A Second Corticotropin-Releasing Hormone Gene (*CRH2*) Is Conserved Across Vertebrate Classes and Expressed in the Hindbrain of a Basal Neopterygian Fish, the Spotted Gar (*Lepisosteus oculatus*)

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ABSTRACT

To investigate the origins of the vertebrate stress-response system, we searched sequenced vertebrate genomes for genes resembling corticotropin-releasing hormone (*CRH*). We found that vertebrate genomes possess, in addition to *CRH*, another gene that resembles *CRH* in sequence and syntenic environment. This paralogous gene was previously identified only in the elephant shark (a holocephalan), but we find it also in marsupials, monotremes, lizards, turtles, birds, and fishes. We examined the relationship of this second vertebrate *CRH* gene, which we name *CRH2*, to *CRH1* (previously known as *CRH*) and *urocortin1/urotensin1* (*UCN1/UTS1*) in primitive fishes, teleosts, and tetrapods. The paralogs *CRH1* and *CRH2* likely evolved via duplication of *CRH* during a whole-genome duplication early in the vertebrate lineage. *CRH2* was subsequently lost in both teleost fishes and eutherian mammals but retained in other lineages. To determine where *CRH2* is expressed relative to *CRH1* and *UTS1*, we used in situ hybridization on brain tissue from spotted gar (*Lepisosteus oculatus*), a neopterygian fish closely related to teleosts. In situ hybridization revealed widespread distribution of both *crh1* and *uts1* in the brain. Expression of *crh2* was restricted to the putative secondary gustatory/secondary visceral nucleus, which also expressed *calcitonin-related polypeptide alpha* (*calca*), a marker of parabrachial nucleus in mammals. Thus, the evolutionary history of *CRH2* includes restricted expression in the brain, sequence changes, and gene loss, likely reflecting release of selective constraints following whole-genome duplication. The discovery of *CRH2* opens many new possibilities for understanding the diverse functions of the CRH family of peptides across vertebrates. J. Comp. Neurol. 523:1125–1143, 2015.

INDEXING TERMS: neuropeptides; evolution; genome duplication; fishes; pons; parabrachial nucleus

Corticotropin-releasing hormone (CRH), also known as corticotropin-releasing factor (CRF), a neuropeptide found in all vertebrates, plays a central role in regulating physiological and behavioral responses to stressors (Vale et al., 1981; Korosi and Baram, 2008). Vertebrate CRH is homologous to invertebrate diuretic hormone (Kataoka et al., 1989), suggesting an ancient origin of these peptides before 500 million years ago, when ancestors of vertebrates and insects diverged (Lovejoy and Barsyte-Lovejoy, 2010). In most vertebrate species, urotensin 1 (*UTS1*), referred to as urocortin 1 (UCN1) in mammalian species, is the paralogous gene thought to be most closely related to the *CRH* gene. *UTS1* shares with *CRH* a simple two-exon structure, a 41-amino-acid processed peptide, and important roles in stress regulation (Vaughan et al., 1995). The peptide UTS1 also has potent corticotropin-releasing activity (Lederis et al., 1982). Because of its sequence and functional similarity to CRH, UTS1 is regarded as the closest ortholog of CRH in vertebrates.

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Recently, two predicted *crh* genes were identified in the elephant shark (*Callorhinchus milii*) genome (Nock et al., 2011). Because only one *CRH* gene has been identified in other vertebrate genomes, these duplicated *crh* genes were proposed to have arisen by gene or genome duplication in the elephant shark lineage more recently than the second round of vertebrate whole-genome duplication (Lovejoy and de Lannoy, 2013). To test this hypothesis and increase our understanding of vertebrate *CRH* evolution, we asked whether homologs of *crh2* as well as *crh1* are present in genomes of other vertebrates, including fishes and tetrapods.

Many gene families in vertebrates were generated during multiple rounds of whole-genome duplication that occurred early in vertebrate evolution (Ohno, 1970; Abi-Rached et al., 2002; Dehal and Boore, 2005). Recently, genome sequences have become available for several basal vertebrates, including spotted gar (*Lepisosteus oculatus*; Amores et al., 2011; Flicek et al., 2014). Although spotted gar (Neopterygii; superorder Ginglymodi) is closely related to teleost fishes (Neopterygii; superorder Teleostei), the chromosomal organization of its genome more closely resembles that of humans. Neopterygian fishes, including gars, lack the teleost-specific genome duplication that the teleost lineage experienced prior to its massive ecological and evolutionary radiation (Christoffels et al., 2004; Hoegg et al., 2004; Jaillon et al., 2004; Amores et al., 2011). The evolutionary position of gars as a sister group to teleosts was proposed based on anatomical data but has remained controversial. Recent genomic studies using multiple gene sequences or ultraconserved elements have confirmed that the infraclass Holostei [orders Lepisosteiformes and Amiiformes; i.e., gars (*Lepisosteus* and *Atractosteus* spp.) and the bowfin (*Amia calva*)] forms a monophyletic group that is an outgroup to the teleosts (Broughton et al., 2013). There are only seven extant species of gar (and one bowfin) compared with over 30,000 extant teleosts. Despite this paucity of extant species, Lepisosteiformes are a hardy group with a fossil record that stretches back over 100 million years to the Albian age (Gayet et al., 2002). Thus, with their ecological and evolutionary proximity to teleosts and the recently sequenced genome of the spotted gar (*L. oculatus*), gars are a particularly valuable group for understanding mechanisms of vertebrate molecular evolution.

The brain anatomy of the spotted gar has been characterized in relatively few histological and immunohistochemical studies. Some spotted gar neuropeptide expression patterns, including neuropeptide Y and gonadotropin-releasing hormone, have been described via immunohistochemistry (Chiba, 2005). Other gar species in the genus *Lepisosteus*, however, have received more attention from neuroanatomists, who have generated descriptions that are useful for obtaining a comparative understanding of gar neuroanatomy (Källen, 1950; Bradshaw et al., 1969; Northcutt and Butler, 1976, 1980, 1993; Platel et al., 1977; Parent and Northcutt, 1982; Collin and Northcutt, 1995; Chiba and Oka, 1999). The recent sequencing of the spotted gar genome has also opened the door for analysis of specific mRNA expression by in situ hybridization (ISH). Thus, the taxonomic position and available neuroanatomical and genomic resources for gars allow us to localize conserved genes in the spotted gar brain to further our understanding of the evolution and function of vertebrate neuropeptide signaling systems. In the present study, we first employed comparative genomic and phylogenetic analyses to identify the evolutionary relationships of the CRH family of genes: *crh1*, *uts1*, and the newly identified *crh2*. We next used chromogenic ISH in the brain of the spotted gar to characterize and analyze expression patterns of these genes. Our analysis reveals new insights into the evolution of the CRH family, with potential functional implications for stress-regulating systems across vertebrates.

MATERIALS AND METHODS

Animals

Juvenile spotted gar (sexually immature) were purchased from Rainforest International (53–85 mm standard length [SL]) or caught from the Atchafalaya Basin, Louisiana (24–26 cm SL). In total nine spotted gar were used for the in situ hybridization (ISH) experiments. Animal experiments were performed according to regulations established by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University.

Sequence analysis

Throughout this article, we use standard gene nomenclature. For fishes, gene names and symbols are italicized and protein names 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 vertebrate CRH gene family members, *CRH1*, *CRH2*, and *UTS1/UCN1* genes were located by the following searches: TBLASTN searches in Pre-Ensembl for spotted gar and painted turtle, TBLASTN searches of the elephant shark (*Callorhinchus milii*) genome database (Venkatesh et al., 2014), GenBank (RRID:nif-0000–02873) for fruitfly (*Drosophila melanogaster*), peregrine falcon (*Falco peregrinus*; Zhan et al., 2013), Burton's

TABLE 1.

Sources for Nonteleost CRH Family Genes Described in This Article[1]

Species	*CRH1 (or DHLP)*	*CRH2*	*UTS1/UCN1*
Anolis carolinensis	ENSACAG00000016674	Chr4:156090387–156090806	
Callorhinchus milii	scaffold_48:2540395–2540874	JX061316.1	scaffold_124:1277589–1277948
Chrysemys picta bellii	JH584532.1:1218763–1219284	JH584590.1:1252468–1252896	
Drosophila melanogaster	AAF54421.4		
Falco peregrinus	XM_005240928	XM_005239946	
Homo sapiens	ENSG00000147571		ENSG00000163794
Latimeria chalumnae	ENSLACG00000002627	JH129487.1:64083–64421	ENSLACG00000009866
Lepisosteus oculatus	ENSLOCG00000017813	LG18:10117226–10117678	ENSLOCG00000017992
Mus musculus	ENSMUSG00000049796		ENSMUSG00000038676
Ornithorhynchus anatinus	ENSOANG00000002306	Ultra337:3053769–3054077	
Sarcophilus harrisii	GL841248.1:1200305–1200907	GL834659.1:2576366–2576701	GL856719.1:1191900–1192289
Taeniopygia guttata	ENSTGUG00000011277	Chr20:8814638–8815027	
Xenopus laevis	crh-a: NM_001172210 crh-b: NM_001172209		NM_001092960

[1]Gene sequences from Ensembl are indicated by the Ensembl identification numbers. For predicted genes that have not been annotated in Ensembl, the location of the gene CDS on a chromosome (or contig for *Callorhinchus milii*) is indicated with the form "chromosome:1st codon position-stop codon position." Where sequences are not present in a genome, that cell in the table has been left blank.

mouthbrooder (*Astatotilapia burtoni*), and medaka (*Oryzias latipes*) sequences, and TBLASTN searches in Ensembl (RRID:nif-0000–21145) for all other species (Tables 1, 2). In Tables 1 and 2, Ensembl identification numbers or GenBank accession numbers are listed when available; otherwise, the position on a chromosome, contig, or scaffold is given. *CRH* open reading frames (ORFs) were predicted from genomic DNA using the GENSCAN web server at MIT (RRID:OMICS_01494; Burge and Karlin, 1997). For comparisons of spotted gar *crh1*, *crh2*, and *uts1*, multiple sequence alignment was carried out using MAFFT (RRID:OMICS_00979; Katoh and Standley, 2013) in Geneious software version 5.1.7 (Biomatters), with the following settings: algorithm = E-INS-i; scoring matrix = BLOSUM62; gap-open penalty = 1.53.

