006). In particular, qrfp expression in mammals has been localized to the ventromedial hypothalamus, periventricular nucleus of the hypothalamus, and lateral hypothalamic area. Analysis in goldfish using RT-PCR identified QRFP expression in the hypothalamus, optic tectum, thalamus, and testis, but these results were not validated by ISH (Liu et al., 2009). To determine where qrfp is expressed in zebrafish, we performed ISH using a qrfp-specific probe. We first observed qrfp expression at 120 hpf, at which time zebrafish larvae exhibit sleep/wake behaviors (Zhdanova et al., 2001; Prober et al., 2006), we observed qrfp expression in a bilateral population of 10–15 neurons in the hypothalamus (Fig. 2B). This expression pattern persists in adult zebrafish, which contain ~120 qrfp-expressing neurons (Fig. 3D). At all developmental stages analyzed, qrfp expression in the brain was restricted to the hypothalamus.
expression was observed in stable transgenic lines. Expression in muscle in transient injection experiments and stable lines could be eliminated by including two copies of a neuron-restrictive silencing element (NRSE; Bergeron et al., 2012; Xie et al., 2013) immediately 5′ to the qrfp promoter (data not shown). We were able to identify a stable transgenic line using the 1 kb promoter without NRSE elements that produced strong EGFP expression in qrfp-expressing neurons. To determine the specificity of this promoter, we performed FISH using a qrfp-specific probe together with immunohistochemistry using an EGFP-specific antibody (Fig. 2C). We found that 97 ± 1% of EGFP-positive neurons were also positive for qrfp mRNA, and 98 ± 1% of qrfp mRNA-positive neurons were also positive for EGFP (n = 113 neurons in 10 brain hemispheres), indicating that this transgenic line is specific for qrfp-expressing neurons. Using Tg(qrfp:EGFP) (data not shown) and Tg(qrfp:Brainbow) (Fig. 2D, Movie 1), we observed extensive QRFP neuron projections in the hypothalamus, sparse projections to the forebrain, and projections to the hindbrain that extend down the spinal cord.

To characterize qrfp-expressing neurons in more detail, we performed double FISH using probes specific for qrfp, and markers for specific neurotransmitters and neuropeptides. We found that all qrfp-expressing neurons colocalize with markers for glutamatergic neurons, including vlglut2b (Fig. 2E), vlglut1 (data not shown), and vlglut2a (data not shown), and do not colocalize with markers for GABAergic neurons, including gad67 (Fig. 2F) and gad65 (data not shown). These results suggest that qrfp neurons produce the excitatory neurotransmitter glutamate but not the inhibitory neurotransmitter GABA. To compare the localization of larval zebrafish qrfp-expressing neurons to other hypothalamic neuron subtypes (Fig. 3), we performed double FISH using WT larvae or single FISH using Tg(qrfp:EGFP) larvae, using probes specific for qrfp and avp, ccka, cckb, crh, hypocretin (hcrt), oxt, sr1.1, vip, and the dopaminergic neuron marker thl. We found that qrfp-expressing neurons are of sleep bouts during the day or night. Time of heat shock is indicated by arrows and gaps in activity (A) and sleep (B) line graphs on Day 6. Line and bar graphs represent the mean ± SEM. n indicates the number of larvae analyzed. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons of Tg(hsp:QRFP) and WT larvae using Student’s t test.
located dorsal relative to neurons expressing several of these genes in the hypothalamus, including arvp, cckα, cckβ, slt1.1, and vip. qrfp expression is also observed slightly caudal to oxt and slightly rostral to the hypothalamic cluster of th1-expressing neurons. qrfp-expressing neurons are also intermingled with neurons that express cphb and hcr. We occasionally observed colocalization of qrfp and cphb in one or two neurons, but never observed colocalization of qrfp and hcr (n > 50 brains). We also examined qrfp-expressing neurons, and compared their location to that of hcr-expressing neurons, in adult Tg(qrfp:EGFP); Tg(hcrm:RFp) double transgenic animals (Liu et al., 2015) that were fixed and cleared using PACT (Yang et al., 2014; Fig. S3). Similar to larval stages, we observed that qrfp neurons (~120 cells) and Hcr neurons (~60 cells) are localized near each other in the hypothalamus, but do not colocalize. These results indicate that qrfp is expressed in a population of hypothalamic neurons that is distinct from several other hypothalamic cell types, suggesting that these neurons may have a distinct function.

