Identification of multiple vasotocin receptor cDNAs in teleost fish: Sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge

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ABSTRACT

Vasopressin and its homolog vasotocin regulate hydromineral balance, stress responses, and social behaviors in vertebrates. In mammals, the functions of vasopressin are mediated via three classes of membrane-bound receptors: V1a-type, V1b-type and V2-type. To date, however, only a single class of vasotocin receptor has been identified in teleost fish. Here, cDNAs encoding three putative vasotocin receptors – two distinct V1a-type receptor paralogs (*V1a1* and *V1a2*) and a previously undescribed V2-type receptor (*V2*) – and a single isotocin receptor were isolated and sequenced from the Amargosa pupfish (*Cyprinodon nevadensis amargosae*). RT-PCR revealed that mRNAs for these receptors differed in expression patterns with *V1a1* mRNAs abundant in the brain, pituitary and testis, *V1a2* transcripts at greatest levels in brain, heart and muscle, *V2* transcripts most common in the gills, heart and kidney, and isotocin receptor mRNAs abundant in the midbrain, pituitary and gonads. In response to an acute hyperosmotic challenge, pro-vasotocin and *V2* mRNA levels in the hypothalamus decreased, while transcripts of *V1a1* in the hypothalamus and *V1a2* in the gills increased. Partial transcripts for structurally related V2-type, as well as multiple V1a-type, receptors were also identified in other teleosts, suggesting that multiple vasotocin receptors may be present in many Actinopterygii fishes.

1. Introduction

The neurohypophysial hormone arginine vasopressin (VP) is a well established regulator of osmotic balance, vasoconstriction, and social and affiliative behaviors in mammals. In teleost fish, VP's non-mammalian homolog – arginine vasotocin (VT) – plays similar roles by regulating salt and water balance, cardiovascular function, social behaviors and the physiological response to stressors (reviewed by Balment et al., 2006). In teleosts, VT has been shown to control osmotic balance by acting on the kidney and gills (Balment et al., 1993; Warne et al., 2002). VT induces an antidiuretic effect in the kidney by decreasing glomerular filtration rates resulting in a net increase in water reabsorption and decrease in urine flow (Amer and Brown, 1995), while concurrently acting on the gills to alter Cl⁻ permeability (Guibbolini and Avella, 2003; Avella et al., 1999). Accordingly, teleost VT receptor binding, immunoreactivity and mRNAs have been localized to kidney and gill tissues in several teleost species (Guibbolini et al., 1988; Mahlmann et al., 1994; Guibbolini and Avella, 2003; Warne, 2001; Warne et al., 2005; An et al., 2008a,b). VT receptor binding has also been localized to the

nervous system of fish (Moons et al., 1989), where VT acts within the brain to regulate behaviors associated with sociality and reproduction (Goodson and Bass, 2001) and on the pituitary gland to modulate adrenocorticotrophic hormone (ACTH) release (Baker et al., 1996).

Receptors for VT/VP – as well as for the structurally related nonapeptides oxytocin, mesotocin and isotocin (IT) – are G-protein coupled membrane receptors characterized by seven transmembrane helices connected via alternating extracellular and intracellular loops, as is typical of class A G-protein coupled membrane receptors including rhodopsin (Barberis et al., 1998; Darlison and Richter, 1999; Palczewski et al., 2000). In mammals, VP acts through three distinct receptor types: V1a-type, V1b-type and V2-type. The amino acid structures of these three receptor types have been described in several species including rats, mice and humans (e.g., Thibonnier et al., 1994; Bösel et al., 2009). In birds, three distinct VT receptors have been also identified; these receptors appear to belong to the V1a-type, V1b-type and, tentatively, the V2-type mammalian VP receptor families (Tan et al., 2000; Cornett et al., 2003; Jurkevich et al., 2005). The functional roles of these avian receptors in osmoregulation, stress responses, and oviposition appear to parallel those of VP/VT receptors identified in other vertebrates (Baeyens and Cornett, 2006). Recently, three VT receptors belonging to the mammalian V1a-type, V1b-type and

V2-type receptor classes were also identified in the firebelly newt (*Cynops pyrrhogaster*) (Hasunuma et al., 2007). In Actinopterygian fishes, however, only a single type of VT receptor has been identified to date.

VT receptors have now been isolated and described from several teleost fishes ranging from flatfishes to trout (Mahlmann et al., 1994; Conklin et al., 1999; Warne, 2001; An et al., 2008a). All these receptors constitute a single type of VT receptor that groups within the V1a-type class of mammalian VP receptors, based on the receptors' amino acid structural identities and intracellular signal transduction mechanisms. Mahlmann et al. (1994) first demonstrated that the mammalian V1-type VP receptor-specific antagonist [d(CH₂)₅,Tyr(Me)²,Arg⁸]vasopressin inhibited VT-induced membrane currents using the white sucker (*Catostomus commersoni*) VT receptor expressed in *Xenopus laevis* oocytes. In a separate study using cultured gill epithelium from European sea bass (*Dicentrarchus labrax*), VT and the V1-type receptor agonist, [Phe²,Orn⁸]-oxytocin, were found to stimulate Cl⁻ secretion, while a mammalian V2-type receptor agonist failed to have any effect (Guibbolini and Avella, 2003). Likewise, flounder (*Platichthys flesus*) VT receptor expressed in *Xenopus laevis* oocytes induced a VT-like inward voltage current following application of the mammalian V1-type agonist [Phe²,Orn⁸]-oxytocin, while the mammalian V2-type receptor agonist [deamino¹,Val⁴,D-Arg⁸]-vasopressin had no effect (Warne, 2001). This flounder VT receptor was shown to act via a phospholipase C (PLC) and inositol-1,4,5-triphosphate (IP₃) intracellular signaling pathway (Warne, 2001), which is characteristic of the transduction pathways used by mammalian V1-type receptors, but distinct from the adenylate cyclase and cAMP intracellular signaling pathways used by mammalian V2-type receptors (Wargent et al., 1999).