To create a phylogenetic tree of CRH family members, CRH predicted amino acid sequences were generated from ORFs and aligned in Geneious using MAFFT with the following settings: algorithm = E-INS-i; scoring matrix = BLOSUM62; gap-open penalty = 3. A neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was

Abbreviations

A	anterior thalamic nucleus	PGZ	periventricular gray zone of tectum
AC	anterior commissure	Pit	pituitary gland
aCb	cerebellar auricle	PMm	magnocellular part of the magnocellular preoptic area
CC	cerebellar crest	POA	preoptic area
Ce	cerebellum	PPa	anterior part of the parvocellular preoptic area
CG	central gray	PPp	posterior part of the parvocellular preoptic area
CP	central posterior nucleus	PPv	ventral part of the periventricular pretectal nucleus
Dd	dorsal part of the dorsal telencepalon	PVO	paraventricular organ
Dl	lateral part of the dorsal telencephalon	Ri	inferior reticular formation
Dm	medial part of the dorsal telencephalon	Rs	superior reticular formation
DON	descending octaval nucleus	SC	suprachiasmatic nucleus
Dp	posterior part of the dorsal telencephalon	SGn	secondary gustatory nucleus
DP	dorsal posterior nucleus	smn	spinal motor neurons
DTN	dorsal tegmental nucleus	SR	superior raphe nucleus
ECL	external cell layer of olfactory bulb	SVn	secondary visceral nucleus
FR	fasciculus retroflexus	Te	tectum
GL	glomerular layer of olfactory bulb	Tel	telencephalon
Hd	dorsal zone of the periventricular hypothalamus	Teg	tegmentum
Hv	ventral zone of the periventricular hypothalamus	TL	torus longitudinalis
HYP	hypothalamus	TLa	torus lateralis
ICL	internal cell layer of olfactory bulb	TP	posterior tuberal nucleus
IL	inferior lobe of the hypothalamus	TPp	periventricular nucleus of posterior tuberculum
IP	interpeduncular nucleus	TS	torus semicircularis
IR	inferior raphe nucleus	V	ventricle
LC	locus coeruleus	Va	cerebellar valvula
LR	lateral recess	Vd	dorsal nucleus of the ventral telencephalon
LT	lateral tegmental nucleus	Vs	supracommissural nucleus of the ventral telencephalon
mlf	medial longitudinal fasciculus	Vv	ventral nucleus of the ventral telencephalon
MON	medial octaval nucleus	VL	vagal lobe
nMLF	nucleus of medial longitudinal fasciculus	Vm	trigeminal motor nucleus
OC	optic chiasm	VM	ventromedial thalamic nucleus
OpT	optic tract	Xm	vagal motor nucleus
PC	posterior commissure	VIIm	facial motor nucleus

TABLE 2.
Sources for Teleost CRH Family Genes Described in This Article

Teleost species	crha	crhb	uts1
Astatotilapia burtoni	XM_005946397.1	EF363131	XM_005949377
Danio rerio	ENSDARG00000093401	ENSDARG00000027657	ENSDARG00000014927
Gasterosteus aculeatus	scaffold_120: 91757–91374	ENSGACG00000002971	ENSGACG00000006113
Oryzias latipes	XM_004078828.1	AB070613	AB196781.1

TABLE 3.
Primer Binding Site Sequences Used To Generate Templates for the Synthesis of Riboprobes

Gene	Forward primer[1]	Reverse primer[1]	Probe length (bp)
crh1	AGCTCAATTTCCTTGTTTCTACTGC	CATCATTTTTCTGTTGCTGTGCGC	479
crh2	GCCATGAGCAAGTTGCTTCTCCTG	CAGCTTTAGCCATTTCTAGGAACTC	400
uts1	GCCACCATACTCCTCCTATCACAC	CAACTATCACTTGCCAACCTCATC	462
calca	CGTTACAGCACAGAAACGAGCCTGC	TCTCCTTCGCCTCCCAAATGCT	145

[1]T3 transcription initiation sequence (aattaaccctcactaaaggg) was added to the reverse primer (for antisense probes) or forward primer (for sense control probes).

created from the protein alignment in Geneious using a Jukes-Cantor genetic distance model and checked via 1,000 bootstrap iterations. *Drosophila melanogaster* diuretic hormone was set as the outgroup (Cabrero et al., 2002). The tree displays consensus support percentages from bootstrap values. *CRH* gene synteny in humans and zebrafish was compared by using the Synteny database (Catchen et al., 2009) and Ensembl.

In situ hybridization

To localize mRNAs of *crh1*, *crh2*, *uts1*, and *calca* in the brain of the spotted gar, we performed chromogen-based in situ hybridization with riboprobes on cryosectioned tissue. Templates for riboprobes were generated by PCR amplification (Platinum PCR SuperMix; Life Technologies, Grand Island, NY) of spotted gar genomic DNA using commercially synthesized (Life Technologies) gene-specific primers (Table 3) and the following reaction conditions: 95 C for 1 minute; 40 cycles of 95 C for 15 seconds, 55 C for 15 seconds, 72 C for 1 minute; and 72 C for 1 minute. Purified PCR products (MinElute PCR Purification Kit; Qiagen, Valencia, CA) were then used as the template for the transcription reaction. Probes were designed to be complementary to the longest exon for each gene and were transcribed from the T3 polymerase transcription initiation sequence (aattaaccctcactaaaggg) that was added to the reverse (for anti-sense probes) or forward (for sense control probes) template primer. Digoxigenin-labeled antisense and sense riboprobes for *Lepisosteus oculatus crh1, crh2, uts1*, and *calca* were transcribed in vitro by using T3 RNA Polymerase (Fermentas) and DIG RNA labeling mix (Roche Molecular Biochemicals, Indianapolis, IN), with RNase Out (Life Technologies), and were then treated with Turbo DNAse (Ambion Inc., Austin, TX) and purified with illustra ProbeQuant G-50 Micro Columns (GE Healthcare, Fairfield, CT). 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 (for probe sizes see Table 3).

Juvenile spotted gars were anesthetized in 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 were then removed from the head and cryoprotected in 30% sucrose at 4 C for 2–3 days until sectioning.

Spotted gar brains were mounted in Tissue-Tek OCT (Sakura) and sectioned at 20 μm in the transverse plane with a cryostat (Leica CM1850). Sections from each brain were collected onto three sets of alternate charged slides (VWR Superfrost Plus), dried at room temperature overnight, and then stored at −80 C prior to use in ISH. With an ImmEdge hydrophobic barrier pen (Vector Laboratories, Burlingame, CA), a hydrophobic barrier was drawn on the edges of each slide and allowed to dry at room temperature (30 minutes). Sections were rehydrated with 3 × 5-minute washes in PBS, fixed with 4% PFA for 20 minutes at room temperature, washed for 2 × 5 minutes with PBS, and permeabilized with proteinase K (10 μg/ml) for 15 minutes at room temperature. Sections were then washed for 1 × 5 minutes with PBS at room temperature, postfixed with 4% PFA for 15 minutes at room temperature, and

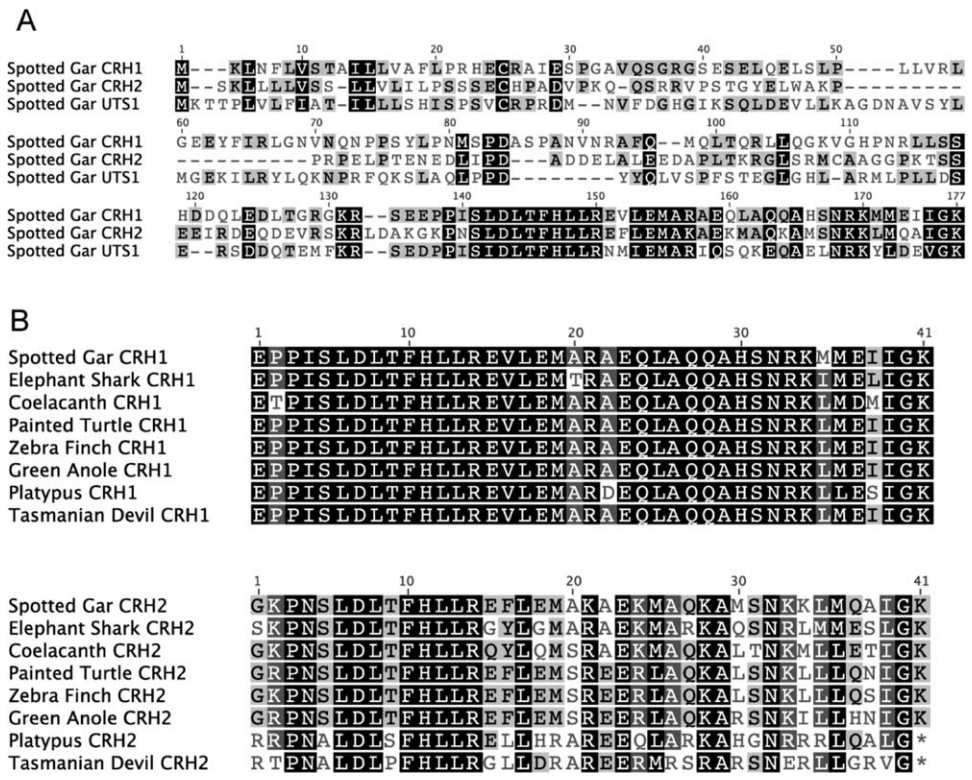

Figure 1. CRH1 and CRH2 sequences are conserved across vertebrate evolution. We aligned both full-length translated peptide sequences and predicted processed peptide sequences in MAFFT software with the EINS-i algorithm and the default settings. Amino acid similarity was determined as follows: score matrix = BLOSUM62; threshold = 2. **A:** Alignment of full-length translated CDS of spotted gar CRH1, CRH2, and UTS1. Black, amino acids similar in all three sequences; gray, similar to one corresponding residue; white, not similar to either corresponding residue. **B:** Vertebrate CRH1 and CRH2 alignments. The predicted 41-amino-acid processed peptide sequences for CRH1 and CRH2 were aligned separately for seven vertebrate species that possess both genes. Black, 100% of residues are similar across species; dark gray, 80–100% of residues are similar; light gray, 60–80% of residues are similar; white, less than 60% of residues are similar. *Stop codon.

