Divergent evolution of two corticotropin-releasing hormone (CRH) genes in teleost fishes

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Genome duplication, thought to have happened twice early in vertebrate evolution and a third time in teleost fishes, gives rise to gene paralogs that can evolve subfunctions or neofunctions via sequence and regulatory changes. To explore the evolution and functions of corticotropin-releasing hormone (CRH), we searched sequenced teleost genomes for CRH paralogs. Our phylogenetic and synteny analyses indicate that two CRH genes, crha and crhb, evolved via duplication of crh1 early in the teleost lineage. We examined the expression of crha and crhb in two teleost species from different orders: an African cichlid, Burton's mouthbrooder, (Astatotilapia burtoni; Order Perciformes) and zebrafish (Danio rerio; Order Cypriniformes). Furthermore, we compared expression of the teleost crha and crhb genes with the crh1 gene of an outgroup to the teleost clade: the spotted gar (Lepisosteus oculatus). In situ hybridization for crha and crhb mRNA in brains and eyes revealed distinct expression patterns for crha in different teleost species. In the cichlid, crha mRNA was found in the retina but not in the brain. In zebrafish, however, crha mRNA was not found in the retina, but was detected in the brain, restricted to the ventral hypothalamus. Spotted gar crh1 was found in the retina as well as the brain, suggesting that the ancestor of teleost fishes likely had a crh1 gene expressed in both retina and brain. Thus, genome duplication may have freed crha from constraints, allowing it to evolve distinct sequences, expression patterns, and likely unique functions in different lineages.

Keywords: corticotropin-releasing factor (CRF), genome duplication, subfunctionalization, cichlid, zebrafish, spotted gar, brain, retina

Introduction

Corticotropin-releasing hormone (CRH), also referred to as corticotropin-releasing factor (CRF) (Hauger et al., 2003), is known primarily for its central role in regulating neuroendocrine responses to stressors via release from the hypothalamus (Vale et al., 1981; Korosi and Baram, 2008). Yet CRH and other neuroendocrine releasing hormones are also expressed more broadly throughout the brain (Swanson et al., 1983). CRH immunoreactivity has also been found in retinal amacrine cells, including displaced amacrine cells, and ganglion cells in several species, i.e., chicken (Kiyama et al., 1984), rat (Skofitsch and Jacobowitz, 1984), and turtle (Williamson and Eldred, 1989). CRH-expressing neurons play diverse roles throughout the brain and in the retina, but many of these have not been fully explored (Kovács, 2013).
Many gene families in vertebrates were generated during two rounds of whole-genome duplication early in vertebrate evolution (Ohno, 1970; Abi-Rached et al., 2002; Dehal and Boore, 2005). Recently, we found that an ancestral vertebrate CRH gene experienced a duplication early in vertebrate evolution, giving rise to two orthologs—paralogous genes that originated via whole-genome duplication—CRH1 and CRH2 (Grone and Maruska, 2015). We found both CRH1 and CRH2 in many groups of vertebrates, including ray-finned fishes, reptiles, birds, and mammals (Grone and Maruska, 2015), in addition to the elephant shark (Callorhinchus milii), where it was originally reported (Nock et al., 2011). Although CRH1 and CRH2 genes share sequence homology, CRH1 protein sequences are much more conserved compared to the highly variable CRH2 sequence, suggesting that the CRH1 protein retained essential structure and functions. Furthermore, expression of crh2 mRNA in spotted gar brain is much more restricted compared to crh1 mRNA (Grone and Maruska, 2015). Spotted gar crh1 and crh2 expression has not, however, been examined in the retina. Indeed, expression of crh2 has not been examined in the retina of any species.

No identifiable crh2 gene ortholog is found in any sequenced teleost species, suggesting that crh2 was lost early in teleost evolution (Grone and Maruska, 2015). Teleost fishes, however, experienced an additional (third) whole genome duplication (WG3) prior to their ecological and evolutionary radiation (Christoffels et al., 2004; Hoegg et al., 2004; Jaillon et al., 2004; Amores et al., 2011). Many teleost genes are thus present in duplicate compared to their mammalian homologs. In the course of describing crh1 and crh2, we also showed that many teleost species’ genomes contain two crh1 orthologs: crha and crhb (Grone and Maruska, 2015). These two teleostean crh orthologs encode different predicted 41-amino-acid processed peptides. Only crhb has been studied, while crha has gone unremarked. In fact, until recently, only one crh gene was thought to exist in zebrafish and many other teleosts (Chandrasekar et al., 2007; Lovejoy et al., 2014).