The QRF receptors Gpr103a/QRFPR and Gpr103b/QRFPRB are conserved in zebrafish
Several studies have shown that the G-protein-coupled receptor Gpr103 (also known as AQ27 and SP9155) acts as a receptor for QRF-derived peptides (Fukusumi et al., 2003; Jiang et al., 2003; Takayasu et al., 2006). The human genome contains a single gpr103 gene (Lee et al., 2001), whereas two paralogs, gpr103a and gpr103b, are present in rodents (Kampe et al., 2006; Takayasu et al., 2006). Similar to rodents, we found that the zebrafish genome contains two gpr103 paralogs, denoted gpr103a (qrfpra) and gpr103b (qrfprb; Fig. 4). The zebrafish paralogs share 80% amino acid identity with each other, and each zebrafish paralog is ~60% identical to each mouse paralog.

gpr103a and gpr103b are expressed in distinct neuronal populations in the larval zebrafish brain
To determine the expression patterns of the putative zebrafish QRFP receptors, we performed ISH using probes specific for gpr103a and gpr103b. gpr103b expression was first detected at 48 hpf in two distinct hypothalamic nuclei (Fig. S5A–C). At 120 hpf, we observed expression in several brain regions, including a large hypothalamic expression domain (Fig. 5D, E), a series of rostral–caudal stripes in the hindbrain (Fig. 5D), and several scattered neuronal populations in the midbrain and hindbrain (Fig. 5D, E). We were unable to detect gpr103a expression until 120 hpf, at which point we observed expression in two clusters of neurons in the ventral hypothalamus (Fig. 5F) and dorsally as four paired clusters of neurons in the lateral midbrain (Fig. 5G). Thus, similar to rodents (Fukusumi et al., 2003; Kampe et al., 2006; Takayasu et al., 2006; Bruzzone et al., 2007), the zebrafish gpr103 paralogs are expressed in apparently nonoverlapping regions of the hypothalamus and hindbrain. These regions are consistent with QRFP signaling playing a role in regulating a variety of homeostatic functions, possibly including sleep.

To characterize gpr103b-expressing neurons in more detail, we performed double FISH using probes specific for gpr103b and several neurotransmitter subtype markers. We found that the broad domain of hypothalamic gpr103b-expressing cells contains two territories. Rostral gpr103b-expressing cells colocalize with a GABAergic marker (Fig. 6), whereas caudal gpr103b-expressing cells colocalize with a glutamatergic marker (Fig. 7). We also examined the rostral–caudal stripes of gpr103b-expressing cells in the hindbrain because this expression pattern appeared to be similar to previously described alternating stripes of glutamatergic and GABAergic neurons in the larval zebrafish hindbrain (Higashijima et al., 2004). We found that many of these gpr103b-expressing cells colocalized with GABAergic markers (Fig. 6), whereas few of these cells colocalized with glutamatergic markers (Fig. 7). We were unable to perform a similar analysis for gpr103a because its expression was too low to obtain robust FISH labeling. These results indicate that gpr103b is expressed in both excitatory and inhibitory neurons in regions of the larval zebrafish brain that are consistent with the regulation of sleep.

QRFP overexpression decreases daytime locomotor activity
The expression of QRFP in the hypothalamus, and of its receptors in the hypothalamus and brainstem, suggested that QRFP signaling might affect locomotor activity and/or sleep. Indeed, several studies in rodents (do Rego et al., 2006; Kampe et al., 2006; Moriya et al., 2006; Takayasu et al., 2006) have shown that intracerebroventricular injection of QRFP peptides can affect locomotor activity, although the effects were transient and inconsistent in different studies. The discrepancies may arise from the use of QRFP peptides that were synthesized and modified in vitro, and thus may differ from mature peptides that are generated from the full-length protein in vivo. Furthermore, the technically challenging and invasive nature of these injections, the small number of animals studied, and the transient effects on behavior may confound the results.