washed with 2 × 5-minute 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. After acetic anhydride treatment, sections were washed for 1 × 5 minutes with PBS at room temperature and then incubated in prewarmed hybridization buffer in a sealed, humidified chamber to prehybridize at 60–65 C for 3 hours. When prehybridization 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 (*crh1*, n = 4; *crh2*, n = 5; *uts1*, n = 4; *calca*, n = 2). 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 hours) at 60–65 C in sealed, humidified chambers. Sections were then washed for 2 × 30 minutes at 60–65 C in prewarmed 2× SSC:formamide with 0.1% Tween-20, for 2 × 15 minutes at 60–65 C in a 1:1 mixture of 2× SSC and maleic acid buffer with 0.1% Tween-20 (MABT), and for 2 × 10 minutes at 60–65 C with MABT. Slides were then washed for 2 × 10 minutes with MABT at room temperature, and nonspecific binding was blocked by incubation in MABT with 2% bovine serum albumin (BSA) for 3 hours at room temperature. Slides were then incubated with alkaline-phosphatase-conjugated anti-DIG Fab fragments (Roche; RRID:AB_514497) diluted 1:5,000 in MABT-2% BSA overnight at 4 C in a sealed, humidified chamber. After antibody incubation, slides were washed with MABT (3 × 30 minutes) at room temperature, rinsed for 2 × 5 minutes in alkaline phosphatase buffer at room temperature, and then developed with nitroblue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP) substrate (Roche) at 37 C in the dark for 1–3 hours. Slides were then washed in PBS at room temperature to stop the reaction (3 × 5 minutes), fixed in 4% PFA for 10 minutes, washed in PBS (3 × 5 minutes), and coverslipped with Aqua-Mount media (Thermo Scientific).

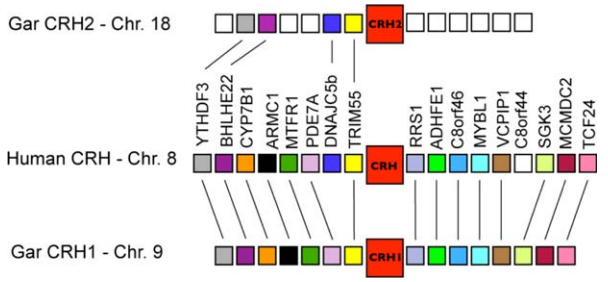

Figure 2. Spotted gar *crh2* shares conserved synteny with human CRH and spotted gar *crh1*. Protein-coding genes are depicted as boxes placed along a line in the same order in which they are arranged on the corresponding chromosome. On the middle line, the genes immediately adjacent to human CRH on chromosome 8, including eight genes more proximal to the centromere than CRH and nine more distal genes, are depicted as color-coded squares adjacent to the central square representing CRH. Filled squares represent genes with homologs on either spotted gar chromosome 18 (near *crh2*), spotted gar chromosome 9 (near *crh1*), or both. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

To test for probe specificity, sense control probes were applied to one set of alternate sections (transverse 25 µm) for each gene and run simultaneously with antisense probes applied to another set of alternate slides. None of these sense controls showed any labeling. To facilitate identification of neuroanatomical structures in the gar brain, we also stained one set of alternate slides with cresyl violet acetate (0.1%; Acros Organics; Thermo Scientific), followed by dehydration in an ethanol series (50–100%), clearing in xylene, and coverslipping with Cytoseal 60 (Thermo Scientific).

Imaging and analysis

To map the expression patterns of genes in the spotted gar brain, slides were visualized on a Nikon Eclipse Ni microscope and photographs taken with a color digital camera (Nikon DS-Fi2) controlled by Nikon Elements software. Stained sections were viewed in brightfield and phase-contrast or darkfield illumination to facilitate visualization of neuroanatomical landmarks and brain nuclei in relation to DIG-labeled cells. Images were sharpened and adjusted for contrast, brightness, and levels as needed in Photoshop CS6 (Adobe Systems). Distribution maps were created in Photoshop by tracing the outline of the left side of representative transverse sections along with relevant neuroanatomical structures, duplicating it and creating a mirror image to represent a full transverse section, and then marking the cell locations for each gene as a consensus determined from all individuals. The following neuroanatomical articles were used to identify and label regions of the gar brain: Northcutt and Butler (1980, 1993), Northcutt (1982), Parent and Northcutt (1982), Braford and Northcutt (1983), Chiba (2005), and Morona et al. (2013).

RESULTS

Sequence analysis

A search of the spotted gar (*Lepisosteus oculatus*) Pre-Ensembl genome database revealed an unannotated ORF sequence with homology to CRH. This newly identified gene shows homology with the recently described *crh2* gene from elephant shark, *Callorhinchus milii*, and throughout this article we refer to this gene as *crh2*. Accordingly, we refer to the previously described *crh* gene in *L. oculatus* and other vertebrates as *crh1*. As in other CRH genes, the full coding sequence of *L. oculatus crh2* is contained in a single exon. The *L. oculatus crh2* sequence is located on linkage group 18 and contains an ORF spanning bases 10,117,226-10,117,678 that encodes a predicted 151-amino-acid Crh2 protein sequence. To characterize this amino acid sequence, we aligned it with and compared with to the sequences of spotted gar Crh1 and urotensin 1 (Uts1; Fig. 1A). Across the full length of the predicted peptides, Crh2 shares 33.3% pairwise identity with Crh1 but only 24.3% pairwise identity with Uts1. In the C-terminal 41-amino-acid region containing the predicted processed peptides, Crh2 is 68.3% identical to Crh1 and 43.9% identical to Uts1.

To determine whether orthologs of this spotted gar *crh2* gene are also found in other taxa, the sequence was used to query other genome databases, including Ensembl vertebrate genome databases. Uncharacterized CRH gene homologs were found in several species, including green anole (*Anolis carolinensis*), painted turtle (*Chrysemys picta bellii*), zebra finch (*Taeniopygia guttata*), Tasmanian devil (*Sarcophilus harisii*), coelacanth (*Latimeria chalumnae*), elephant shark (*Callorhinchus milii*), and platypus (*Ornithorhynchus anatinus*). The average pairwise identity among all eight CRH2 predicted amino acid sequences in Figure 1B is 63.9%, with 15 residues (36.6%) invariant among all eight sequences.

The CRH1 sequences, in contrast, are more highly conserved across vertebrate classes and species. The average pairwise identity among all eight CRH1 predicted amino acid sequences in Figure 1B is 94.3%, with 34 residues (82.9%) invariant among all eight sequences. Several tetrapod species share perfect identity of CRH1 sequence. These species include green anole (*Anolis carolinensis*), painted turtle (*Chrysemys picta bellii*), zebra finch (*Taeniopygia guttata*), and

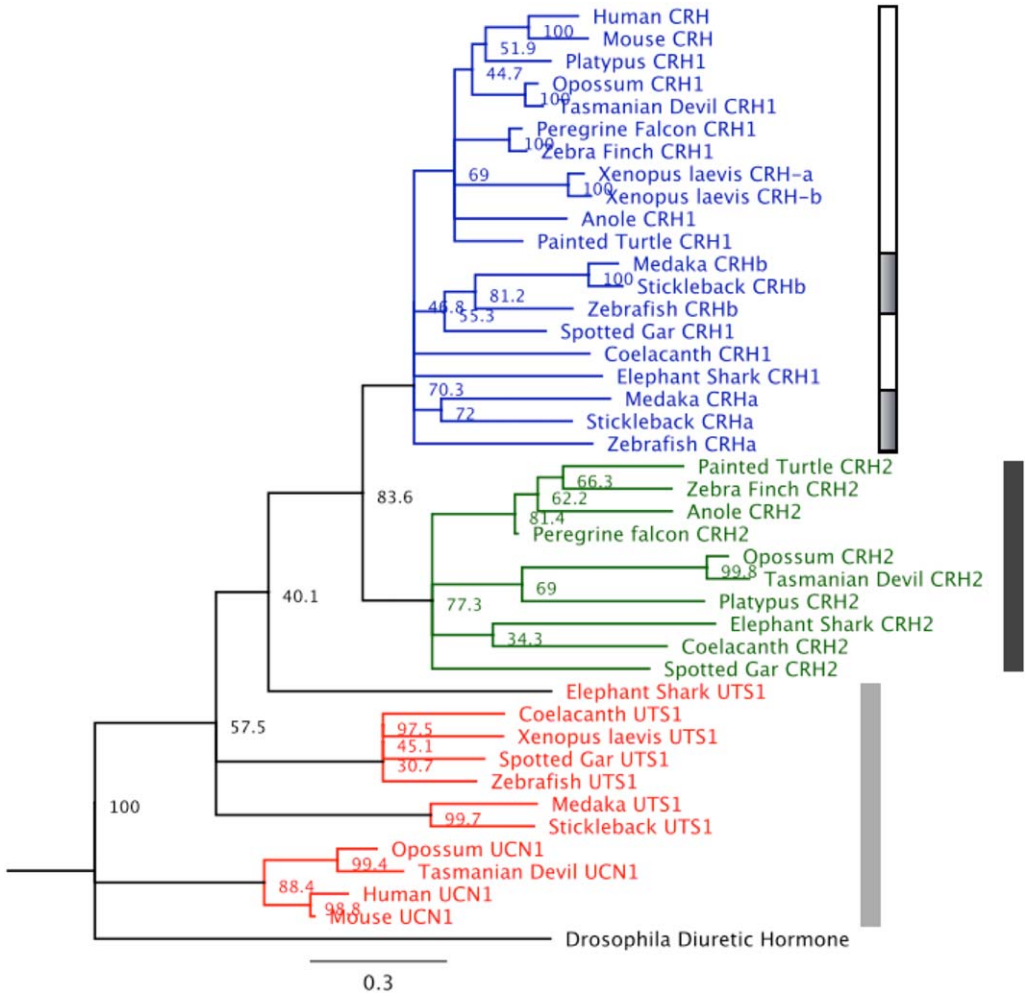

Figure 3. Phylogenetic tree of CRH family neuropeptides in vertebrates, based on predicted protein sequences, including newly described CRH2, as well as teleost Crha and Crhb. The tree reveals three major groups of CRH family genes. The top group of genes (white vertical bar) forms the CRH1 cluster, which includes the two CRH1 genes, crha and crhb, found in teleost fishes (gray shaded bars). The middle group of genes (black vertical bar) forms the CRH2 cluster, with representatives of several vertebrate classes but no eutherian mammals or teleost fish. The bottom group of genes (gray vertical bar) forms the UTS1 cluster, which includes UTS1/UCN1 genes from all classes of vertebrates. Drosophila melanogaster diuretic hormone was used as an outgroup. Protein sequences translated from the ORFs of each gene were aligned in MAFFT software (for details see Materrials and Methods). For each species, all available forms of CRH family peptides were included in the tree. Nodes are labeled with percentage bootstrap support after 1,000 bootstrap replicates. Branch length is proportional to number of substitutions per site, and the scale bar indicates length of 0.3 substitutions/site. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Tasmanian devil (*Sarcophilus harisii*; Fig. 1B), as well as human (*Homo sapiens*; sequence not shown). On the other hand, CRH1 amino acid sequences from coelacanth (*Latimeria chalumnae*), elephant shark (*Callorhinchus milii*), and platypus (*Ornithorhynchus anatinus*) all have nearly, but not exactly, perfect identity with this standard tetrapod CRH1 amino acid sequence (Fig. 1B).