Genome sequences are now available for several teleosts, including zebrafish (Howe et al., 2013), medaka (Kasahara et al., 2007), three-spined stickleback (Jones et al., 2012), Atlantic cod (Star et al., 2011), rainbow trout (Berthelot et al., 2014), and several African cichlid species (Brawand et al., 2014). The synteny, sequences, and phylogenetic relationships of the duplicated CRH genes in these species have not been previously studied, and expression patterns of crha have not been reported for any species. Comparing duplicated genes in teleosts to the orthologs in spotted gar, a primitive non-teleost ray-finned fish, can generate insights regarding diverse evolutionary processes including gene duplication, gene loss, sequence evolution, and regulatory changes (Braasch et al., 2015; Gehrke et al., 2015).

In the present study, we first employed comparative genomic and phylogenetic analyses to identify the evolutionary relationships of the teleost CRH genes, crha and crhb, to other CRH-family peptides. We then used in situ hybridization in the brain and retina of two teleosts, the zebrafish, Danio rerio, and an African cichlid fish, Astatotilapia burtoni, as well as the spotted gar, Lepisosteus oculatus, to characterize and analyze expression patterns of CRH genes. Our analyses reveal a diverse range of evolutionary outcomes following CRH gene duplication, with potential functional implications for stress-regulating systems and neuromodulation of brain and retinal function across vertebrates.

**Materials and Methods**

**Animals**

Juvenile Lepisosteus oculatus were purchased from Rainforest International (Bloomington, IN) or caught from the Atchafalaya Basin, LA. Danio rerio were purchased from Arizona Aquatic Gardens (Oro Valley, AZ). Astatotilapia burtoni (Fernald, 1977) originally derived from a wild-caught population were maintained at LSU. Juvenile Lepisosteus oculatus (n = 5; Standard Length (SL) = 69.6 ± 12.4 mm (mean±sd), adult Danio rerio (n = 3 males, 2 females; SL = 25.8 ± 1.3 mm), and adult Astatotilapia burtoni (n = 5 males; SL = 45.4 ± 5.1 mm) were used for the in situ hybridization (ISH) experiments. All experiments were performed in accordance with the recommendations and guidelines stated in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 2011. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA.

**Sequence Analysis**

Throughout this paper, we use standard gene nomenclature. For fishes, gene symbols are italicized and protein symbols are capitalized. For other vertebrates, human conventions are used: gene symbols in all capitals and italicized, protein symbols in all capitals.

To determine the phylogenetic relationships among fish CRH gene family members, sequences for CRH-family genes were located by searches in Ensembl for spotted gar (Lepisosteus oculatus), Atlantic cod (Gadus morhua), and zebrafish (Danio rerio), and in GenBank for Burton’s mouthbrooder (Astatotilapia burtoni), medaka (Oryzias latipes), rainbow smelt (Osmerus mordax), Atlantic salmon (Salmo salar), and bicolor damselfish (Stegastes partitus) (Table 1). Zebrafish urotensin 1 (uts1) sequence was retrieved from GenBank (NP_001025351.1). In Table 1, Ensembl identification numbers or GenBank accession numbers are listed, except for the case of Gasterosteus aculeatus crha, for which the position on a scaffold is given. ORFs for unannotated crha genes were predicted from genomic DNA using GENSCAN (Burge and Karlin, 1997). Predicted amino acid sequences were then generated from ORFs.

All multiple sequence alignments were carried out using MAFFT (Katoh and Standley, 2013) in Geneious software version 5.1.7 (Kearse et al., 2012), with the following settings: Algorithm = E-INS-i; scoring matrix = BLOSUM62; gap-open penalty = 1.53.

MEGA5 identified the best amino acid substitution model as JTT+G+I (Tamura et al., 2011), according to comparisons of the Bayesian Information Criteria for 48 different amino acid models. Therefore, we used the Jones-Taylor-Thornton model (JTT) (Jones et al., 1992), with non-uniformity of evolutionary rates among sites modeled by estimating a discrete Gamma
**TABLE 1 | Sources for teleost CRH family genes described in this paper.**

| Teleost species                  | Common name                        | crha     | crhb     |
|----------------------------------|------------------------------------|----------|----------|
| Astatotilapia burtoni            | Burton’s mouthbrooder              | XM_005946397 | EF383131 |
| Danio rerio                      | Zebrabfish                         | ENSDARG000000093401 | ENSDARG00000027657 |
| Gadus morhua                     | Atlantic cod                       | ENSMGG000000020152 | ENSMGG000000020107 |
| Gasterosteus aculeatus           | Three-spined stickleback           | scaffold_120: 91757-91374 | ENSGACG000000002971 |
| Cryzias latipes                  | Medaka                             | ENSORL00000006816 | ENSORL00000008503 |
| Osmerus mordax                   | Atlantic salmon                    | crha1: EG786316 crha2: EG808138 | crhb1: DY733168 crhb2: BT057824 |
| Stegastes partitus               | Bicolor damselfish                 | XM_008303879.1 XM_008288433.1 | XM_008288433.1 |
| Takifugu rubripes                | Fugu                               |          |          |