To avoid these potential problems, we used a genetic approach to overexpress the full-length QRFP gene in zebrafish larvae using an HS-inducible promoter (hsp; Halloran et al., 2000), and monitored effects on behavior using a previously described locomotor activity assay (Prober et al., 2006). This approach is noninvasive, generates the full-length QRFP protein that is processed and modified in vivo, and allows the behavior of many animals to be studied before and after transgene overexpression. We found that Tg(hsp:QRFP) larvae were somewhat less active than WT larvae

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**Figure 9.** Tg(hsp:QRFP) larvae exhibit leaky qrfp mRNA expression in the absence of a heat shock. Representative whole-mount brains from 5 dpf Tg(hsp:QRFP) larvae and their nontransgenic WT siblings are shown. A—D. Larvae were fixed 1 h after a 1 h heat shock at 37°C (A, B) and were compared with non-heat shocked larvae that were maintained at 28.5°C (C, D). Transgenic heat shocked larvae exhibit robust and nearly ubiquitous qrfp expression (B) compared with identically treated WT siblings (A). Transgenic larvae that were not heat shocked exhibit mildly elevated qrfp mRNA levels throughout much of the brain (D) compared with identically treated WT siblings (C), indicating leaky qrfp expression in non-heat shocked Tg(hsp:QRFP) larvae. Endogenous qrfp is not visible because development of the ISH reaction was stopped before endogenous qrfp could be detected. Transgenic and nontransgenic sibling larvae were processed for ISH in the same tubes and genotyped after imaging, thus allowing comparison of relatively subtle differences in qrfp mRNA levels. Scale bar, 300 μm. Large spots (in A, C, D) are pigment cells.
**Figure 10.** *qrfp* mutant larvae are more active and sleep less during the day. A, C, D, *qrfp* 
+/− larvae (black) are more active during the day (A, C) and slightly more active at night (D) compared with their *qrfp* +/− siblings (gray). E, F, *qrfp* −/− larvae are also more active when awake compared with controls. B, G, *qrfp* −/− larvae sleep less during the day pre-HS (11% decrease; *p < 0.05 by Student’s *t* test; Fig. 8A, C), likely due to leaky expression of the transgene from the hsp promoter (Fig. 9). However, locomotor activity levels were more significantly reduced in Tg(hsp:QRFP) larvae compared with WT larvae immediately following heat shock (24% decrease; *p < 0.001 by Student’s *t* test; Fig. 8A, C), and the effect was even stronger the next day (30% decrease; *p < 0.001 by Student’s *t* test; Fig. 8A, C). Comparison of WT and Tg(hsp:QRFP) locomotor activity levels before (Day 5) and after (Day 7) heat shock revealed a significant genotype–heat shock interaction (two-way ANOVA, *p < 0.05), indicating that locomotor activity is affected by heat shock differently in Tg(hsp:QRFP) and WT larvae, and consistent with heat shock significantly decreasing locomotor activity in Tg(hsp:QRFP) larvae. QRFP-overexpressing larvae also exhibited less activity while awake (30% decrease; *p < 0.001 by Student’s *t* test; Fig. 8E) during the day. There was no effect of QRFP overexpression on locomotor activity or activity while awake at night (Fig. 8E) during the day. We also observed that *qrfp* +/− siblings, consistent with the QRFP overexpression phenotype, we hypothesized that *qrfp* mutant larvae would exhibit increased locomotor activity. Indeed, we found that *qrfp* −/− larvae exhibited significantly more locomotor activity (Fig. 10A, C, D) and locomotor activity while awake (Fig. 10E, F) compared with their *qrfp* +/− and WT siblings, consistent with the QRFP overexpression phenotype, this effect was stronger during the day (Fig. 10A, C–F; day locomotor activity: 8% increase, *p < 0.001; night locomotor activity: 5% increase, *p < 0.05; day locomotor activity while awake: 8% increase, *p < 0.001; night locomotor activity while awake: 5% increase, *p < 0.01; by Student’s *t* test). We also observed that *qrfp* −/− larvae slept significantly less during the day (18% decrease, *p < 0.01 by Student’s *t* test; Fig. 10B, G), but not at night (Fig. 10H). Compared with their *qrfp* +/− siblings. The *qrfp* −/− sleep defect was due to a decrease in the number of sleep bouts (Fig. 10I), with no effect on the
lengths of these bouts (Fig. 10K), indicating that daytime bouts of wakefulness are consolidated in gpr103<sup>−/−</sup> larvae compared with controls. We conclude that endogenous qrfp is required to maintain normal daytime levels of sleep and locomotor activity.