While these studies suggest that the teleost VT receptor acts through a PLC/IP₃ intracellular signaling pathway similar to mammalian V1a-type receptors, there is also biochemical evidence that teleost fish may possess a V2-type receptor, or at least a VT receptor that acts by altering intracellular cAMP levels. In isolated renal tubules from rainbow trout, Perrott et al. (1993) found that VT causes a dose-dependent induction of intracellular cAMP production, a result consistent with the V2-type transduction pathway for VP in mammals (Wargent et al., 1999). Similarly, in trout hepatocytes and gill membranes, VT has been shown to alter adenylate cyclase activity and inhibit cAMP accumulation induced by glucagon (Guibbolini and Lahlou, 1987; Guibbolini et al., 2000). This inhibition appears to occur via VT induction of an inhibitory G-protein pathway (Guibbolini and Lahlou, 1992). Taken together, these studies suggest that some teleosts may possess a VT receptor that acts by altering cAMP intra-cellular signaling.

Recently, two VT receptors, one mammalian V1a-type and one mammalian V2-type, were also identified in a Sarcopterygian fish, the African lungfish (*Protopterus annectens*) (Konno et al., 2009). This lungfish V2 receptor acts by modulating intracellular cAMP concentrations via a transduction pathway homologous to that of mammalian V2-type receptors, and consistent with the biochemical evidence for a V2-type receptor in teleosts (Guibbolini and Lahlou, 1987, 1990). If lungfish possess VT receptors from both the V1a-type and V2-type families, then when did the multiple nonapeptide hormone receptors presently seen in tetrapods first evolve? Did a single VT/VP receptor evolve into the distinct V1a-type, V1b-type and V2-type receptors prior to the divergence of the modern day tetrapod lineage from fish?

Here, cDNAs for VT and IT receptors were isolated and sequenced from the brain of the teleost Amargosa River pupfish, *Cyprinodon nevadensis amargosae*. The VT system has been studied previously in this species for its role in the regulation, and subsequent evolutionary diversification, of aggression and other behaviors in allopatric populations inhabiting the Death Valley

region of California and Nevada, USA. VT modulates aggression in male Amargosa pupfish (Lema and Nevitt, 2004a), and in previous work, Lema and Nevitt (2004b) discovered that wild allopatric populations of this species differ in the size of hypothalamic VT-immunoreactive neurons. This population-level variation in neural VT expression results from the interacting effects of variation in habitat conditions (e.g., salinity, temperature) and an evolved genetic difference between populations in how the VT system responds to environmental conditions (Lema, 2006).

In the current study, cDNAs encoding putative receptors for VT and IT were isolated and sequenced from *C. n. amargosae*, as a first step toward exploring how receptor regulation at the level of target tissues may contribute to evolutionary divergence in VT-mediated social behaviors, as has been seen in mammalian systems (e.g., Insel et al., 1994). Using degenerate primer PCR, three putative VT receptor cDNAs, and one IT receptor cDNA, were identified. Two of these VT receptor cDNAs appear to be paralogs that show greatest homology to mammalian V1a-type receptors, while the third cDNA encodes a previously undescribed teleost VT receptor with closest homology to mammalian V2-type receptors.

2. Materials and methods

2.1. cDNA cloning of vasotocin and isotocin receptors

2.1.1. Total RNA isolation

Amargosa River pupfish (*Cyprinodon nevadensis amargosae*) adults were collected from the Amargosa River near Dumont Dunes, San Bernardino County, California, USA, on 29 May, 2007 (salinity, 1.3 ppt; temperature, 22.2 °C). An adult male pupfish (body mass, 1.81 g; standard length, 40.05 mm) was euthanized in tricaine methanesulfonate (MS222; Argent Chemical, Redmond, WA), and the whole brain with connected pituitary dissected and stored in RNAlater (Applied Biosystems, Inc., Foster City, CA). Total RNA was extracted using Tri-Reagent RT (Molecular Research Center, Cincinnati, OH) with bromoanisole as the phase separation reagent. The resulting total RNA was DNase I treated (Invitrogen, Carlsbad, CA) and then quantified (260:280 ratio of 2.02; NanoDrop Technologies, Wilmington, DE). RNA quality was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

2.1.2. Determination of partial VT and IT receptor cDNA sequences

To obtain partial cDNA sequences, first strand cDNA was synthesized in a 20 µl reverse transcription reaction by incubating 2 µg of total RNA template (4.83 µl) with 1.0 µl annealing buffer, 1.0 µl of random primers, and 1.17 µl of RNase-free H₂O (Sigma, St. Louis, MO) at 65 °C for 5 min. Subsequently, 10 µl of 5× buffer and 2 µl of Superscript III Reverse Transcriptase Enzyme Supremix (Invitrogen) were added, and the mixture was incubated at 25 °C for 10 min followed by 50 °C for 50 min and 85 °C for 5 min.

PCR was performed using degenerate primers designed from consensus regions of cDNA sequences for VT and IT receptors from fish and amphibians. For the V1-type teleost VT receptor, one set of nested degenerate primers was designed to VT receptor cDNAs described previously from European flounder (*Platichthys flesus*, GenBank accession no. AF184966) (Warne, 2001) and cichlid (*Astatotilapia burtoni*, accession no. AF517936). For the V2-type VT receptor, BLASTX searches of GenBank using cDNA sequences for the V2 receptor from the amphibians *Taricha granulosa* (EF567078) and *Cynops pyrrhogaster* (AB274038) identified two putative teleost V2 receptor sequences. These two sequences – an EST sequence (GenBank no. XM.001345969) from zebrafish (*Danio rerio*) and a genomic DNA sequence (GenBank no. CAAE01014991) comprising part of chromosome 9 of puffer (*Takifugu rubripes*) – were aligned (Sequencher, v.4.8, Gene Codes Corp., Ann Arbor, MI) and the resulting alignment used to design nested degenerate primers. For the isotocin receptor, nested primers were designed to the IT receptor cDNA sequence from white sucker (*Catostomus commersoni*, accession no. X87783) (Hausmann et al., 1995) and deduced sequence from zebrafish genomic DNA (XM.001341471). Degenerate primers for all receptors are provided in Table 1.