The *L. oculatus crh1* syntenic environment on chromosome 9 strongly resembles that in *H. sapiens* in that many of the same genes are present in the region on *H. sapiens* chromosome 8 surrounding human *CRH* (Fig. 2). The region of *L. oculatus* chromosome 18 that flanks *crh2* also shows clear evidence of conserved synteny, but some genes have been conserved and others lost. Among 17 protein-coding genes adjacent to *H. sapiens CRH*, 15 are found in the syntenic block of genes near *L. oculatus crh1* (Fig. 2). Two genes (*dnajc5b* and *C80rf44)* found near *H. sapiens CRH* are not present in the gar *crh1* syntenic region. Among the same 17 protein-coding genes adjacent to *H. sapiens CRH*, only four are found near *L. oculatus crh2*: *trim55*, *dnajc5b, bhlhe22, and ythdf3* (Fig. 2).

Phylogenetic analysis of amino acid sequences revealed that CRH1 and CRH2 cluster in separate

TABLE 4.

Presence and Absence of CRH Family Genes in the Genomes of Amphibians, Reptiles, and Birds[1]

	CRH1	*CRH2*	*UTS1*
Amphibians			
Xenopus laevis	+ (2)	–	+
Reptiles			
Alligator mississippiensis	+	–	+
Anolis carolinensis	+	+	–
Chrysemys picta bellii	+	+	–
Birds			
Meleagris gallapavo	+	–	–
Gallus gallus	+	–	–
Anas platyrhynchos	+	+	–
Taeniopygia guttata	+	+	–
Falco peregrinus	+	+	–
Melopsittacus undulatus	+	–	+

[1]+, Present; –, absent; (2), two paralogs present.

groups that are more closely related to each other than to UTS1 (Fig. 3). One hundred percent of bootstrap replicates identified the crh1/crh2 group as being separate from the *uts1* gene group, and 35.2% of replicates identified CRH1 and CRH2 as adjacent gene groups.

CRH2 was not identified in the genomes of eutherian mammals (i.e., alpaca, armadillo, bushbaby, cat, chimpanzee, cow, dog, dolphin, elephant, ferret, gorilla, gibbon, guinea pig, hedgehog, horse, human, hyrax, kangaroo rat, lesser hedgehog tenrec, macaque, marmoset, megabat, microbat, mouse, mouse lemur, orangutan, panda, pig, pika, rabbit, rat, sheep, shrew, sloth, squirrel, tarsier, and tree shrew), teleost fishes (i.e., cave fish, cod, platyfish, medaka, tetraodon, stickleback, and zebrafish), or some lineages of birds, including the sequenced galliformes, turkey (*Meleagris gallapavo*) and chicken (*Gallus gallus*), and the budgerigar (*Melopsittacus undulatus*). However, we did find *CRH2* in the genomes of the related passerines and falcons (zebra finch and peregrine falcon) and duck (*Anas platyrhynchos*).

CRH1 is broadly conserved, but *CRH2* and *UTS1* are not found in all genomes and may have been separately lost in different lineages (see Table 4). For example, the green anole, *Anolis carolinensis*, has *CRH1* and *CRH2*, but has no identified *UTS1* homolog. The American alligator, *Alligator mississippiensis*, on the other hand, has *CRH1* and *UTS1*, but not *CRH2*. The African clawed frog, *Xenopus laevis*, has two *CRH1* homologs, likely as a result of whole-genome duplication (Bisbee et al., 1977), but does not have an identifiable *CRH2* homolog.

Several teleost genomes we examined, including zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), and Burton's mouthbrooder (*Astatotilapia burtoni*), contained two homologs of *crh1, crha* and *crhb*, but did not contain identifiable homologs of *crh2*. In our phylogenetic tree, teleost *crha* and *crhb* cluster with gar *crh1*, whereas gar *crh2* is more distantly related (Fig. 3). The known and highly conserved *crh1* gene homolog in teleosts is referred to as *crhb* in zebrafish, whereas the novel CRH is annotated as *crha*, because of the nomenclature guidelines, which indicate that a novel paralog in zebrafish is given the suffix "a" or "b" based on the "a" or "b" designation of other nearby genes on the same chromosome. In zebrafish, *crha* is located on chromosome 2, whereas *crhb* is located on chromosome 24, in accordance with the finding that chromosomes 2 and 24 are duplicates arising from the teleost whole-genome duplication (Woods et al., 2005).

Expression of the CRH gene family in the spotted gar brain

Antisense riboprobes for *crh1*, *crh2*, and *uts1* all showed strong and specific positive labeling in the gar brain, whereas negative control sense probes for each gene showed no label (Fig. 4). In general, each gene showed distinct regionally specific expression, but some brain nuclei showed expression of both *crh1* and *uts1*. *crh1* showed the most abundant and widespread staining of all three genes, followed by *uts1*. Both *crh1* and *uts1* mRNAs were found throughout spotted gar brain from the forebrain to the hindbrain. Expression of *crh2*, however, was restricted to a single nucleus in the pontine brain and did not show coregionalization with either *crh1* or *uts1*. Specific localization patterns for *crh1*, *crh2*, *uts1*, and *calca* are described in detail below.

CRH1

Forebrain

Some scattered *crh1*-expressing cells were found in the olfactory bulbs, primarily at the peripheral margins within the glomerular layer (Fig. 5A). In the dorsal telencephalon, cells were observed predominantly in the medial (Dm) and dorsal (Dd) parts (Figs. 5C,D), with occasional scattered cells seen in the posterior (Dp) and central (Dc) parts in some individuals. In the ventral telencephalon, *crh1*-expressing cells were abundant along the midline ventricle in the dorsal (Vd), ventral (Vv), and supracommissural (Vs) nuclei (Figs. 5B,C, 6A).

In the diencephalon, there was a strong hybridization signal in several regions of the preoptic area, including the anterior part of the parvocellular preoptic area (PPa), magnocellular part of the magnocellular preoptic area (PMm), and the posterior part of the parvocellular preoptic area (PPp; Figs. 5C–F, 6B). Labeled cells were also seen in the suprachiasmatic nucleus (Fig. 5E, 6C). Abundant *crh1*-expressing cells were seen throughout the thalamic nuclei, including the anterior thalamic nucleus (A), ventromedial thalamic nucleus (VM), and dorsal posterior (DP) and

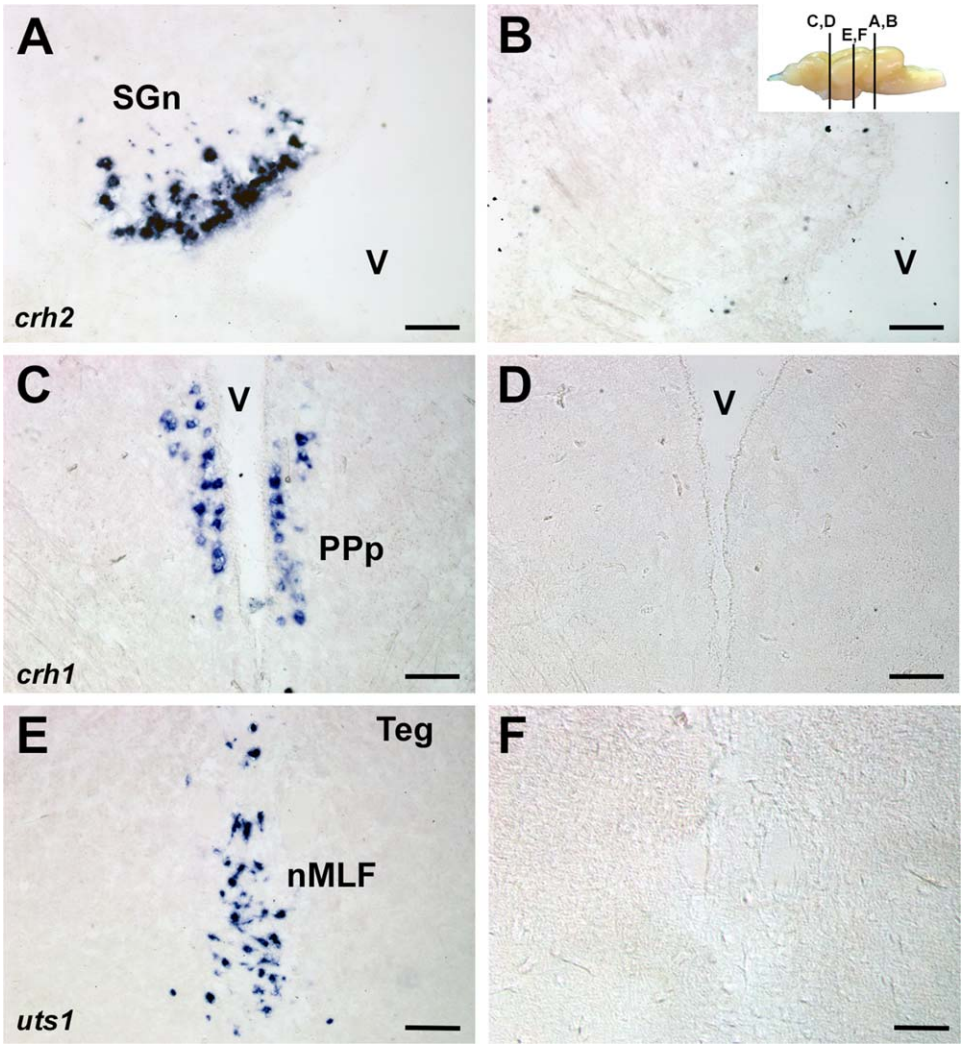

Figure 4. Representative examples of in situ hybridization staining in the spotted gar brain with antisense and sense control probes for *crh2* (**A,B**), *crh1* (**C,D**), and *uts1* (**E,F**). Brightfield photomicrographs of antisense (A,C,E) and sense (B,D,F) probes for each gene were taken on alternate adjacent 25-μm transverse sections from the same brain. Sense controls did not show any positive labeling for any of the candidate genes. **Inset** shows the approximate location of each section. For abbreviations see list. Scale bars = 25 μm in A–D; 100 μm in E,F. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