**gpr103a; gpr103b double-mutant zebrafish larvae are more active and sleep less during the day**

To determine the downstream signaling pathway involved in QRFP-regulated sleep, we generated zebrafish containing mutations in gpr103a and gpr103b using the TALEN (Sander et al., 2011) and ZFN (Foley et al., 2009) methods, respectively (Chen et al., 2013). We isolated a gpr103a mutant containing an 11 bp deletion, which introduces a shift in the reading frame before the first predicted transmembrane domain, resulting in an early stop codon and generating a predicted 105 aa protein (Fig. 4A). We isolated a gpr103b mutant containing a 4 bp deletion, which introduces a shift in the reading frame in the predicted fifth transmembrane domain, resulting in an early stop codon, and generating a predicted 220 aa protein (Fig. 4A). Both mutant proteins should be unable to properly insert into the cell membrane due to the loss of transmembrane domains, and should therefore be nonfunctional. We found that zebrafish homozygous for each single mutant, as well as double-homozygous mutants, were viable and fertile, with no obvious developmental defects (data not shown). To determine whether these genes are required for normal levels of locomotor activity and sleep, we compared the behavior of gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae to that of related WT larvae (see Materials and Methods). Similar to qrfp mutants, gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae exhibited increased locomotor activity (17% increase; p < 0.001 by Student’s t test; Fig. 11A,C), increased locomotor activity while awake (17% increase; p < 0.001 by Student’s t test; Fig. 11E) and decreased sleep (45% decrease; p < 0.001 by Student’s t test; Fig. 11B) during the day compared with WT controls. Consistent with qrfp mutant larvae, the gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> sleep phenotype was mainly due to a decrease in the number of sleep bouts (Fig. 11I), with less or no effect on the length of these bouts (Fig. 11K). In contrast to qrfp mutant larvae, gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae also exhibited decreased locomotor activity (11% decrease; p < 0.001 by Student’s t test) and locomotor activity while awake (10% decrease; p < 0.001 by Student’s t test) at night (Fig. 11D,F) compared with controls. This observation suggests that Gpr103a and Gpr103b may have a QRFP-independent function at night. To control for any potential effects of a heat shock on behavior, and thus to allow comparison with the QRFP overexpression experiment, we performed a heat shock during the afternoon on Day 6, and monitored behavior before and after the heat shock. We found that the mutant phenotype compared with WT controls was similar before and after the heat shock (Fig. 11A–L). Together, these results indicate that gpr103a and gpr103b are required to maintain normal daytime levels of sleep and locomotor activity, similar to qrfp, consistent with Gpr103a and Gpr103b functioning as receptors for QRFP in vivo.

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**Figure 11.** gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae are more active and sleep less during the day. A, C, E, gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae (black) exhibit more locomotor activity (A, C) and locomotor activity while awake (E) during the day compared with related WT larvae (gray). B, G, gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae also sleep less during the day. I, K. The sleep phenotype is primarily due to a decrease in the number of sleep bouts (K), with less or no effect on the length of sleep bouts (I). D, F, At night, gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae exhibit less locomotor activity (D) and locomotor activity while awake (F) compared with WT larvae. The amount of sleep (K) and number (I) and length (L) of sleep bouts are largely normal for gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> larvae at night. A heat shock applied during the afternoon on Day 6 (indicated by arrows and gaps in activity (A) and sleep (I) line graphs) has no effect on the mutant locomotor activity or sleep phenotypes compared with WT larvae. Line and bar graphs represent the mean ± SEM. n indicates the number of larvae analyzed. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons of gpr103a<sup>−/−</sup>; gpr103b<sup>−/−</sup> and WT larvae using Student’s t test.
The QRFP overexpression behavioral phenotype is abolished in gpr103a; gpr103b double-mutant larvae