Partial cDNAs for β -actin and elongation factor-1 α (EF-1 α) were also isolated and sequenced from Amargosa River pupfish for use as control genes (Table 1). Degenerate primers for β -actin were designed from zebrafish (AF057040), mud carp (*Cirrhinus molitorella*; DQ007446), goldfish (*Carassius auratus*; AB039726) and the carp *Spinibarbus denticulatus* (DQ656598), and to the β -actin gene sequence from *Rivulus marmoratus* (AF168615). For EF-1 α , degenerate nested primers were designed to consensus regions of EF-1 α cDNAs from *Oryzias latipes* (NM.001104662), *Pagrus major* (AY190693), *Carassius auratus* (AB056104) and *Seriola quinqueradiata* (AB032900). These primer sets amplified a 697-bp partial sequence of β -actin (EU886377) and a 827-bp partial sequence of EF-1 α from Amargosa pupfish (EU906930).

Table 1

Degenerate primers used for isolation of partial cDNAs for VT/IT receptors, β -actin and EF-1 α .

Receptor	Primer name	Direction	Sequence (5' to 3')
V1-type	V1-F1	Sense	CACAGAGGGGAGTGCAT
	V1-R1	Antisense	CTCGCTCAGGGAGAAGATGAA
	V1-N1	Sense	GTGGT(C/T)GC(C/T)TTCTTCCAGGT
	V1-N2	Antisense	CGGT(G/T)ATCCAGGTGAT(A/G)TACC
V2-type	V2-F1	Sense	ATGGTCAA(C/T)CTGTGTGTGC(A/C)GA
	V2-R1	Antisense	ACCTGACA(A/G/T)(A/C/T)(A/G)GTGAT(A/G)AT(A/G)(A/T)G
	V2-N1	Sense	GTTTGC(A/C/T)TCCTC(C/T)TACATGATTGT
	V2-N2	Antisense	GTCATCCAGGT(A/G)AC(A/G)TAGGC
ITR	ITR-F1	Sense	ATGAAGCACCTCAGCATTGC
	ITR-R1	Antisense	AACATGGAGATGATAAAGGGCAT
	ITR-N1	Sense	CCTCAACTCATTGGGACATCA
	ITR-N2	Antisense	GTCATTTTAACGGTGGTATTGTTGG
β -actin	β -actin-F1	Sense	ATCATGTT(C/T)GAGACCTTCAACACCC
	β -actin-R1	Antisense	TACTCCTGCTTGT(A/G)ATCCACAT
	β -actin-N1	Sense	GTGACATCAAGGAGAAGCT(G/C)TGCTA
	β -actin-N2	Antisense	GCAATGCC(A/G)GGGTACATGGT
EF-1 α	EF1a-F1	Sense	GGGAAAGGAAAA(A/G)(A/C/T)CCACAT
	EF1a-R1	Antisense	C(C/T)TTGAC(A/G)GACACGTTCTT(G/C)A
	EF1a-N1	Sense	CACAT(C/T)AACATCGTGGT(C/T)ATTGGC
	EF1a-N2	Antisense	ACGTTGTACCAGG(A/C/G)(A/G)(C/T)(A/G)GC

For each gene of interest, first-strand cDNA was amplified in a 50 μ l PCR reaction containing 2 μ l of reverse-transcribed cDNA, 3.0 μ l MgCl₂ (25 mM), 10 μ l of 10 $\times$ Flexi buffer, 1.0 μ l dNTP, 0.25 μ l GoTaq polymerase (Promega, Madison, WI), and 31.75 μ l RNase-free H₂O under a thermal profile of 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 50–52 °C for 30 s, and 72 °C for 1 min, and ending with 72 °C for 10 min on a PT-100 Thermal Cycler (MJ Research). When electrophoresis of the PCR product on a 1.2% agarose gel revealed a band of predicted size, the cDNA was purified (QIAquick PCR Purification Kit, Qiagen, Inc., Valencia, CA) and sequenced on a ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Inc.) using ABI Big Dye Terminator Cycle Sequencing Kit 3.1. Consensus sequences for each partial length cDNA were assembled using Sequencher™ v.4.8 (Gene Codes Corp., Ann Arbor, MI).

2.1.3. Cloning and sequencing of full-length cDNAs

Nested gene-specific primers for all identified VT and IT receptors from Amargosa River pupfish were designed from the partial cDNA sequences obtained above. These primers were then used to acquire the full-length cDNA sequences by 5'- and 3'-rapid amplification of cDNA ends (BD SMART RACE cDNA Amplification Kit, Clontech Laboratories, Inc., Mountain View, CA). Gene-specific primers for the RACE reactions are provided as part of the accompanying on-line Supplemental Materials, Table 1. First-strand cDNA was amplified in 50 μ l reactions with the following reaction components: 5 μ l of 10 $\times$ High Fidelity PCR Buffer, 2 μ l of MgSO₄ (50 mM), 1 μ l of dNTPs (10 mM), and 0.2 μ l of Platinum® Taq High Fidelity Polymerase (Invitrogen, Inc.), along with 3 μ l of cDNA template. Amplification occurred under a thermal profile of 5 cycles of 94 °C for 30 s followed by 72 °C for 2 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 2 min, and then 25 cycles of 94 °C for 30 s, 68 °C

for 30 s, and 72 °C for 2 min. Electrophoresis of the 5'- and 3'-RACE products on 1.2% agarose gels revealed single bands for some receptors; if a single band was not evident after the initial PCR, the nested gene-specific primers were used to amplify the 5'- and 3'-RACE products. The resulting cDNAs were cloned (TOPO TA Cloning Kit, Invitrogen) using ampicillin resistant bacteria, and the resulting plasmids were extracted, purified (Purelink™ Quick Plasmid Miniprep Kit, Invitrogen) and sequenced. Full-length cDNA sequences were obtained by aligning overlapping 5'- and 3'-RACE products (Sequencher™ v.4.8 software) and compared against other neurohypophyseal receptor sequences using BLAST.