central posterior (CP) thalamic nuclei (Figs. 5E–G, 6C–E). The periventricular nucleus of the posterior tuberculum (TPp), paraventricular organ (PVO), and tuberal hypothalamic region also showed *crh1*-labeled neurons (Figs. 5G,H, 6D–G). In the hypothalamus, *crh1* cells were localized primarily to the ventral hypothalamic nucleus (Hv) beneath the ventricle, whereas only occasional scattered cells were seen in the dorsal hypothalamic nucleus (Hd), primarily in rostral sections (Figs. 5G–K, 6E,G).

Midbrain

In the rostral midbrain, *crh1*-positive cells were found in the nucleus of the medial longitudinal fasciculus (nMLF; Fig. 5H), in the dorsal tegmental nucleus (DTN; Fig. 5H), in the torus semicircularis (TS; Fig. 5H–K), and scattered along the ventral aspect of the tectal ventricle (Fig. 5G–K). The most conspicuous *crh1*-labeling in the midbrain was in the large lateral tegmental nucleus (LT; Figs. 5I, 6H). Scattered individual *crh1* neurons were located throughout the periventricular gray zone (PGZ) of the tectum (Figs. 5E–K), but were less abundant than *uts1*-expressing cells in this same region. In the caudal midbrain and isthmal region, there were also several distinct populations of *crh1*-expressing cells dorsal and lateral to the medial longitudinal fasciculus (mlf) and in the central gray region lining the fourth ventricle (Figs. 5J,K).

Hindbrain

In the hindbrain, *crh1*-expressing cells were seen in the trigeminal motor nucleus (Vm; Figs. 5L, 6I), putative

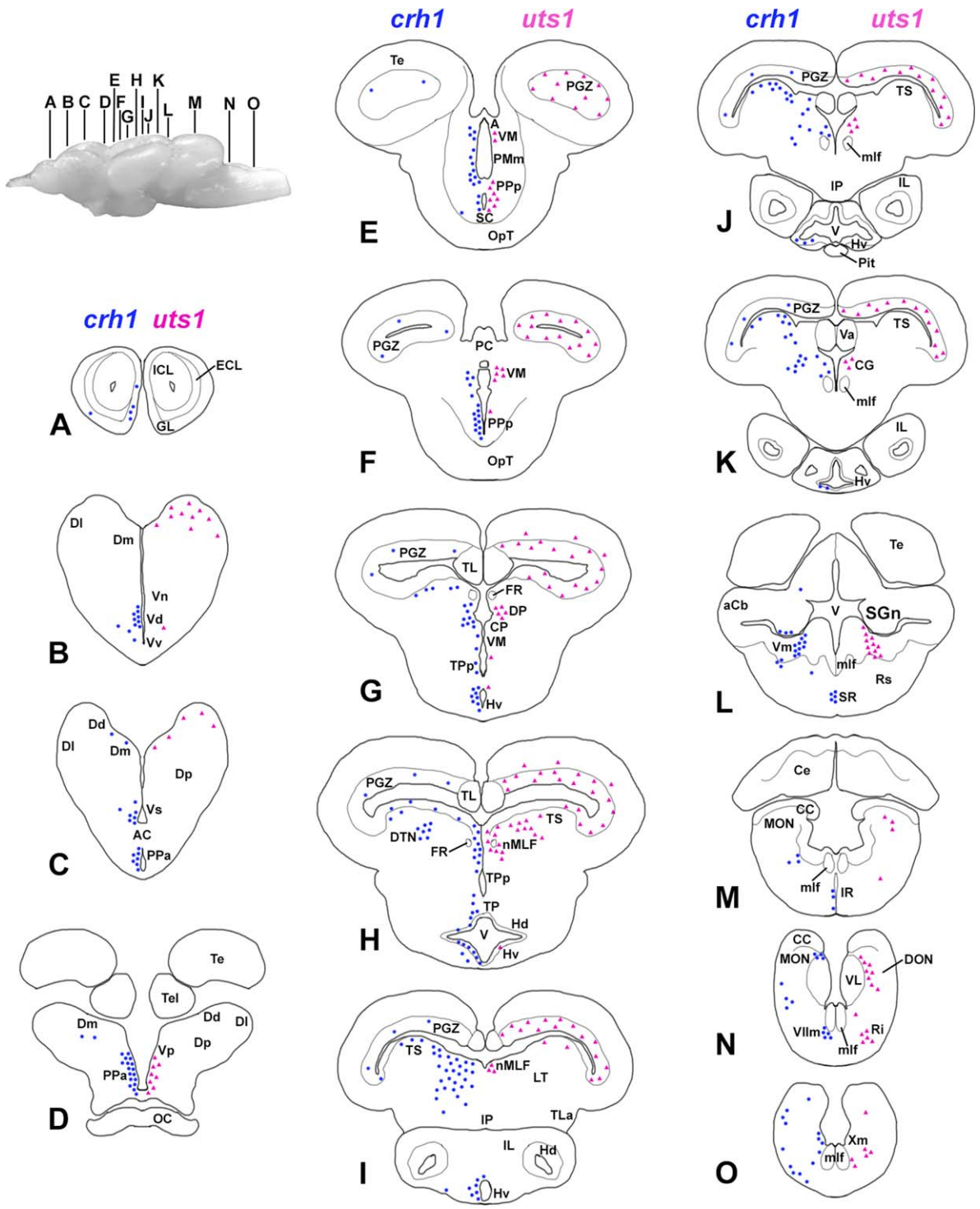

Figure 5. Localization of *crh1* (left side; blue dots)- and *uts1* (right side; magenta triangles)-expressing cells in the brain of the spotted gar. Diagrams of representative transverse sections (right sides are traced mirror images of left sides) are shown from rostral to caudal (**A–O**). **Inset** shows the approximate location of each section. See list for abbreviations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

locus coeruleus (LC; Fig. 6I), inferior (IR) and superior (SR) raphe nuclei (Figs. 5L,M, 6I), facial motor nucleus (VIIm; Figs. 5N, 6K), and vagal motor nucleus (Xm; Fig. 5O). Occasional single cells were also seen in the cerebellar auricle at the level of the rostral hindbrain. In the caudal hindbrain, several *crh1* cells were consistently seen along the fourth ventricle at the dorsal aspect of the vagal lobe (Figs. 5N, 6J), scattered in the region of