To test whether Gpr103a and Gpr103b are functional receptors for QRFP, we compared the effect of QRFP overexpression on locomotor activity in WT larvae to gpr103a^-/-; gpr103b^-/- larvae (Fig. 12). Consistent with our previous experiments, QRFP overexpression decreased locomotor activity during the day of heat shock and the following day (day pre-HS: 12% decrease, p = 0.42; day post-HS: 27% decrease, p < 0.01; following day: 25% decrease, p < 0.01, by Tukey’s HSD test), whereas gpr103a^-/-; gpr103b^-/- double-mutant larvae exhibited increased daytime locomotor activity (day pre-HS: 56% increase, p < 0.001; day post-HS: 24% increase, p < 0.05; following day: 29% increase, p < 0.01, by Tukey’s HSD test). Strikingly, the QRFP overexpression-induced decrease in daytime locomotor activity was abolished in gpr103a^-/-; gpr103b^-/- larvae (p = 0.92, by Tukey’s HSD test), suggesting that Gpr103a and Gpr103b are functional receptors for QRFP in zebrafish.

QRFP expression does not exhibit a circadian oscillation

Because QRFP gain-of-function and loss-of-function phenotypes were largely restricted to the day, we hypothesized that endogenous qrfp expression, and thus QRFP signaling, might be regulated in a circadian manner. To test this hypothesis, we raised larvae under standard 14 h/10 h light/dark conditions until 6 dpf, and then transferred them to constant dark. In this “free-running” condition, WT larvae maintain molecular and behavioral circadian rhythms in the absence of daily light/dark cues (Kaneko and Cahill, 2005). After 24 h in constant dark, we isolated samples every 6 h for 24 h, and assayed qrfp levels using quantitative RT-PCR (qRT-PCR). In contrast to the circadian clock gene period 1b (per1b), whose expression oscillated in a circadian manner, we failed to observe a circadian oscillation of qrfp expression (Fig. 13A). Consistent with this result, using ISH we observed similar qrfp mRNA levels during both subjective day and subjective night in larvae that were entrained in standard light/dark conditions and fixed after transfer to constant dark (Fig. 13B). These observations suggest that qrfp expression is not regulated by the circadian clock.

QRFP overexpression decreases locomotor activity and increases sleep in larvae that lack behavioral circadian rhythms

Although we found that the circadian clock does not regulate qrfp expression, the fact that QRFP gain-of-function and loss-of-function phenotypes are more prominent during the day suggested that the circadian clock might modulate QRFP function. Alternatively, the ability of overexpressed QRFP to suppress daytime locomotor activity may depend on the presence of light and be independent of the circadian clock. To test this hypothesis, we raised and tested larvae in constant light conditions, which prevents the development of behavioral circadian rhythms (Hurd and Cahill, 2002). We found that QRFP overexpression decreased locomotor activity (20% decrease; p < 0.001 by Student’s t test) and locomotor activity while awake (19% decrease; p < 0.001 by Student’s t test) compared with nontransgenic WT siblings during the 30 h that we monitored behavior following heat shock (Fig. 14A, C, D). QRFP overexpression also increased sleep under these conditions (34% increase; p < 0.05 by Student’s t test; Fig. 14B, E). This result suggests that the QRFP overexpression phenotype is specific to the day due to the presence of light and not due to circadian regulation of QRFP function.

As an additional test of whether the circadian clock modulates QRFP function, we raised and tested larvae in constant dark conditions. Similar to larvae raised and tested in constant light, QRFP overexpression in constant dark decreased locomotor activity (16% decrease; p < 0.01 by Student’s t test) and locomotor activity while awake (16% decrease; p < 0.001 by Student’s t test) compared with nontransgenic WT siblings (Fig. 15A, C, D). However, in contrast to constant light, QRFP overexpression had no effect on the amount of sleep in constant dark (Fig. 15B, E), although it did affect sleep architecture (Fig. 15F, G). Together, these results suggest that the circadian clock does not modulate QRFP function, but rather that the effects of QRFP overexpression on behavior are stronger in light than in dark.