**TABLE 2 | Primer sequences<sup>a</sup> used to generate templates for the synthesis of riboprobes used for in situ hybridization.**

| Gene                  | Forward primer (5′ → 3′)     | Reverse primer (5′ → 3′) | Probe length (bp) |
|-----------------------|------------------------------|--------------------------|-------------------|
| L. oculatus crh1      | AGCTCAATTTCCTTCTTCTACTGC     | CATCATTCTCTGTGTGCTGCGC   | 479               |
| L. oculatus crh2      | GCCATGAGCAAAGTCCCTCCTCTCTG  | CAGCTTACCTTCTAGGAACCT    | 400               |
| A. burtoni crha       | CAGACTGGCCTCTGAGTAGGAAACGC   | TCTTCTCACCTCTGAGGATATCC  | 416               |
| A. burtoni crhb       | GACTCGAACCTTCTCCACATC        | ATGAAGAAGGCGATATCTCTAC   | 545               |
| D. rerio crha         | GGACGATTAGAAATGAAAGGC        | TGTTATTAAAAATAGG         | 238               |
| D. rerio crhb         | CCTGGCCACCTTTTGACATGAAACGC  | CTCTTGACAAACAAATATTAGG   | 904               |

<sup>a</sup>T3 transcription initiation sequence (aattaaccctcactaaggg) was added to the reverse primer (for anti-sense probes) or forward primer (for sense control probes).

distribution (+G) and by assuming that certain sites are evolutionarily invariant (+I).

An unrooted Maximum Likelihood phylogenetic tree was created from the protein alignment using PhyML (Guindon and Gascuel, 2003) in Geneious software with the JTT+G+I model. The following parameters were used: Tree topology search: NNIs; Initial tree: BioNJ; Number of substitution rate categories: 4. The tree was checked via 1000 bootstrap iterations.

Gene synteny for teleost crha and crhb genes was compared to the syntenic region near spotted gar crh1 using Genomicus (Louis et al., 2013).

**Colorimetric in situ Hybridization**

To localize crha and crhb mRNA in the brain and retina of Burton’s mouthbrooder and zebraﬁsh, and crh1 and crh2 in the brain and retina of spotted gar, we performed chromogen-based in situ hybridization with riboprobes on cryosectioned tissue. DNA templates for riboprobes were generated by PCR ampliﬁcation (Platinum PCR SuperMix, Life Technologies) of genomic DNA from the corresponding species using commercial primers (Table 2) and the following reaction conditions: 95°C for 1 min, 40 cycles of: (95°C for 15 s, 55°C for 15 s, 72°C for 1 min), and 72°C for 1 min. Purified PCR products (MinElute PCR Purification Kit, Qiagen) were then used as templates for the transcription reactions. Probes were designed to be complementary to the longest exon (exon 2) for each gene and were transcribed from the T3 poly(A) template primer. Digoxigenin (DIG)-labeled antisense and sense riboprobes for L. oculatus crh1 and crh2 (as described previously Grone and Maruska, 2015) and for A. burtoni and D. rerio genes, crha and crhb, were transcribed in vitro using T3 RNA Polymerase (Fermentas) and DIG RNA labeling mix (Roche Molecular Biochemicals), with RNAse Out (Life Technologies), and were then treated with Turbo DNase (Ambion Inc.) and purified using illustra ProbeQuant G-50 Micro Columns (GE Healthcare). PCR products and final probes were checked on a 1% agarose gel after each step and veriﬁed to be single bands of the correct size (see Table 2 for probe sizes).

Fish were anesthetized using ice water and decapitated. All solutions used for tissue preparation and ISH were made with RNAse-free 0.25-µm-ﬁltered water. The top of the skull was removed to expose the brain, and the brain was ﬁxed in the head overnight at 4°C with 4% paraformaldehyde (PFA) made in phosphate buffered saline (PBS, pH 7.2). Heads were then transferred to PBS and washed overnight at 4°C. Brains and left eyes were then removed from the heads (lenses were removed from the eyes by making a small slit in the dorsal portion of the eye) and cryoprotected in 30% sucrose at 4°C for 1–3 days until sectioning.