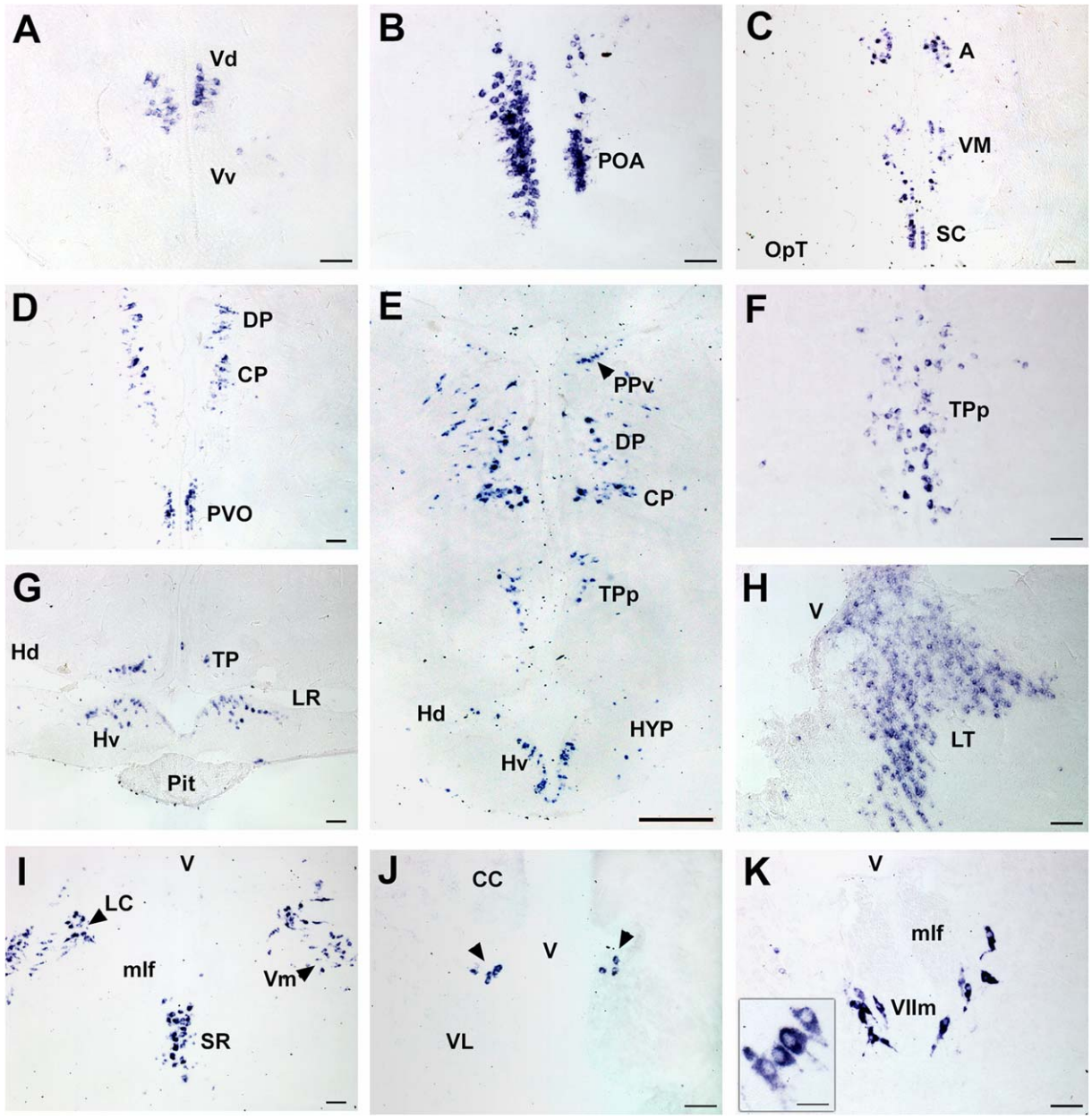

Figure 6. Representative brightfield photomicrographs of *crh1*-expressing neurons in the brain of the spotted gar. **A:** Ventral telencephalon area. **B:** Caudal preoptic area (POA). **C:** Rostral thalamic area dorsal to the optic tract (OpT). **D:** Thalamic area just caudal to C. **E:** Caudal thalamic area and rostral hypothalamus. **F:** Posterior tuberculum area. **G:** Tuberal hypothalamic area dorsal to the pituitary gland. **H:** Lateral tegmental nucleus (LT) of the mesencephalon. **I:** Hindbrain in the area of the superior raphe nucleus (SR) and trigeminal motor nucleus (Vm). **J:** Several *crh1*-expressing cells (arrowheads) are located on the dorsal aspect of the vagal lobes (VL). **K:** Caudal hindbrain showing large facial motor neurons lateral to the tracts of the mlf. **Inset** shows high magnification of several labeled motor neurons. Photomicrographs were taken from 20-μm transverse sections and are depicted from rostral to caudal (A–K). For abbreviations see list. Scale bars = 20 μm in A,B,F,H,J,K,inset; 50 μm in C,D,G,I; 200 μm in E. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the descending octaval nucleus (DON; Fig. 5N), and scattered along the lateral margins of the hindbrain (Fig. 5O).

UTS1

Forebrain

No *uts1* labeling was observed in the olfactory bulbs. In the telencephalon, *uts1*-expressing cells were found primarily in dorsal parts, including Dm, Dd, and Dl (Figs. 5B,C). In these regions, *uts1*-expressing cells were more abundant lining the dorsomedial aspect of the dorsal telencephalon in rostral sections compared with caudal sections. Only occasional single, scattered cells were seen in ventral nuclei of the telencephalon (primarily Vd and Vs regions; Fig. 5B).

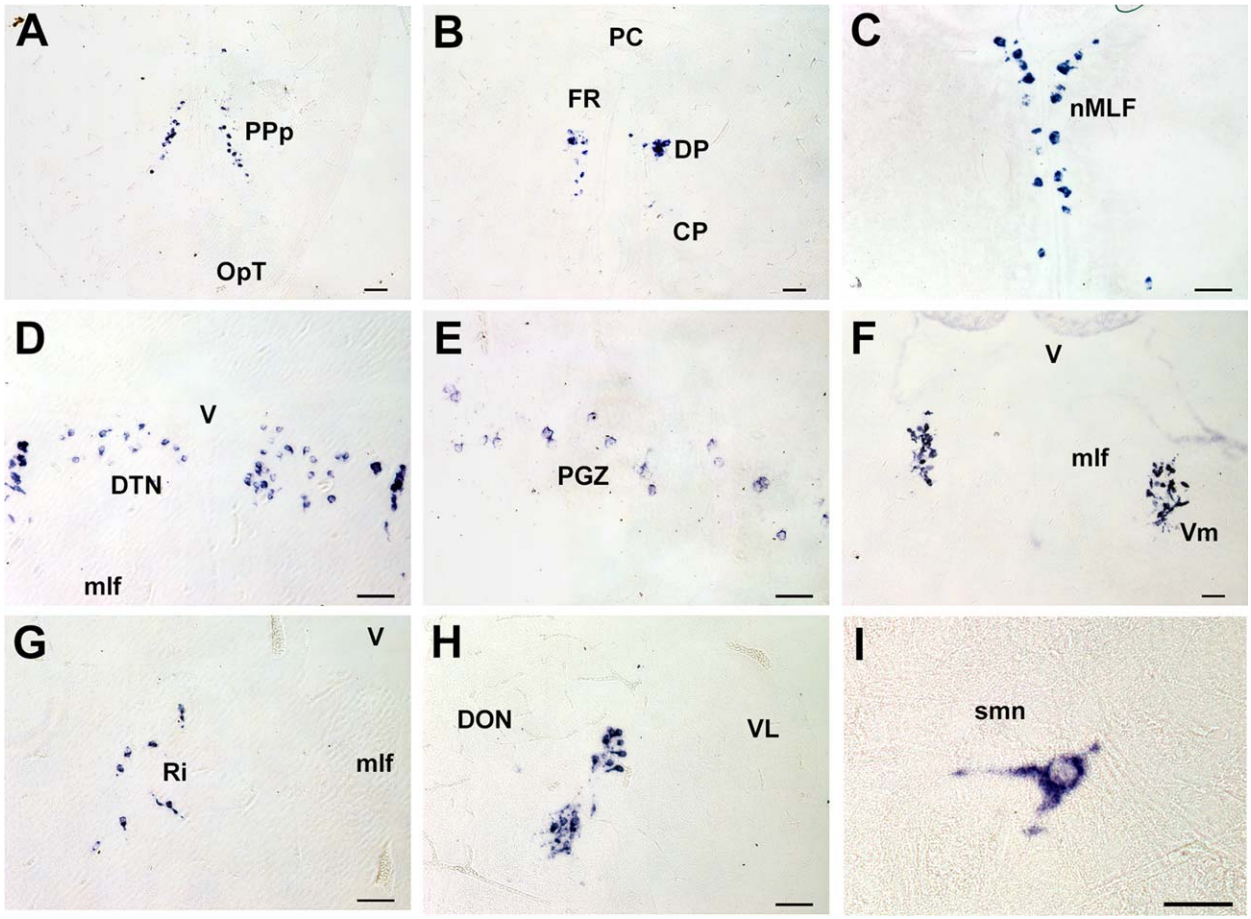

Figure 7. Representative brightfield photomicrographs of *uts1*-expressing neurons in the brain of the spotted gar. **A:** Posterior part of the parvocellular preoptic area (PPp) above the optic tract (OpT). **B:** Rostral thalamus in the region of DP and CP. **C:** Mesencephalon in the region of nMLF. **D:** Dorsal tegmental nucleus (DTN) beneath the ventricle. **E:** Scattered cells in the periventricular gray zone (PGZ) of the tectum. **F:** Hindbrain in the region of the trigeminal motor nucleus (Vm). **G:** Caudal hindbrain in the region of the inferior reticular formation (Ri). **H:** Caudal hindbrain near the vagal lobe (VL) and descending octaval nucleus (DON). **I:** Large neuron in the region of spinal motor neurons (smn). Micrographs were taken from 20-μm transverse sections and are depicted from rostral to caudal (A–I). For abbreviations see list. Scale bars = 50 μm in A,B,F; 20 μm in C–E,G–I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In the diencephalon, strong *uts1* signal was seen in both the anterior (PPa) and the posterior (PPp) preoptic areas (Figs. 5D–F, 7A), with a few cells in the suprachiasmatic nucleus. Distinct *uts1*-labeled cells were also evident in VM, DP, and TPp (Figs. 5E–G, 7B). In the hypothalamus, a few scattered cells were seen in Hv (Fig. 5G,H) but not in Hd.

Midbrain

There was a conspicuous *uts1*-labeled cell population along the midline in the region of the nMLF (Figs. 5H,I, 7C) and in the region of the dorsal tegmental nucleus (DTN; Fig. 5H, 7D). More caudally, *uts1*-expressing cells were found in the central gray region along the ventral lining of the fourth ventricle (Figs. 5J,K). In the tectum, *uts1*-expressing cells were numerous and scattered throughout the periventricular gray zone from its rostral to its caudal extent (Figs. 5E–K, 7E). There were also occasional scattered cells in more superficial layers of the tectum near the PGZ (Figs. 5G,H, 7E) and a few scattered cells in the torus semicircularis (Fig. 5H,I).

Hindbrain

In the hindbrain, a distinct, large population of *uts1* cells was evident in the trigeminal motor nucleus (Figs. 5L, 7F). There were scattered cells in the region of the medial octaval nucleus beneath the cerebellar crest (Fig. 5M), a large dorsoventrally elongated population at the border of the vagal lobe and DON (Figs. 5N, 7H),