To more directly test the hypothesis that QRFP overexpression inhibits locomotor activity in a light-dependent manner, we entrained larvae for 4 d in standard light/dark conditions, and on the morning of the fifth day of development we exposed them to alternating 1 h periods of lights on and off for 72 h (Fig. 16). We performed a heat shock during the afternoon of the sixth day of development, and compared the locomotor activity of Tg(hsp:QRFP) larvae to their WT siblings during light and dark periods.
before and after heat shock. Before heat shock, there was no significant difference in the amount of locomotor activity between transgenic and WT larvae during either the light (\(p = 0.98\), by Tukey’s HSD test) or dark (\(p = 0.68\), by Tukey’s HSD test) periods (Fig. 16A, B, D, E). In contrast, QRFP-overexpressing larvae were significantly less active than their WT siblings during light periods (\(p < 0.05\), by Tukey’s HSD test), while there was no difference during dark periods (\(p = 0.83\), by Tukey’s HSD test; Fig. 16A, C, D, E). Because the amount of locomotor activity for both genotypes varied depending on the circadian time, we also calculated the ratio of transgenic/WT locomotor activity during each 1 h light or dark trial, and compared these ratios before and after heat shock. For dark trials, there was no significant difference post-HS (1.09 ± 0.03) compared with pre-HS (1.14 ± 0.04; \(p = 0.74\), by Tukey’s HSD test; Fig. 16F). In contrast, for light trials there was a significant decrease after heat shock compared with before heat shock (pre-HS, 0.97 ± 0.01; post-HS, 0.84 ± 0.01; \(p < 0.05\), by Tukey’s HSD test; Fig. 16F). These results indicate that QRFP overexpression decreases locomotor activity in the context of light, but not dark, conditions.
Our studies of QRFP/Gpr103 function in zebrafish have several advantages over previously described studies in rodents. First, we use a noninvasive heat shock-induced genetic overexpression approach, which allows for more reproducible overexpression among animals compared with intracerebroventricular injection. These injections provide more specific peptide targeting compared with heat shock-induced overexpression in zebrafish, which induces gene overexpression throughout the animal. However, the zebrafish QRFP overexpression phenotype is unlikely to be an artifact because the zebrafish *qrfp* and *gpr103* mutants exhibit the opposite behavioral phenotype. Second, we observed gain-of-function and loss-of-function phenotypes that persisted for multiple days. In contrast, phenotypes induced by QRFP peptide injection in rodents lasted only a few hours, and are thus less robust and more subject to artifacts caused by animal handling. Third, because our study monitored the behavior of 96 zebrafish larvae simultaneously, we were able to assay many more animals than is possible in rodent experiments, which may produce more reproducible data. Fourth, rodent studies used QRFP peptides that were synthesized and modified in vitro. Because QRFP undergoes cleavage and modification in vivo, peptides synthesized in vitro might not be properly modified, which could result in reduced or abnormal function. In contrast, we overexpressed the full-length *qrfp* gene in zebrafish *in vivo*, which allows the protein to be cleaved and modified by endogenous mechanisms. As a result, QRFP that is overexpressed in zebrafish is...
more likely to be similar to naturally produced QRFP. Together, we suggest that our analysis of QRFP/Gpr103 function in zebrafish provides several advantages over rodent studies and has provided novel insights into QRFP function.

The human qrfp gene encodes a 136 aa prepropeptide that is cleaved to form the mature 26RFa and 43RFa peptides (Chartrel et al., 2011; Ukena et al., 2014). While the mammalian 26RFa peptide is well conserved in zebrafish, it is unclear whether zebrafish produce a peptide similar to mammalian 43RFa. The zebrafish QRFP preproprotein contains an Arg-Arg motif, as well as a Lys-Arg motif that is conserved with goldfish, which could generate N-terminal elongated peptides of 54 or 60 aa, respectively. However, these extended sequences have low homology with the mammalian peptides (Fig. 1B), so it is unclear whether these zebrafish peptides are generated in vivo. Additional studies are required to identify the mature QRFP peptides that are produced in zebrafish and to determine their functions. However, regardless of whether zebrafish produce these elongated peptides, our