Brains and eyes were mounted in Tissue-Tek OCT (Sakura) and sectioned at 20µm in the transverse plane with a cryostat (Thermo, Cryostar NX50). Sections from each brain and eye were collected onto 2–4 sets of alternate charged slides (VWR, SuperFrost Plus), dried at room temperature for 2 days, and then stored at −80°C prior to use in ISH. Using an ImmEdge Hydrophobic Barrier Pen (Vector Laboratories), a hydrophobic barrier was drawn on the edges of each slide and allowed to
dry at room temperature (30 min). Sections were rehydrated with 3 × 5 min washes in PBS, fixed with 4% PFA for 20 min at room temperature, washed 2 × 5 min with PBS, and permeabilized with proteinase K (10 μg/ml) for 10 min at room temperature. Sections were then washed 1 × 5 min with PBS at room temperature, postfixed with 4% PFA for 15 min at room temperature, and washed with 2 × 5-min PBS washes and one short rinse in water. Sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0, which was mixed and vortexed immediately before use. Following acetic anhydride treatment, sections were washed 1 × 5 min with PBS at room temperature, and then incubated in pre-warmed hybridization buffer in a sealed humidified chamber to pre-hybridize at 60–65°C for 3 h. When pre-hybridization was complete, the hybridization solution was removed and replaced with hybridization buffer containing gene-specific probes, to generate multiple complete series of brain sections for each gene studied. Slides were covered with HybriSlip hybridization covers (Life Technologies) to help prevent evaporation and to distribute the probe evenly on the slide, and hybridization was carried out overnight (12–16 h) at 60–65°C in sealed humidified chambers. Sections were then washed 2 × 30 min at 60–65°C in pre-warmed 2X SSC in 50% Formamide with 0.1% Tween-20, 2 × 15 min at 60–65°C in 1:1 mixture of 2X SSC and maleic acid buffer with 0.1% Tween-20 (MABT), and 2 × 10 min at 60–65°C with MABT. Slides were then washed 2 × 10 min with MABT at room temperature, and non-specific binding was blocked by incubation in MABT with 2% bovine serum albumin (BSA) for 3 h at room temperature. Slides were then incubated with alkali-phosphatase-conjugated anti-DIG Fab fragments (Roche) diluted 1:5000 in MABT 2% BSA overnight at 4°C in a sealed humidified chamber. Following antibody incubation, slides were washed with MABT (3 × 30 min) at room temperature, rinsed 2 × 5 min in alkaline phosphatase buffer at room temperature, and then developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Roche) at 37°C for 5 min in alkaline phosphatase buffer at room temperature to stop the reaction (3 × 5 min), fixed in 4% PFA for 10 min, washed in PBS (3 × 5 min), and coverslipped with Aqua-Mount media (Thermo Scientific).

For each species, staining with anti-sense probes was strong, specific (minimal background), and consistent across all individuals examined. To test for probe specificity, sense control probes were applied to one set of alternate sections (transverse, 20 μm) for each gene and run simultaneously with anti-sense probes applied to another set of alternate slides. None of these sense controls showed any labeling.

### Double Fluorescent in situ Hybridization (FISH)

Fluorescent probe preparation was similar to that described above except that fluorescein-labeled nucleotides (FITC-labeling mix, Roche) were incorporated into the nucleic acid sequence during the transcription reaction. Prior to hybridization, the double FISH protocol was also identical to that described above for chromogenic ISH. Hybridization for double FISH was performed by simultaneously applying two probes (DIG-labeled *crha* and fluorescein-labeled *crhb*) to the tissue followed by incubation at 60–65°C overnight in sealed chambers as described above. Slides were then washed with 2 × SSC: formamide with 0.1% tween-20 (2 × 30 min) at 60–65°C, followed by room temperature incubation in Tris-NaCl-Tween (TNT) buffer (2 × 10 min), quenching in TNT + 0.3% H2O2 (10 min), and rinsing in TNT buffer (3 × 5 min). Non-specific binding was then blocked with 0.5% blocking reagent (Roche) in TNT (1.5 h) followed by incubation with Anti-Fluorescein-POD Fab fragments (Roche; 1:250 dilution) overnight at 4°C. Slides were next rinsed with TNT buffer (4 × 10 min), incubated with Alexa Fluor 488 tyramide (2 h), washed with TNT buffer (2 × 5 min), quenched with TNT + 0.3% H2O2 (15 min), washed with TNT buffer (2 × 5 min) and blocked with 0.5% blocking reagent in TNT (1 h). Slides were then incubated with anti-DIG Fab fragments (Roche; 1:5000 dilution) for 2 h at room temperature, washed with TNT buffer (2 × 5 min), and developed with Fast Red (1–2 h at 37°C; Roche). Reactions were stopped by washing in TNT buffer (2 × 5 min), and slides were mounted with Fluorogel II containing DAPI nucleic acid counterstain (Electron Microscopy Services) and coverslipped.

### Imaging and Analysis

To identify the expression patterns of genes in the brain and retina, DIG-labeled sections were imaged using brightfield illumination and FISH-labeled sections were imaged using fluorescence on a Nikon Eclipse Ni microscope. Photographs were taken with a color (Nikon DS-Fi2; for DIG-labeled) or monochrome (Nikon DS-Qi1Mc; for fluorescent-labeled) digital camera controlled by Nikon Elements software. Images were sharpened and adjusted for contrast, brightness, and levels as needed in Photoshop CS6 (Adobe Systems).

### Results

#### Sequence Analyses

In teleost fishes, the amino acid sequence of Crha is much more similar to Crhb than to Uts1 (Figure 1A). Zebrafish (*Danio rerio*) Crha and Crhb full-length peptide sequences share 45.1% pairwise identity, with 70.7% pairwise identity in the 41-amino-acid processed peptide region. Zebrafish Crha and Uts1, on the other hand, share only 29.2% pairwise identity of their full-length sequences and 56.1% pairwise identity in the 41-amino-acid processed peptide region.