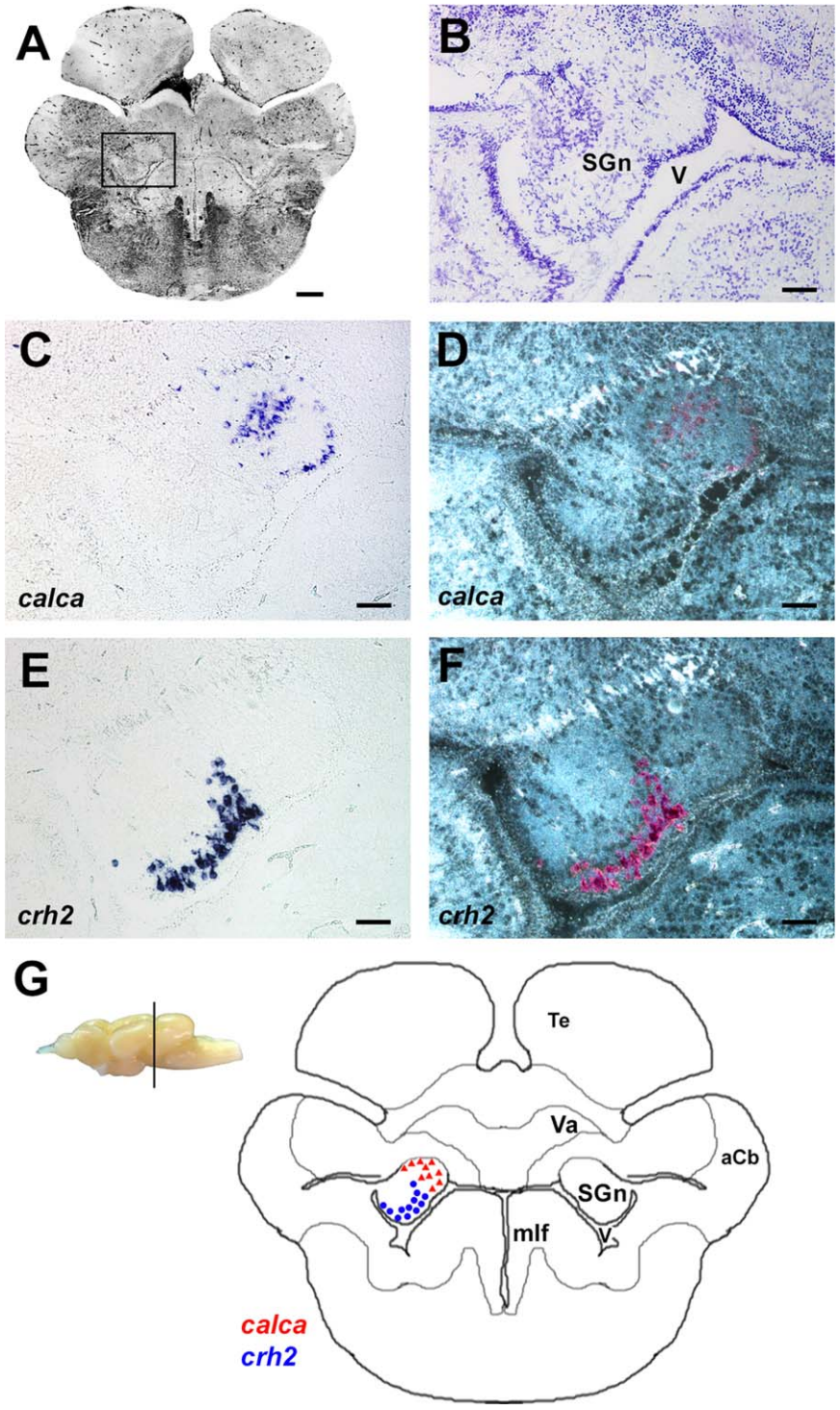

Figure 8. Localization of *crh2*- and *calca*-expressing cells in the brain of the spotted gar. **A:** Representative transverse section to illustrate the region of *crh2*- and *calca*-expressing neurons in the pontine/isthmic region of the brain. **B:** Higher magnification of the boxed secondary gustatory nucleus (SGn) region in A stained with cresyl violet. **C,D:** *calca*-Expressing cells in the dorsal portion of the SGn in brightfield (C) and phase contrast (D). **E,F:** *crh2*-Expressing neurons in the ventral region of the SGn in brightfield (E) and phase contrast (F). **G:** Summary diagram to illustrate the distribution of nonoverlapping *crh2*-expressing (blue dots) and *calca*-expressing (red triangles) neurons within the SGn. **Inset** shows the approximate location of the sections. Photomicrographs in B–F were taken from adjacent 20-μm sections stained with cresyl violet (B), *calca* (C,D), or *crh2* (E,F) from the same brain. For abbreviations see list. Scale bars = 200 μm in A; 50 μm in B–F. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

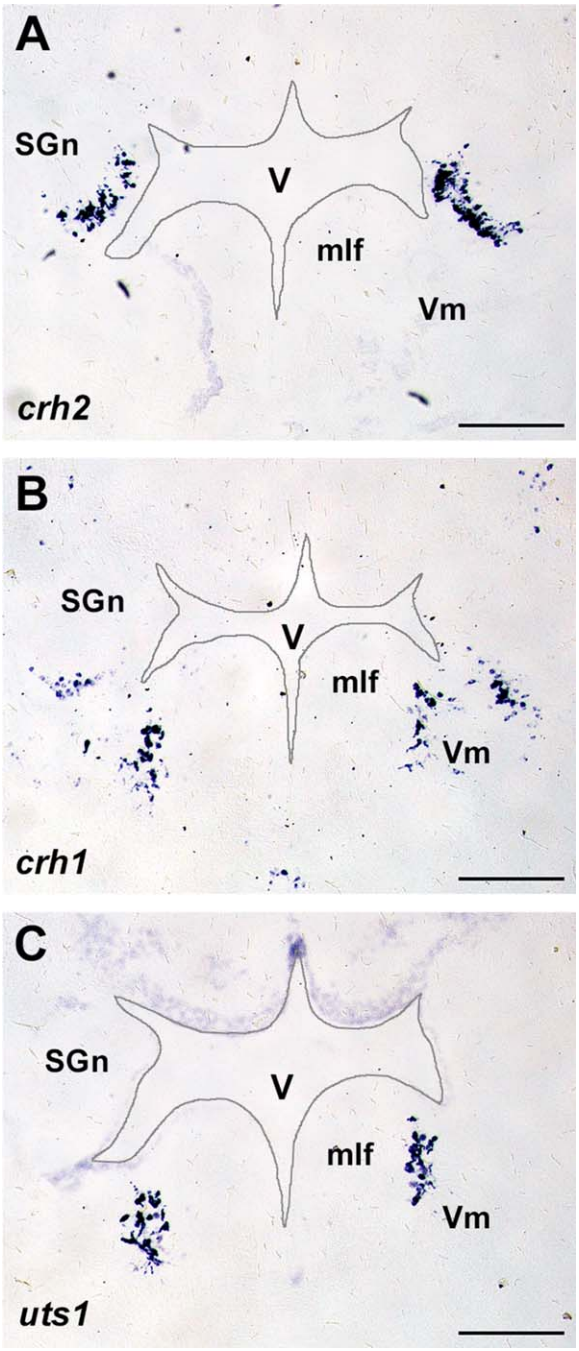

Figure 9. Representative brightfield photomicrographs of transverse sections through the pontine/isthmal brain of the spotted gar to illustrate the relationship among *crh2*-, *crh1*-, and *uts1*-expressing cells. Photographs were taken from alternate adjacent 20-μm transverse sections stained for *crh2* (A), *crh1* (B), and *uts*1 (C) in the same brain. The ventricle (V) is outlined in gray in each section for reference. Scale bars = 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and some cells within several reticular formation nuclei (Figs. 5L–O, 7G). Several large putative spinal motor neurons were also labeled in the caudal hindbrain and rostral spinal cord (Fig. 7I).

CRH2 and CALCA

Expression of *crh2* was restricted to a single nucleus in the pontine/isthmic region of the spotted gar brain that lines the lateral edge of the fourth ventricle (Fig. 8). Based on labeled brain atlases of other gar species (Northcutt and Butler, 1980; Parent and Northcutt, 1982; Morona et al., 2013), these *crh2*-expressing cells appear to be localized to the secondary gustatory nucleus (SGn) or secondary visceral nucleus (SVn; Fig. 8E–G). To facilitate identification of the SGn and SVn, we also performed ISH for *calca*, a gene that encodes both calcitonin and calcitonin gene-related peptide (CGRP) by alternative splicing and has been used as a molecular marker for the parabrachial nucleus in mammals (putative homolog to the SGn in fishes; Rosenfeld et al., 1983; Kawai et al., 1985). The *calca*-expressing neurons in the gar were labeled within what appeared to be a different subdivision of the same nucleus that contains the *crh2*-expressing cells, but in a more dorsal position (Figs. 8C,D,G). Labeling of alternate brain sections from a single individual revealed absence of coregionalization of these *crh2* cells with *crh1* and *uts1* (Fig. 9). No *crh2* signal was detected in any other brain region, including primary visceral areas reaching caudally to the spinal cord.

DISCUSSION

Our phylogenetic analysis revealed several fascinating and previously unappreciated features of vertebrate CRH evolution. First, and most importantly for this report, we found that two genes homologous to *CRH* are present across at least five classes of vertebrates, Chondrichthyes, Osteichthyes, Reptilia, Aves, and Mammalia. Therefore, our results support a revised nomenclature for CRH genes, with *CRH1* and *CRH2* designating the two conserved paralogs, analogous to the regularization of gonadotropin-releasing hormone (GnRH) nomenclature to include two orthologs, *GnRH1* and *GnRH2*, that are conserved across vertebrate classes (Fernald and White, 1999). Second, although *CRH1* is present in all sequenced vertebrate genomes and displays a remarkably low degree of sequence variability across species, *CRH2* has been lost independently in multiple vertebrate lineages, including teleosts and eutherian mammals, and has relatively high sequence variability. Third, *CRH1* has been reduplicated in teleosts. Our identification and characterization of CRH family gene duplications provides an important step toward understanding the evolution of vertebrate neuropeptide systems via ancient gene duplications.

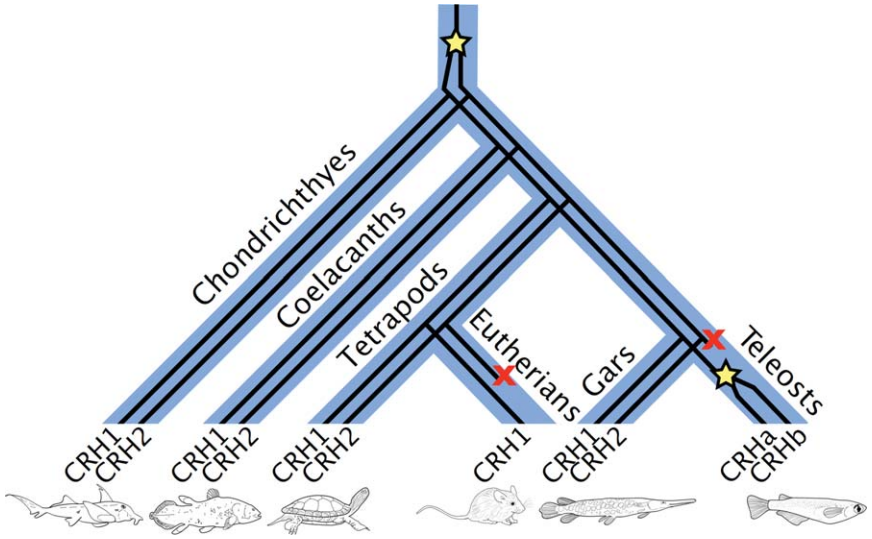

Figure 10. A proposed history of *CRH* gene duplications and losses in vertebrates represented by a generalized gene tree. Wide shaded lines represent diverging vertebrate lineages; narrow black lines represent the *CRH* gene lineage. Given the new *CRH* sequence data presented here, we propose several revisions to the history of *CRH* evolution in vertebrate lineages. *CRH1* and *CRH2* are present in species representatives from several lineages, including tetrapod classes such as reptilia (e.g., green anole), aves (e.g., zebra finch), and mammalia (e.g., Tasmanian devil) as well as chondrichthyes (e.g., elephant shark), sarcopterygii (e.g., coelacanth), and actinopterygii (e.g., spotted gar). Because *CRH2* homologs are not found in available teleost or eutherian species databases, and these two clades have been sequenced relatively extensively, we propose that both teleostei and eutheria have lost *CRH2* (indicated by X). Teleostei, however, also experienced a duplication of *CRH1*, likely during a third whole-genome duplication early in teleost evolution (indicated by a star on the teleost branch). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CRH family evolution in vertebrates