The full-length sequence for each cDNA was confirmed by PCR using primers designed to the 5' and 3' untranslated regions (UTRs) of each transcript. Primers used in these full-length PCRs are provided as on-line Supplemental Material, Table 2. Reverse-transcribed total RNA was amplified in 50 μ l reactions containing 2 μ l of cDNA template, 21 μ l nuclease-free H₂O, 25 μ l of Taq DNA polymerase (GoTaq Colorless Master Mix, Promega), and 1 μ l each of forward and reverse primer under a profile of 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 4 min, and then 72 °C for 5 min. The resulting PCR products were examined on 0.8% agarose gels, and then purified (QIAquick PCR Purification Kit, Qiagen, Inc.) and sequenced.

Amino acid alignments between the resulting pupfish VT/IT receptors and homologous receptors for vertebrate neurohypophyseal hormones were made using the ClustalW method (Lasergene software; DNASTAR, Inc., Madison, WI). The secondary structure of each membrane receptor was predicted using the Predict Transmembrane Topology (MEMSTAT v.3) program on the PSIPRED Protein Structure Prediction Server (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Jones et al., 1994; Jones,

Table 2

Nucleotide sequences for primers and Taqman probes used in quantitative real-time RT-PCR.

Transcript	Primer or probe	Sequence (5' to 3')	Amplicon size (nucleotide bp)	PCR efficiency (avg. %)
V1a1	forward primer	CTGCTCATCGGAAATCTCCTGCAA	155	101.1%
	probe	ACCTAACGCAGAAGCAGGAGATTGGA		
	reverse primer	TGATTTCGATCTTGCCACCTCT		
V1a2	forward primer	GGTGCAAATGTGGTCAGTGTGGGATA	140	105.1%
	probe	TTGCCAGTCTCAACAGCTGCTGCAA		
	reverse primer	GAAGGTGGCCGCTAAAGATCATGT		
V2	forward primer	CGCCCTCATCATTACCATCTGTCA	101	104.5%
	probe	CCGCGAGATTCAACAACATCTACCTG		
	reverse primer	AGCTCAGCCATCACTATCCTCT		
ITR	forward primer	CACCTGAGCATCGCAGATCTTGTT	179	102.9%
	probe	ATGTTTGCGTCCACTACATGCTGGT		
	reverse primer	GGCTGGCAGACTGCTAAACATCTA		
pro-VT	forward primer	ATCAGAACCAGCAGCCATGCATCA	89	106.3%
	probe	TGTGCGTCTGGGACTCTCGCCCTCT		
	reverse primer	AGTTCTGGATGTAGCAGGCGGA		
EF1 α	forward primer	TACAAGTGCGGAGGAATCGACAAG	162	102.2%
	probe	TGGACAAACTGAAGGCCGAGCGTGA		
	reverse primer	GGTCTCAAACCTCCACAGAGCGAT		

Table 3

Amino acid identity (%) of the Amargosa pupfish VT receptors and IT receptor with other members of the vasopressin/vasotocin and oxytocin/mesotocin/isotocin receptor family.

	Accession no.	V1a1 pupfish	V1a2 pupfish	V2 pupfish	ITR pupfish
V1a1 pupfish	GQ981412	–	69.74	32.99	50.50
V1a2 pupfish	GQ981413	69.74	–	34.07	47.81
V2 pupfish	GQ981414	32.99	34.07	–	33.76
ITR pupfish	GQ981415	50.50	47.81	33.76	–
VTR flounder	AF184966	70.69	85.16	33.48	49.48
VTR cichlid	AF517936	70.59	84.11	31.72	48.83
VTR white sucker	X76321	70.95	73.25	33.26	51.02
V1a lungfish	AB377531	64.11	68.57	33.97	49.75
V1/V1a firebelly newt	AB274037	58.94	64.16	32.92	48.77
V1a chicken	NM001110438	59.33	62.79	33.20	45.07
V1a mouse	NM.016847	54.59	58.69	33.00	44.53
V1a human	NM000706	53.81	58.31	32.79	45.68
V3/V1b firebelly newt	AB284503	49.88	52.32	33.61	44.17
V1b chicken	NM001031498	49.88	46.90	36.34	46.76
V1b mouse	NM.011924	50.49	51.25	35.55	47.25
V1b human	NM000707	49.64	50.37	36.70	47.19
V2 lungfish	AB377532	42.08	41.96	47.46	41.07
V2 firebelly newt	AB274038	41.32	44.48	44.35	39.89
V2 chicken	NM001031479	44.59	44.39	35.81	44.00
V2 mouse	NM.019404	39.53	39.40	41.54	38.22
V2 human	NM000054	39.22	38.59	41.58	36.41
ITR white sucker	X87783	48.39	50.26	33.12	73.15
MTR roughskinned newt	DQ186599	46.80	49.47	33.83	62.69

Abbreviations: VTR, vasotocin receptor; V1a, vasopressin/vasotocin V1a receptor; V1b, vasopressin/vasotocin V1b receptor; V3/V1b, vasotocin V3/V1b receptor; V2, vasopressin/vasotocin V2 receptor; ITR, isotocin receptor; MTR, mesotocin receptor.

2007), and hydrophobicity plots for each deduced receptor amino acid sequence were generated on the Kyte-Doolittle scale (Kyte and Doolittle, 1982).

2.2. Sequencing of VT and IT receptor genes from genomic DNA

Genomic DNA was isolated from the liver using the DNeasy Cell and Tissue Kit (Qiagen) prior to being treated with RNase (Promega). Genomic DNA was amplified in a 50 µl PCR reaction containing 2 µl of DNA template (~200 ng), 25 µl GoTaq Colorless Master Mix (Promega), 21 µl nuclease-free H₂O, and 1 µl each of forward and reverse primer (10 µM) using a thermal profile of 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 3–4 min, followed by a final extension of 72 °C for 10 min. Primer pairs were identical to those used above for full-length cDNA sequence confirmation, and are provided as on-line Supplemental Material (Table 2). The resulting PCR products were visualized on 0.8% agarose gels with ethidium bromide (Invitrogen, Inc.) and then purified (QIAquick PCR Purification Kit, Qiagen, Inc.) and sequenced.