Crha peptides have a higher degree of sequence variability across species than do Crhb peptides. The 7 Crha predicted 41-amino-acid peptide sequences we compared (Figure 1B) show on average 80.6% pairwise identity and 24 of 41 (58.5%) invariant amino acid residues. The 8 predicted Crhb processed 41-amino-acid peptide sequences we compared (Figure 1C) show on average 90.0% pairwise identity and 30 of 41 (73.2%) invariant amino acid residues.

The C-terminal region of the Crha peptides showed much greater sequence variability than the N-terminal portion. The 18 C-terminal amino acid residues have 64.8% average pairwise identity across the species examined, and only 6 of 18 (33.3%) are invariant across species. On the other hand, the 23 N-terminal amino acid residues have 93.0% average pairwise identity, and 18 of 23 (78.3%) are invariant across species.
Crhb, in contrast to Crha, had low sequence variability across species even in the C-terminal region. The 18 C-terminal amino acid residues have 85.3% average pairwise identity and 11 of 18 (61.1%) are invariant across species. In Crhb, the N-terminal 23 amino acid residues were less variable, with 93.3% average pairwise identity among species and 18 of 23 (78.3%) invariant residues across species.

No crha gene was identifiable in some teleost genomes. In particular, fugu (Takifugu rubripes) and pufferfish (Tetraodon nigroviridis) appear to have lost crha. A region of the tetraodon genome containing trim55a (Chromosome 15_random:114509–145684) did not contain an identifiable crha homolog. Similarly, BLAST searches of the tetraodon genome revealed no crha.

The Atlantic cod crhb gene is currently listed in Ensembl as a pseudogene (ENSGMOG00000002107), but Genscan predicts that it encodes a peptide with homology to Crhb. Further supporting its presence as a true gene, mRNA transcripts of this crhb gene in Atlantic cod have been identified from cDNA (GU828012.1).

**Synteny**

Teleost crha and crhb genes are found on chromosomes adjacent to similar genes (Figure 2). In tilapia (Oreochromis niloticus), medaka, and fugu, crhb genes are in regions of more highly conserved synteny compared to crha genes. For example, of 20 genes adjacent to tilapia crhb (10 upstream and 10 downstream), 17 have homologs among the group of 30 genes adjacent to spotted gar crh1 (15 upstream and 15 downstream). In contrast, only 3 of the 20 genes adjacent to tilapia crha have homologs among the group of 30 genes adjacent to spotted gar crh1.

![FIGURE 1](image1.png) FIGURE 1 | Two CRH genes, crha and crhb, are conserved across diverse teleost species. (A) Alignment of full-length translated CDS of zebrafish Crha, Crhb, and Uts1. Black, amino acids similar in all three sequences; Gray, similar to one corresponding residue; White, not similar to either corresponding residue. (B) Teleost Crha alignment. The predicted 41-amino-acid processed peptide sequences for Crha were aligned for seven teleost species. (C) Teleost Crhb alignment. The predicted 41-amino-acid processed peptide sequences for Crhb were aligned for eight teleost species, including fugu. Black, 100% of residues are similar; Dark Gray, 80–100% of residues are similar; Light Gray, 60–80% of residues are similar; White, less than 60% of residues are similar. Sequences were aligned using MAFFT software with the E-INS-i algorithm and the default settings. Amino acid similarity: Score Matrix, BLOSUM62; Threshold, 2.

![FIGURE 2](image2.png) FIGURE 2 | Teleost crha and crhb genes share synteny with spotted gar crh1. The central pentagons (surrounded by a vertical rectangle) represent CRH genes. Spotted gar crh1 is at the top center. Directly below are medaka, tilapia, fugu, and zebrafish crhb genes. Below these are medaka, tilapia, and zebrafish crha genes. For each CRH gene, 10 flanking genes on each side are represented by colored pentagons. Each color identifies a set of orthologous genes, and the pentagons point in the direction of transcription. Figure modified from Genomicus phyloview output.
Phylogenetics
Teleost Crhb and Crha sequences are more closely related to Crh1 than to Crh2 sequences from non-teleost fishes (Figure 3). Teleost Crhb protein sequences form a distinct clade (Figure 3). Teleost Crha protein sequences, on the other hand, are much more divergent in sequence and are recovered as a related grade in our tree. Predicted species relationships are reflected in the tree, as the spiny-rayed fishes (acanthomorpha), including cichlid, medaka, bicolor damselfish, fugu, and three-spined stickleback, form related groups for both Crha and Crhb. Sequences from the other fishes are more distantly related, as expected. Furthermore, as expected from the greater sequence variability of Crha, the branch length for Crha proteins is on average longer than for Crhb proteins.

crh Localization in the Retina
In the spotted gar retina, crh1-expressing cells were found exclusively in the inner nuclear layer (INL), near the border of the inner plexiform layer (IPL) (Figure 4B). Based on morphology and location, these are likely a type of amacrine cell. On the other hand, no crh2 mRNA was found in the spotted gar retina (Figure 4C).