It appears likely that *CRH1* and *CRH2* were generated by duplication of the chromosome containing an ancestral *CRH* gene, possibly during the second round of vertebrate whole-genome duplication. This hypothesis is supported by the facts that *CRH1* and *CRH2* share features of their chromosomal syntenic environments and that no other known peptide shares greater sequence similarity to *CRH1* than *CRH2*. The phylogenetic tree shown in Figure 2 is based on very divergent amino acid sequences for a family of small genes that have experienced differential directional selection over the course of approximately half a million years of evolution. Therefore, the position of each gene in relation to its orthologs in other species does not perfectly match the known species phylogenies. Nevertheless, the phylogenetic analysis provides strong support for the hypothesis that *CRH1* and *CRH2* genes arose via duplication. Intriguingly, teleost fishes appear to have lost *CRH2* but had a separate duplication of *CRH1*, likely during the teleost-specific third round of whole-genome duplication (Fig. 10).

The earliest tetrapod ancestor had a *CRH2* ortholog, because the *CRH2* gene is found in representatives of lizards, birds, monotremes, and marsupials. Although *CRH1* is retained in all sequenced vertebrate genomes, *CRH2* appears to have been lost independently multiple times during vertebrate evolution. The loss of *CRH2* in several lineages, along with the greater sequence variability among species compared with *CRH1*, suggests that stabilizing selection on *CRH2* was relaxed after whole-genome duplication. However, *CRH2* was retained for hundreds of millions of years of evolution in species as diverse as the elephant shark (*Callorhinchus milii*) and the gray short-tailed possum (*Monodelphis domestica*), suggesting that it served physiologically important roles. Because we did not find a *CRH2* paralog in any teleost or eutherian genome, or in the single sequenced amphibian genome of the Western clawed frog (*Xenopus tropicalis*), we hypothesize that it was lost in the teleost, amphibian, and eutherian lineages (Fig. 10). It remains possible, however, that some eutherian mammals, amphibians, or teleost fishes have retained a *CRH2* gene that existing genome sequence data have not yet revealed.

We did not identify *CRH2* in the sea lamprey *Petromyzon marinus*. It is possible that all agnathans lack *CRH2*, but future availability of other agnathan genomes may reveal *CRH2* orthologs. We found only one *CRH* gene in the genome of *P. marinus*, but the identity of this gene is difficult to determine with confidence because its homology with *CRH1*, *CRH2*, and *UTS1* is

distant. The lack of a second *P. marinus CRH* or *UTS1* is despite the evidence that the *P. marinus* genome shares both rounds of vertebrate genome duplication (1R and 2R) with tetrapods (Smith et al., 2013). The singularity of *P. marinus CRH* is, however, in concordance with the genome-wide paucity of genes involved in neuropeptide signaling observed in *P. marinus* (Smith et al., 2013). In the Ensembl *P._marinus_*7.0 genome assembly, the contigs are short and are missing large portions in the syntenic regions predicted to contain *CRH* genes, suggesting that further *CRH* genes could actually be present in the *P. marinus* genome.

The proposition that CRH2 arose during the second round of vertebrate genome (2R) duplication further suggests the likelihood that urotensin (*UTS1*) arose earlier by duplication of a joint *CRH/UTS1* ancestor, likely in the first round of vertebrate whole-genome duplication (1R). The tunicate diuretic hormone-like peptide (*DHLP*) gene may be homologous to the common ancestor of *CRH1*, *CRH2*, and *UTS1* (Lovejoy and Barsyte-Lovejoy, 2010). Urocortin 2 and urocortin 3 are other members of the *CRH* family that likely diverged from a common ancestor of CRH1 and UTS1 (Lovejoy and de Lannoy, 2013).

Expression patterns of CRH1, UTS1, and CRH2 in spotted gar

In the spotted gar, both *crh1* and *uts1* cells were widely distributed throughout the brain in patterns similar to those of other fishes (Zupanc et al., 1999; Pepels et al., 2002; Alderman and Bernier, 2007) as well as other vertebrates (Swanson et al., 1983; Jozsa et al., 1984; Merchenthaler et al., 1984; Bons et al., 1988; Mancera et al., 1991; Morin et al., 1999; Yao et al., 2004), suggesting diverse functional roles in neuroendocrine regulation, behavior, sensory integration, stress coping, feeding, and autonomic system modulation. In contrast to the widespread distribution of *crh1* and *uts1*, *crh2*-expressing cells were restricted to a single nucleus in the isthmic region of the hindbrain, suggesting a more specialized function in stress-related feeding or autonomic regulation.

In addition to the *crh1*- and *uts1*-expressing cells in the telencephalon and preoptic area that are well-described from immunostaining and ISH in fishes (e.g., salmon, brown ghost knifefish, tilapia, and zebrafish; Matz and Hofeldt, 1999; Zupanc et al., 1999; Pepels et al., 2002; Alderman and Bernier, 2007), the spotted gar also showed abundant *crh1* and *uts1* mRNA expression in several more caudal brain regions, such as the thalamus, mesencephalon, isthmus, and rhombencephalon, not typically reported in other fish species. Similar caudal distributions of CRH- and/or UTS1/UCN1-expressing neurons, however, have been described for some amphibians (Yao et al., 2004), reptiles (Mancera et al., 1991), birds (Bons et al., 1988), and mammals (Swanson et al., 1983; Bons et al., 1988; Morin et al., 1999). The widespread distribution of *crh1* and *uts1* in gar further supports the idea that these peptides likely serve conserved and diverse neurotransmitter or neuromodulator roles. Although there were several brain regions in spotted gar where *crh1* and *uts1* cell distributions overlapped, such as POA, thalamus, nMLF, isthmal nuclei, and PGZ of the tectum, the widespread and unique distribution patterns of *crh1*, *uts1*, and *crh2* suggest diverse functional roles for these neuropeptides. Future localization and functional studies in additional species are needed to understand fully the significance of these distribution patterns.

Given the neuroanatomical landmarks and observed expression patterns of *calca* and the family of *crh* genes, we interpret the *crh2*-expressing nucleus in the gar as SGn or possibly SVn. Although pontine/isthmic nuclei have not been thoroughly mapped in spotted gar, they have been described in some teleost and mammalian species for comparison. The *calca* gene, which is expressed in nuclei of the pons in teleosts as well as mammals and is often used as a marker of the parabrachial nucleus (PBN), encodes both calcitonin and CGRP by alternative splicing (Jacobs et al., 1981). Calcitonin is a 32-amino-acid peptide that regulates calcium balance, a function in which CGRP also participates. CGRP, on the other hand, is a potent 37-amino-acid vasodilator (Brain et al., 1985) that regulates signals of peripheral pain following inflammation (Bennett et al., 2000; Zhang et al., 2001). In mice, CGRP neurons in the parabrachial nucleus suppress feeding by participating in a circuit with inputs from agouti-related peptide (AgRP) neurons in the hypothalamus and outputs to the central amygdala (Carter et al., 2013). The PBN in mammals contains subpopulations of neurons that regulate different functions, including taste (Rosen et al., 2011; Tokita and Boughter, 2012). In teleosts, Cgrp-immunoreactive neurons in the pons project to the hypothalamic inferior lobe, which is also involved in gustation (Kanwal et al., 1988; Batten and Cambre, 1989; Lamb and Caprio, 1993). Immunohistochemistry revealed Cgrp-positive somata in the SVn, but not the SGn, of goldfish (*Carassius auratus*) and catfish (*Ictalurus punctatus*; Finger and Kanwal, 1992). In catfish, the Cgrp-containing SVn is located rostrolateral to the SGn. In the spotted gar, the *calca*-expressing neurons are located dorsomedial to the *crh2*-positive neurons in the putative SGn, suggesting a different positional relationship between the SGn and SVn in this basal actinopterygian. Calcitonin and calcitonin-related peptides have been proposed to be the

genes most closely related to the CRH family of peptides (Lovejoy and de Lannoy, 2013). Expression of *crh2* and *calca* in adjacent brain regions of the spotted gar thus suggests functional partitioning between CRH family and calcitonin family as well as within the CRH family.

CONCLUSIONS

The existence of two distinct vertebrate *CRH* paralogs has gone unnoticed since the original discovery of *CRH* in 1981, despite over 10,000 published articles on CRH indexed in PubMed to date. Recent expansion of genome sequencing into nontraditional model organisms creates the possibility of rapidly making discoveries about the evolution of diverse vertebrate lineages that were not possible before or possible only through laborious cloning and sequencing efforts for single genes. Our discovery of this novel *CRH2* in multiple vertebrate genomes and its restricted distribution in the hindbrain of spotted gar lays the framework for future comparative studies on its function(s). For example, what are the functional implications of the loss of *CRH2* in teleost fishes and placental mammals? The story of *CRH* evolution following gene duplication appears to reflect the release of selective constraints on one copy, *CRH2*, which diverges in structure, expression pattern, and possibly function, whereas the other copy, *CRH1*, retains a highly conserved sequence and broad expression pattern. Understanding the regulation and function of *CRH2* as well as *CRH1* will enrich our understanding of how diverse vertebrates respond to their ever-changing environments.

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CONFLICT OF INTEREST STATEMENT

The authors have no identified financial, personal or other conflicts of interest.

ROLE OF AUTHORS

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

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