2.3. Phylogeny construction

Full length amino acid sequences for all identified pupfish VT and IT receptors were aligned to the sequences of receptors in the VP/VT, OT/MT/IT, and related neuropeptide families from other vertebrates and invertebrates obtained from GenBank. Amino acid sequences were aligned using ClustalX (Larkin et al., 2007). Phylogenetic analyses were conducted using MEGA v.4.1beta (Tamura et al., 2007), and a tree was assembled by the Neighbor-Joining method (Saitou and Nei, 1987) using a p-distance model. All positions containing alignment gaps were eliminated only in pairwise sequence comparisons (pairwise deletion of gaps). Confidence values for clusters of associated taxa were obtained by bootstrap tests (1000 replicates).

2.4. Tissue distribution patterns of VT and IT receptors

One adult male (body mass, 2.22 g; SL, 41.50 mm) and one adult female (1.65 g, 37.40 mm) pupfish were collected by minnow trap on 28 May, 2007 from the Amargosa River. Pupfish were immediately euthanized in MS222, and the brain, pituitary gland, gills, heart, gonads, liver, kidney, gastrointestinal (GI) tract and a piece of skeletal muscle from the side of the caudal peduncle were dissected. The dissected brain was further subdivided into the forebrain (olfactory bulb and telencephalon), midbrain (optic tectum and hypothalamus), cerebellum, and hind-brain (medulla oblongata). All tissues were stored individually in RNAlater (Applied Biosystems, Inc.). Total RNA was extracted from each tissue using Tri-Reagent (Molecular Research Center, Inc.) and treated with DNase I (Invitrogen) before being quantified by spectrophotometry. First strand cDNA was then synthesized in 15 µl reverse transcription reactions containing 0.72 µg of total RNA template and the RT reaction conditions described above. PCR was performed using GoTaq Master-Mix (Promega) under the following thermal profile: 95 °C for 2 min, 30–34 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 5 min (except for β-actin, where PCR was performed for only 24 cycles). Primers for each transcript are provided in the on-line Supplementary Materials, Table 3. PCR products (range:

224–291 bp nucleotides in size) were visualized on 2% agarose gels with ethidium bromide (Invitrogen Corp, Carlsbad, CA) and photographed using a BioRad Gel Doc 2000 Gel Documentation System (BioRad Laboratories, Hercules, CA).

2.5. Semiquantitative RT-PCR evaluation of gonadal VT/IT receptor mRNA abundance

A total of four ($n=4$) sexually mature female (body mass, 1.42 ± 0.18 g; SL, 37.12 ± 1.88 mm; mean $\pm$ SEM) and four ($n=4$) sexually mature male (body mass, 1.69 ± 0.15 g; SL, 37.37 ± 1.18 mm) pupfish were collected by minnow trap from the Amargosa River on 29 May 2009. Fish were quickly euthanized in MS222, and the testes and ovaries were dissected and stored in RNAlater. Microscopic examination of one gonad from each fish indicated that all fish were sexually mature, and the presence of mature oocytes throughout the ovaries suggested that it is likely that these large pupfish were actively spawning, as would be expected for this population during April–May (Soltz, 1974). Total RNA was extracted from the other gonad of each fish (Tri-Reagent; Molecular Research Center, Inc.), DNase I (Invitrogen) treated, and quantified by spectrophotometry before first strand cDNA for each sample was synthesized in 25 µl reverse transcription reactions containing 1.5 µg of total RNA template under the RT reaction conditions described above. PCR was performed using GoTaq MasterMix (Promega) under the following thermal profile: 95 °C for 2 min, 24–35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 5 min. Primers for each receptor were identical to those described above for assessing tissue distribution patterns of each receptor transcript. PCR products were visualized on 2% agarose gels with ethidium bromide (Invitrogen) and photographed using a BioRad Gel Doc 2000 Gel Documentation System (BioRad Laboratories, Hercules, CA). Relative PCR product band intensity was then quantified in each testis and ovary using ImageJ 1.37v software (National Institutes of Health, USA). Statistical comparisons of gonadal transcript abundance for each VT receptor are expressed as relative levels, so that the tissue (testis or ovary) with the lower mean value obtained a relative value of 1. Statistical comparisons were then calculated between the sexes using t tests (Zar, 1996).

2.6. Salinity regulation of pro-VT and VT/IT receptor mRNAs

2.6.1. Acute salinity challenge

Juvenile Amargosa pupfish (body mass, 0.55 ± 0.08 g; standard length, 24.70 ± 0.78 mm; mean $\pm$ SEM) were collected from the Amargosa River on 30 May 2009 using minnow traps. Physical parameters in the Amargosa River were recorded as a salinity of 2.1 ppt, conductivity of 4060 µS, and dissolved oxygen content of 9.57 mg/l (YSI 85, YSI Inc., Yellow Springs, OH). Pupfish were maintained in 19l, aerated tanks at ambient river temperatures for 7 days before being haphazardly assigned to one of three salinity treatments: 2.1 ppt (control), 17 ppt, or 34 ppt. Salinity changes in the aerated tanks were generated by the gradual addition of hyper-saline water (50 ppt; made from ambient water from the Amargosa River with Instant Ocean®, Aquarium Systems, Sarrebourg, France) over a period of 2 h until experimental salinities (17 ppt or 34 ppt) were achieved. Fish were maintained in these treatment groups for periods of either 5 h or 20 h (after beginning salinity

Technologies (Coralville, IA) and are provided in Table 2. When possible, primers for quantitative RT-PCR reactions were designed to span an intron. Selected real-time RT-PCR products for each of these primer sets were sequenced to confirm specificity of the primer and probe sets.

Quantitative RT-PCR reactions were run in 25 µl volumes with each reaction containing 12.5 µl Master Mix (ABI Universal MasterMix Reagent), 0.5 µl forward primer (45 µM), 0.5 µl reverse primer (45 µM), 0.5 µl probe (10 µM), 8.0 µl ddH₂O (nuclease-free water, Sigma) and 3.0 µl of reverse-transcribed cDNA template. Reactions were run on an ABI 7500 Sequence Detector under a PCR thermal profile of 50 °C for 2 min, 95 °C for 10 min, and then 38 to 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions of total RNA from the experiment were used as a standard curve reference. Samples comprising the standard curve were run in triplicate, and all experimental samples were run in duplicate (average % CV = 0.56%). For each gene measured, DNA contamination was assessed by analyzing a total RNA sample that was not reverse-transcribed, and each run included duplicate samples lacking cDNA template to further control for contamination. *EF-1α* was also quantified as a normalizing gene. Mean *EF-1α* transcript abundance was stable across all salinity treatment groups. Correlation coefficients (r^2) for the standard curve of each gene ranged from 0.989 to 0.996. PCR efficiencies for each transcript were calculated by the following equation: efficiency = $-1 + 10^{(-1/\text{slope})}$, and are provided in Table 2.