Both teleost species we examined showed expression of crhb in the retina, but expression patterns differed between the zebrafish and the cichlid (Figure 4). In the zebrafish, there were abundant crhb-expressing cells in the INL region where amacrine cells are found along the IPL border (Figure 4E). There were also scattered labeled cells in the ganglion cell layer (GCL) of the zebrafish retina, but it is unknown whether these are ganglion cells or displaced amacrine cells. In the cichlid, there were similarly abundant crhb-expressing cells in the amacrine cell region of the INL, but there were also abundant crhb cells found deeper in the INL at the border of the OPL (Figure 4H). Based on their location and morphology, these crhb-expressing cells in the cichlid are likely bipolar cells. In contrast to the zebrafish, crhb was not observed in the GCL of the cichlid retina.

For crha, labeling was absent in the zebrafish retina (Figure 4F), but the cichlid retina contained scattered...
**FIGURE 4 | Localization of crh forms in the retina of fishes.** Photographs of the eye of each species and adjacent 20 µm transverse sections of the retina reacted with each crh anti-sense probe are shown. 
(A–C) The spotted gar *Lepisosteus oculatus* shows crh1 expression in the inner nuclear layer, but crh2 is absent from the retina. 
(D–F) The zebrafish *Danio rerio* expresses crhb in the amacrine cell region of the inner nuclear and in the ganglion cell layer (arrows), but crha is absent from the retina. 
(G–I) The African cichlid fish *Astatotilapia burtoni* shows crhb label in two different cell types within the inner nuclear layer, amacrine and bipolar cells (arrows). crha was also found in the *A. burtoni* retina, but was restricted to the region of amacrine cells in the inner nuclear layer. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PHL, photoreceptor layer; PE, pigmented epithelium. Lines on (A,D,G) indicate the approximate position of the sections. Scale bars = 1 mm (A,D,G); 25 µm (B,C,E,F,H,I).

crha-expressing cells in the amacrine cell region of the INL (Figures 4I, 5). These crha-expressing cells were less numerous than the crhb-expressing cells found in this same layer, and double fluorescent in situ hybridization showed that crha and crhb are not co-localized to the same cells (Figure 5).

**crh Localization in the Brain**

The complete localization of *crh1* and *crh2* in the spotted gar brain was described previously (Grone and Maruska, 2015). Briefly, in the spotted gar, *crh1* is widely distributed throughout the brain, including the ventral hypothalamus (Figure 6). In contrast, *crh2*-expressing cells are absent from the hypothalamus and in fact, are restricted to the secondary gustatory/visceral nucleus of the hindbrain.

Because the distribution of *crhb* in zebrafish and *A. burtoni* brains has been previously described (Alderman and Bernier, 2007, 2009; Chandrasekar et al., 2007; Chen and Fernald, 2008), our focus in the current report was to describe the expression patterns of *crha* in these teleosts. In the zebrafish, *crha*-expressing cells were stained strongly and specifically and were restricted to the lateral tuberal nucleus in the ventral hypothalamus (Figure 6). In *A. burtoni*, there was no *crha* labeling observed anywhere in the brain.

**Discussion**

Our results provide evidence that (1) teleosts have two distinct corticotropin-releasing hormone genes, *crha* and *crhb*, (2) these two genes evolved via a teleost-specific duplication of *crh1*, (3) the ancestral fish *crh1* gene paralog found in the spotted gar is expressed in both retina and brain, and (4) *crha* has been differentially restricted to either retina or brain in *Astatotilapia burtoni* and *Danio rerio*, respectively.
Phylogenetics

We examined a broad range of the available teleost sequences for *crha* and *crhb* genes. Our findings of conserved synteny and conserved sequence across teleost species argue strongly for an origin of the *crha* and *crhb* genes in the teleost-specific whole genome duplication (Figure 7). Our synteny and phylogenetic analyses combine to clearly show that *crha* genes in the species we examined are more similar to each other than to *crhb* genes, despite their higher levels of sequence variability. Our phylogenetic analyses were not able to restrict all Crha protein sequences into a well-defined clade, most likely because of the relatively poor sequence conservation of Crha compared to Crhb (Figure 1). Zebrafish, the only ostariophysan species in our tree, is distantly related to the other teleosts, which is reflected in the relatively large branch lengths of both zebrafish Crha and Crhb. Because Crha and Crhb protein sequences are short and highly variable, it is not surprising that phylogenetic relationships reconstructed using only these proteins do not perfectly match known teleost species relationships.