Figure 16. QRFP overexpression decreases locomotor activity in light but not in dark conditions. A, Tg(hsp:QRFP) larvae (black) and their WT siblings (gray) were entrained for 4 d on a 14 h/10 h light/dark cycle (data not shown), and were then exposed to alternating 1 h periods of lights on and off (indicated by white and black boxes) starting on the morning of the fifth day of development. Larvae were heat shocked during the afternoon of the sixth day of development (indicated by arrow and gap in line graph). B, C, Boxed regions before (B) and after (C) heat shock are shown at higher temporal resolution. D, The average amount of locomotor activity was not significantly different between Tg(hsp:QRFP) and WT larvae during dark periods either before or after heat shock. E, The average amount of locomotor activity was not significantly different between Tg(hsp:QRFP) and WT larvae during light periods before heat shock, but Tg(hsp:QRFP) larvae were significantly less active than WT larvae during light periods after heat shock. F, Because the amount of locomotor activity for both genotypes varied depending on the circadian time (A), we also calculated the ratio of Tg(hsp:QRFP)/WT locomotor activity during each 1 h light or dark trial, and compared these ratios before and after heat shock. For dark trials, there was no significant difference after heat shock compared with before heat shock. However, for light trials there was a significant decrease after heat shock compared with before heat shock. Dashed line indicates a ratio value of 1. Line and bar graphs represent the mean ± SEM. n indicates the number of larvae analyzed. *p < 0.05 for the indicated pairwise comparisons using ANOVA followed by Tukey’s HSD test to correct for multiple comparisons.
loss-of-function studies provide strong evidence that QRFP signaling is required to maintain normal daytime sleep levels.

Our findings indicate that QRFP/Gpr103 signaling regulates sleep primarily during the light phase in a diurnal animal, but this pathway may have a different role in nocturnal animals. Mice containing a targeted knockout of gpr103a exhibit defects in bone formation, but lack additional obvious phenotypes (Baribault et al., 2006), possibly due to redundant function of gpr103b. Mice lacking qrfp or both qrfp and gpr103b will need to be tested to determine whether QRFP/Gpr103 signaling is required to regulate sleep in mammals. In particular, these mutants may reveal whether QRFP signaling regulates sleep during the light phase, when diurnal animals are primarily awake and nocturnal animals are primarily asleep. Alternatively, this pathway may be required to regulate sleep during the time when an animal is primarily awake, which corresponds to the day in diurnal animals and night in nocturnal animals. It is also possible that QRFP signaling only promotes sleep in diurnal animals, or has different functions in mammals and teleosts. If the zebrafish

\[ \text{qrfp} \]

or both

\[ \text{qrfp} \text{ and } \text{gpr103} \]

will need to be tested to understand its role in sleep regulation.

How might QRFP/Gpr103 signaling regulate sleep? Mammalian sleep is thought to be regulated by mutual inhibition of wake- and sleep-promoting neurons, which generates a “flip-flop” switch that ensures a rapid transition between stable wake and sleep states (for review, see Saper et al., 2010; Brown et al., 2012). Several neuronal centers that are thought to promote sleep have been identified. These include neurons in the ventrolateral preoptic nucleus containing the inhibitory neurotransmitter GABA and the inhibitory peptide galanin (Sherin et al., 1996), neurons in the lateral hypothalamus containing GABA and the inhibitory peptide melanin-concentrating hormone (Konadhive et al., 2013; Tsunematsu et al., 2014), GABAergic median preoptic nucleus neurons (Suntsova et al., 2007), GABAergic neurons in the medullary parafacial zone (Anaclet et al., 2014), and somatostatin-positive GABAergic neurons in the basal forebrain (Xu et al., 2015). These neurons are thought to inhibit wake-promoting glutamatergic neurons in the parabrachial nucleus and precerebellar, glutamatergic hypocretin neurons in the lateral hypothalamus, glutamatergic and cholinergic neurons in the basal forebrain, and monoaminergic and cholinergic nuclei of the ascending arousal system, resulting in the promotion of sleep. However, lesion studies suggest that additional sleep-promoting neuronal centers are present (Lu et al., 2000; Fuller et al., 2011). Our results suggest that QRFP neurons may compose one of these sleep-promoting centers. An interesting aspect of QRFP neurons is that they are glutamatergic, and thus excitatory, in contrast to most previously described sleep-promoting neurons, which are inhibitory (Saper et al., 2010; Brown et al., 2012; Anaclet et al., 2014). This distinction suggests that QRFP neurons may interact with the flip-flop switch or other sleep regulatory mechanisms in a manner that is distinct from previously described sleep-promoting neurons. Optogenetic approaches can be used to stimulate and inhibit QRFP neurons to determine whether they are indeed sleep promoting and to test whether they interact with other neurons that are known to regulate sleep.

References


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