Relative VT and IT receptor mRNA expression was subsequently calculated using the serially diluted standard curve, normalized to *EF-1α* mRNA expression in that tissue, and expressed as a relative level by dividing the resulting values to the mean of the designated control group within each sampling time (5 h or 20 h). Normalization of transcript abundance values was conducted within each sampling time because it was found that the pupfish hypothalamus exhibits diel variation in *pro-VT* and *VT/IT* receptor transcript abundance (Lema et al., submitted for publication), which makes the effects of salinity treatment difficult to distinguish when plotted without normalization. Statistical comparisons of mRNA levels were carried out within each sampling time (5 h and 20 h) using one-factor ANOVA models, or a Welch ANOVA model when variances failed to conform to assumptions of normality even after transformation (Zar, 1996). When these models revealed a significant difference, pairwise comparisons were performed between each salinity treatment group and its respective control group using Dunnett's tests. All data are presented as mean ± SEM, and sample sizes (*n*) for each treatment group are provided in the appropriate figures.

3. Results

3.1. Isolation and sequencing of two distinct *V1a*-type receptors from *C. n. amargosae*

Two cDNA fragments encoding putative pupfish VT receptors were identified using RT-PCR with degenerate primers designed to other teleost VT receptors. BLAST analysis of these two fragments confirmed that both cDNAs shared high sequence similarity with VT receptors identified previously in other teleost fishes, but indicated that these two pupfish cDNAs were substantially different from each other. BLAST analysis of the two partial cDNAs revealed greatest homology with two VT receptor gene sequences from puffer (*Takifugu rubripes*, Genbank accession nos. AY027886 and AY027887), suggesting the isolation of two distinct pupfish VT receptor transcripts. Subsequent sequencing of full-length coding regions for these two pupfish cDNAs was conducted using 5'- and 3'-rapid extension of cDNA ends (RACE) PCR. The first full-length cDNA transcript, named *V1a1* (GenBank accession no. GQ981412), was 1634-bp nucleotides in length with a 1236-bp nucleotide open reading frame encoding a predicted protein of 412 amino acids (Fig. 1A). Examination of the secondary structure for this predicted protein revealed the presence of seven transmembrane domains (Fig. 1B), which is structurally characteristic of class A membrane receptors including all VP/VT receptors. The second full-length transcript, named *V1a2* (GenBank accession no. GQ981412), was 1429-bp nucleotide in length with a 1155-bp open reading frame encoding a deduced protein of 385 amino acids (Fig. 1C). Analysis of the secondary structure of this deduced *V1a2* protein again indi-

cated the presence of seven hydrophobic transmembrane helices (Fig. 1D). Full-length PCR using primers designed to the UTRs of each cDNA amplified two *V1*-type receptor genes: one contained a 460-bp nucleotide intron and encoded the *V1a1* transcript (GenBank accession no. GU014233), and a second for the *V1a2* transcript that included a 1555-bp intron (GU014234).

3.2. Isolation and sequencing of a teleost *V2*-type VT receptor cDNA

Degenerate primer PCR identified a partial cDNA fragment for a *V2*-type receptor from *C. n. amargosae*. Extension of this partial cDNA generated a 2230-bp full-length cDNA with an open reading frame of 1491-bp nucleotides encoding a predicted protein of 497 amino acids (GenBank accession no. GQ981414) (Fig. 2A). Subsequent analysis of the secondary structure for the deduced pupfish *V2*-type receptor protein again revealed the presence of seven transmembrane domains (Fig. 2B). Full-length PCR and subsequent sequencing using gene specific primers designed to the 5'- and 3'-UTRs of this *V2* cDNA identified a gene of 3679-bp, with a 1727-bp intron located in the open reading frame (GenBank accession no. GU014235).

3.3. Isolation and sequencing of the isotocin receptor from *C. n. amargosae*

To demonstrate that *C. n. amargosae* contains a distinct IT receptor mRNA in addition to the three VT receptor cDNAs identified above, degenerate primer PCR was used to isolate and sequence a partial cDNA fragment for the IT receptor. Extension of this initial cDNA fragment by RACE PCR generated a full-length transcript of 2295-bp nucleotides containing an 1185-bp nucleotide open reading frame encoding a deduced sequence of 395 amino acids (Fig. 2C) that showed high homology to the IT receptor from white sucker (*Catostomus commersoni*) (Hausmann et al., 1995). Secondary structural analysis of this predicted protein indicated the presence of seven hydrophobic transmembrane domains (Fig. 2D). As above, full-length gene specific primers were designed to the 5'- and 3'-UTRs of this Amargosa River pupfish IT receptor (*ITR*) cDNA (GenBank accession no. GQ981415), and used to isolate and sequence the *ITR* gene from pupfish genomic DNA (Genbank accession no. GU014236).

3.4. Sequence comparison and phylogenetic analysis of teleost VT and IT receptors

Alignment of predicted amino acid sequences for the Amargosa pupfish *V1a1*, *V1a2*, *V2*, and *ITR* cDNAs indicated closest structural similarity between *V1a1* and *V1a2*, although all four putative receptors showed high homology in transmembrane domains 2, 3, 6 and 7 (Fig. 3). Comparison of amino acid sequences likewise revealed strongest identity (69.7%) between the *V1a1* and *V1a2* pupfish receptors, and both of these receptors showed high identity to previously identified VT receptors from other Actinopterygian fishes (70.6–85.2%) (Table 2). The pupfish *V2* receptor showed considerably lower sequence identity to the *V1a1* and *V1a2* receptors (33.0–34.0%), as well as to previously described VT receptors from other teleosts (31.7–33.5%). This pupfish *V2* receptor did, however, show greater amino acid sequence identity to *V2*-type receptors

Fig. 2. *Cyprinodon nevadensis amargosae* *V2* (GenBank accession no. GQ981414) (A) and IT receptor (GQ981415) (C) cDNA receptor sequences and their conceptual translation. Transmembrane segments predicted using MEMSTAT (<http://bioinf.cs.ucl.ac.uk/psipred/>) are underlined in the nucleotide sequence. Start and stop codons are highlighted in black, and polyadenylation signals are bolded. Introns located in the *V2* (GU014235) and *ITR* (GU014236) genes are also shown as lowercase regions of nucleotide basepairs within the open reading frame. Kyte-Doolittle hydrophobicity plots for the *V2* receptor (B) and IT receptor (D) illustrate the seven transmembrane domains within each receptor protein.