Our identification of *crh1* and *crh2* as ohnologs likely arising from the second whole-genome duplication (WG2) suggested that Urotensin 1 (*uts1*) and a proto-CRH gene originated in the first whole-genome duplication (WG1) (Grone and Maruska, 2015), overturning the previously established assumption that *uts1* and *crh* did not diverge until WG2 (Lovejoy and de Lannoy, 2013). Similarly, the elucidation of teleost *crha* and *crhb* clarifies some previous misconceptions about the evolutionary origins of teleost *crh* genes. Some teleost lineages, including salmoniformes and cypriniformes such as carps, have experienced additional genome duplications subsequent to the third one (WG3) common to all teleosts (Ohno et al., 1968; David et al., 2003; Braasch and Postlethwait, 2012). Many of these teleosts, including salmonid and carp species, have duplicated *crhb* genes (Flik et al., 2006), which were sometimes misunderstood to have arisen in the teleost whole-genome duplication (Alsop and Vijayan, 2009). The very high degree of sequence identity between these duplicated *crhb* genes now appears less surprising, as it is apparent that their origins lie in the more recent lineage-specific genome duplications rather than WG3.

In a recent paper attempting phylogenetic analyses of CRH genes in teleosts, the diverse sequences of *crha* and *crhb* genes in teleosts led to misidentification of medaka *crha* as a separate gene distinct from both *crha* and *crhb* (Hosono et al., 2015). Although Hosono et al. mention that *crha* had been identified in zebrafish, they did not include zebrafish *crha* and *crhb* in their phylogeny. Hosono et al. also did not take into account the reduplication of CRH genes in salmonids. Our more detailed look at duplicated CRH genes in fishes has revealed that medaka *crha* is homologous to zebrafish and other teleost *crha* genes (see Figures 1–3 of the current report, and Grone and Maruska, 2015). The available data suggest that two teleost CRH genes, *crha* and *crhb*, exist in many teleosts but have been differentially lost or reduplicated in some teleost lineages. A separate teleost CRH gene, which Hosono et al. called "teleocortin," does not exist.

Interestingly, Hosono et al. found that the predicted medaka *crha* peptide was a potent *in vitro* activator of both CRH receptors (Hosono et al., 2015). They reported high levels of expression of medaka *crha* mRNA in the retina, and *crha* mRNA was also detected in several other tissues, including the brain. Their data support the idea that *crha* may have different functions across teleost species.

Analyses of fossil records and phylogenetic evidence point to an origin for teleosts about 300 million years ago, during the Carboniferous to early Permian geological era (Near et al., 2012). Following the teleost WGD, many genes including neuropeptides were retained in two copies in teleosts, and have evolved differential expression (Glasauer and Neuhauss, 2014). For
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Two CRH genes in teleosts

FIGURE 6 | Localization of **crh** forms in the hypothalamus of fishes. (A) Representative transverse section of the spotted gar (**Lepisosteus oculatus**) brain stained with cresyl violet, with the approximate region of interest indicated (black box). (B) **crh1**-expressing cells were abundant in the ventral hypothalamus (HV) of the gar, in the region above the pituitary (pit). (C) **crh2** expression was absent from the hypothalamus of the spotted gar. (D) Representative transverse section from the zebrafish (**Danio rerio**) brain atlas (Wullimann et al., 1996), with the region of interest indicated (black box). (E) Zebrafish **crhb**-expressing cells were found in the nucleus lateralis tuberis (NLT) (labeled ventral hypothalamus, HV, in atlas), but were also widely distributed throughout the brain. (F) Zebrafish **crha** expression was found exclusively in the NLT, and nowhere else in the brain. (G) Representative transverse section of the cichlid (**Astatotilapia burtoni**) brain stained with cresyl violet, with the approximate region of interest indicated (black box). (H) **crhb**-expressing cells were abundant in the NLT region above the pituitary gland of the cichlid. (I) **crha** expression was not detectable in the cichlid brain. Insets on (A, D, G) show lateral view of the brain with approximate location of transverse sections indicated (line). For each species, panels are adjacent sections labeled with different anti-sense **crh** probes. HV, ventral zone of the periventricular hypothalamus; LH, lateral hypothalamic nucleus; NLTm, medial part of lateral tuberal nucleus; NLTv, ventral part of lateral tuberal nucleus; pit, pituitary; T, tectum. Scale bars = 100 µm (B, C); 25 µm (E, F, H, I).

example the two ohnologs of pro-opiomelanocortin (**pomca** and **pomcb**), which encode adrenocorticotropic hormone (ACTH) as well as other peptides, were differentially restricted to preoptic area and pituitary, respectively, in *Tetraodon nigroviridis* (de Souza et al., 2005).