V1a1	-----MLCSSEISCNITEVFNLTKQKE-----IGAAGSARNLSGKTN	37
V1a2	-----MMGTSPSNSTVHPN	13
ITR	-----MDELLSAQDAWLQNF-----YCNYSHLNKTHTPGNN	31
V2	MERISVETDWDGLGLSSPGASEGNFVSFVTELSFNASRTGGSI FGIFQENGSNTPHS	60
	TM I ICL I TM II	
V1a1	DTDPFGRNEEVAKIEITVLSLAFVAAVGNLSVLLAMYRSRKL SRMHLFMKHLSLADLV	97
V1a2	GSDPFARNEDVAQIEIMVLSITLVVAVIGNVSVLLAMYN TKKMSRMHLFIKHLSLADLV	73
ITR	VVNPLKRNEEVAKVEVTVLVLVLLALTGNLCVLWAIHTTKH SKSRMYF MKHLSIADLV	91
V2	LPQPRTRDQGLARAEIAVLGMVLALTTLGNSFVLWVLLRR RKHNAPMHLFMVNL CVADLV	120
	:*: *	
	↓ ECL I ↓ ↓ TM III ICL II	
V1a1	VAFFQVLPQLCWEVTFRFYGPDFLCRIVKHLQVLGMFAST YMMVMTLD RYIAICHPLQT	157
V1a2	VAFFQVLPQLCWKITDRFYGTDALCRIVKHLQVMGMFAST YMMVMTLD RYIAICHPLKT	133
ITR	VAVFQVLPQLIWDITFRFYGPDII CRLVKYLQVVGMFAS TYMLVLSID RCLAVCQPLRS	151
V2	VALFQVLPQLIWDITERFQGPDLLCRSVKYLQIVGMFAS SYMIVAMTVDRHHAICCP LQA	180
	**: **: *: * *: *: * *: *: *: *: *: *: *: *: *: *: *: *: *	
	TM IV ECL II ↓	
V1a1	LQQ-PTRRAYIMISSTWACSLVLSTPQYCFSLSEVRPGSA VYDCWGHFVEPWGPRAYIT	216
V1a2	LQQ-STKRSYVMIISTWICSLVLSSPQYFIFSMSEIKNGSE VYDCWAHFIEPWGSKAYIT	192
ITR	VHK-GKDR--FCVIGSWILSLVFSTPQAYIFSLREVGNG-- VYDCWGD FVQPWGAKAYIT	206
V2	YRVGAMSRWNTPVMVAWGLALVLSIPQVFIFSRSEVAPG-- EFECWGHFTEPWGLKAYIT	238
	::: : * : : : : *: *: *: *: *: *: *: *: *: *: *: *: *	
	↓ TM V	
V1a1	WITAGIFLVPVAVLVFCYGFICRAIWRNLKCKTR-----	250
V1a2	WMTVGIFLVPVILILCYGFICHSIWKNIKYKKK-----	226
ITR	WMSLSIYIIPVAILSICYGLICFKIWNQINMKT-----	240
V2	WMTVAVFLLPALIITICQIRIFREIHNNIYLKSERIVMAEL KRSENLFRIHGFRT EEDRA	298
	* : : : : *: : *: *: *: * : *	
V1a1	-----	
V1a2	-----	
ITR	-----	
V2	RERERGRQVPRGGGGQLLKDSNLHDCEVGQHYDYVPSVI QYSSCCGQTEGTSLSQE QIP	358
	ICL III TM VI ↓	
V1a1	-----RKSADAVVEATKSGILGRSSVSSVSTISRACLRT VKMTFVIVVAVVVCWAPFFTV	305
V1a2	-----KT VAG---AAGKNGLIGKCSVSSITISRACLRT VKMTFVIVLAYIICWAPFFTV	278
ITR	-----REHFLALTTPRPSKSAHPLSRVSSVRLISKAKIRTV KMTFVVVVIAYIVCWTPFF FV	295
V2	KGSDFRESCTTSPRCSLDCAPPPHPVTPPHSITKAMSKTV RMTLVIVLVYTICSPFFID	418
	: : : : *: *: *: *: *: *: *: *: *: *: *	
	↓ ECL III TM VII	
V1a1	QMWSVWDKTFSWDDSENTAVSLASALLASLNSCCNPWIY MTFSGHLLQDFMHCFS CCYRLR	367
V1a2	QMWSVWDKNFKWDDSENTAVSLASALLASLNSCCNPWIY MTFSGHLLQDFMHCFS CCRKLN	338
ITR	QMWSAWDPAPRE---DMAFTISMLLASLNSCCNPWIYMLF AGHLFHD LKSFCCCCRN-	352
V2	QLWPAWDPPDQ---GVAFTILMLLASLNSCTNPWIYTA FSSSVSRELQNLQCRSRLG	475
	*: *: *: *: : : : : *: *: *: *: *: *: *: *: *: *: *	
V1a1	DKLKKQDSDSSIRRTTLLSRLQG---PRLSKPFRELNYTI KNCPQATPAS	412
V1a2	ADYKKEDSDSSIRRTTLLTRITNRSPTGSSSNWRELDNS PKTSAQTE---	385
ITR	---YVADSSCHVNQECRHKRGSSFTDIKNGSSMRSLTHT SSLGGPTH---	395
V2	RRGSLPDDSTATHTSTTKDNLY-----	497
	: : *: : : : : : : : : : :	