The potential for a wide range of outcomes for genes following WGD has been supported by theoretical considerations as well as genome-wide studies in fishes (Brunet et al., 2006; Kassahn et al., 2009). Following gene duplications, including those caused by whole-genome duplication, genes may experience non-functionalization, sub-functionalization, or neo-functionalization. Sub-functionalization may happen via protein changes (Hughes, 1994) or via regulatory element loss (Force et al., 1999) as described by the “duplication-degeneration-complementation” model, in which ancestral expression domains are differentially lost in different genes (Lynch and Force, 2000). Rapid sub-functionalization may be accompanied by prolonged neo-functionalization, as described by the “subneo-functionalization” model (He and Zhang, 2005). Teleost fish genomes exhibit diverse outcomes following whole genome duplication, as seen, for example, in *pax6* genes (Nornes et al., 1998), *mitf* genes (Lister et al., 2001), homeobox genes (Mungpakdee et al., 2008), and circadian clock genes (Toloza-Villalobos et al., 2015).

It is intriguing that **crh2** has been lost in both teleosts and eutherian mammals, two groups with very successful adaptive radiations in very different environments (Hoegg et al., 2004; Bininda-Emonds et al., 2007). Although we found **crh2** mRNA in the spotted gar hindbrain, it is not clear what the expression pattern (or functions) of **crh2** in a hypothetical teleost ancestor would have been. One possibility is that, early in teleost evolution, the presence of **crha** and **crhb** provided sufficient opportunity for multiple specialized functions of the CRH gene family, which could have allowed **crh2** to be lost.
CRH in the Brain

Many immunohistochemical studies in teleost brains have found CRH-like immunoreactivity that likely corresponds largely to CRH1 peptides (e.g., Batten et al., 1990; Zupanc et al., 1999; Pepels et al., 2002). Teleost CRH1 genes are broadly expressed in the brain. Expression of zebrafish CRH1 in the brain has been documented via in situ hybridization in adults (Alderman and Bernier, 2007) and larvae (Chandrasekar et al., 2007). In A. burtoni, CRH1 is also expressed in many brain regions, including hypothalamus (Chen and Fernald, 2008; Carpenter and Bernier, 2007) and larvae (Chandrasekar et al., 2007). Lesions in the goldfish NLT impaired corticosteroid release in response to stressors (Fryer and Peter, 1977), suggesting a key role for NLT in controlling pituitary adrenocorticotropic hormone (ACTH) release.

Zebrafish Hv or NLT is considered homologous to the mammalian arcuate nucleus (Song et al., 2003; Kurrasch et al., 2009). This region has important roles in nutrient metabolism, feeding, and neuroendocrine regulation. Agouti-related peptide (AGRP) and pro-opiomelanocortin (POMC) mRNA transcripts are found in zebrafish Hv neurons that project to the pituitary (Zhang et al., 2012). The connectivity and molecular identity of the crha-expressing neurons in zebrafish are unknown. Given the known importance of CRH peptides and the Hv nucleus in controlling food intake in fishes, crha neurons in the zebrafish Hv could be involved in regulation of feeding behavior as well as locomotor activity, anxiety, stress-coping, and other behaviors (Backström and Winberg, 2013; Matsuda, 2013).

Conclusions

Powerful genomic resources for diverse fishes, including many teleosts as well as spotted gar, allowed us to examine evolution of ohnologs that arose from whole-genome duplications. Because many neuroanatomical structures, including retina and brain nuclei, are highly conserved, we were also able to compare the diversity of expression domains with precision. Our results highlight the fact that retention of a given ohnolog in multiple lineages does not demonstrate retention of the same function. Different lineages may experience differential regulatory element loss or gain as well as differential gene loss. Studying teleost
crha and crhb genes will deepen our understanding of evolution and function of vertebrate neuropeptide signaling systems. We anticipate that interesting future comparisons will be made between the restricted roles of crh2 in non-teleost vertebrates and crha in teleosts. By examining the restricted or novel functions of duplicated CRH genes, we will come to a greater understanding of CRH neuron development, anatomy, activity, and function. Furthermore, the restriction of crha to the retina in a highly visual species (A. burtonii) but not in another teleost (D. rerio) suggests a more general model in which duplicated neuropeptide genes may evolve expression domains that reflect the different ecological and behavioral specializations of different species.

The two teleost species we examined had divergent expression profiles for crha. Teleost superorders Ostiophysi (including zebrafish) and Acanthopterygii (including cichlids, medaka, sticklebacks, and pufferfish) diverged early in teleost evolution. Ostiophysi and Acanthopterygii have lost different ohnologs for many genes (Garcia de la Serrana et al., 2014). Our data are not sufficient to show at what point in evolutionary history the expression of crha was restricted to the retina in cichlids and to the hypothalamus in zebrafish. Furthermore, it is possible that crha may have additional or complementary expression domains in other teleosts. Future comparative work will be necessary to reveal the functional diversity of crha and crhb genes.

Author Contributions

Both authors had full access to all of the data in the study and take responsibility for its collection and analysis. Study concept and design: BG, KM. Acquisition of data: BG, KM. Analysis and interpretation of data: BG, KM. Drafting of the manuscript: BG, KM. Critical revision of the manuscript for important intellectual content: KM, BG. Obtained funding: KM. Administrative, technical, and material support: KM.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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