Fig. 3. Sequence alignment for amino acid residues of the *V1a1*, *V1a2*, *V2* and *ITR* cDNAs identified from Amargosa pupfish. Putative transmembrane helices (TM I–TM VII) for each receptor were predicted using MEMSTAT (<http://bioinf.cs.ucl.ac.uk/psipred/>) and are delimited by gray boxes. Intracellular loops (ICL) and extracellular loops (ECL) are also illustrated. Consensus between the sequences is indicated by asterisks (four identical residues) or by double dots (conservation between the *V1a1* and *V1a2* paralogs). Conserved residues shown to be important for receptor binding to the VT/IT peptides are marked by arrows. Black arrows indicate conserved residues that form a hydrophobic cleft in the receptor; white arrows indicate residues that form a lipophilic pocket; and gray arrows indicate residues that are involved in specific stacking interactions.

from lungfish and amphibians (44.5–47.4%). The comparatively low sequence identity between the pupfish *V2* receptor and *V2*-type receptors from other vertebrates results from an elongated intracellular membrane domain (ICL III) located between transmembrane helices 5 and 6 in the pupfish *V2* receptor (Fig. 3). This elongated intracellular domain is not present in either the *V1a1* or *V1a2* receptor (Fig. 3), nor in any other previously identified teleost VT receptors.

A phylogenetic tree comparing amino acid sequences for the pupfish *V1a1*, *V1a2*, *V2*, and *ITR* to VP/VT and OT/MT/IT receptors from other vertebrates, as well as to VP/VT-like receptors from invertebrates, is shown in Fig. 4. This phylogeny revealed that the vertebrate VP/VT and OT/MT/IT receptors clustered into four distinct clades: *V1a*-type VP/VT receptors, *V1b*-type VP/VT receptors, OT/MT/IT-type receptors, and *V2*-type VP/VT receptors (Fig. 4). The newly identified pupfish *V1a1* and *V1a2* receptors belonged

Fig. 4. Phylogenetic tree based on alignment of deduced amino acid sequences for vasopressin/vasotocin and oxytocin/mesotocin/isotocin receptors from vertebrates, and for related neuropeptide receptors from invertebrates. Tree was assembled with the neighbor-joining method with pairwise deletion, and bootstrap values from 1000 replicates are indicated at each node. GenBank accession nos. for each sequence are provided following the taxon designation. Arrows indicate the receptors encoded by the pupfish *V1a1*, *V1a2*, *ITR* and *V2* cDNAs ((†) partial protein sequence only; (*) receptor amino acid sequence predicted from genomic DNA) (See Refs. Kawada et al., 2008; Stafflinger et al., 2008).

Fig. 5. Distribution of VT receptor (V1a1, V1a2 and V2) and IT receptor mRNAs in the tissues and organs of the male (A) and female (B) Amargosa pupfish. RT-PCR products were analyzed by electrophoresis on a 2% agarose gel with ethidium bromide staining. PCR product sizes for each transcript are provided.

to the V1a-type VP/VT clade, while the pupfish *ITR* deduced protein grouped within the clade comprised of all vertebrate OT/MT/IT receptors. The pupfish V2 receptor likewise grouped within a clade containing the V2-type receptors from mammals, amphibians, and lungfish. Interestingly, however, the two avian V2-type receptors identified to date – a VT receptor cDNA from chicken (GenBank accession no. NM.001031479 and AF147743) and a putative V2-type VT receptor gene from zebra finch (Genbank accession no. XM.002195382) – do not group within this same V2-type receptor clade, suggesting that these described avian VT receptors may instead belong to a previously unrecognized class of VT receptors distinct from teleost, amphibian and mammalian V2-type receptors (Fig. 4).

3.5. Comparison of tissue expression patterns for VT and IT receptor mRNAs

Receptor mRNA transcript levels, as indicated by RT-PCR, varied among organs and tissues (Fig. 5). *V1a1* mRNAs were most abundant in the midbrain (optic tectum and hypothalamus), cerebellum and pituitary gland of both sexes. *V1a1* transcripts also showed a distinct sex difference in gonadal expression, with *V1a1* mRNAs abundant in the testis but not the ovary ($t = 6.756$, $p = 0.003$) (Fig. 6). The expression pattern of the *V1a2* receptor was distinct from that of the *V1a1* receptor, with expression highest in the midbrain, cerebellum, heart and skeletal muscle (Fig. 5). A fainter amplified *V1a2* cDNA product was also present in gill tissue (Fig. 5). The V2-type receptor transcript, in contrast, was most abundant in the gills, heart, interrenal gland, and liver (Fig. 5).

3.6. Regulation of pro-VT and VT/IT receptor mRNAs in the hypothalamus and gill by salinity

Acute exposure to hyperosmotic conditions (17 ppt or 34 ppt) for 5 h or 20 h altered transcript abundance for *pro-VT*, and for the VT/IT receptors, in the hypothalamus of juvenile pupfish

Fig. 6. Semi-quantitative RT-PCR comparison of VT receptor (V1a1, V1a2 and V2) mRNA levels in the gonads of sexually mature male and female pupfish. RT-PCR was run using gene-specific primers and the resulting PCR products visualized on 2% agarose gels. Relative band intensity was quantified using ImageJ 1.37v software, and *t* tests used to compare the expression of each transcript between ovarian and testicular tissues. Asterisks indicate a statistically significant difference between tissues (* $p < 0.05$, ** $p < 0.01$). The control gene, β -actin, showed similar transcript abundance in testicular and ovarian tissues. Transcript levels are expressed as relative values to the mean of the lesser expressed tissue (testis or ovary). All values are plotted as mean $\pm$ SEM.

Fig. 7. Acute exposure to elevated salinity results in decreased *pro-VT* transcript abundance in the hypothalamus. Results are expressed as normalized transcript abundance with respect to *EF-1 α* transcripts from the same sample. Values are shown as mean $\pm$ SEM, and sample sizes (*n*) are denoted on each bar. Values significantly different from the control of the same treatment time (Dunnett's tests) are indicated by asterisks ($p < 0.05$).

relative to individuals maintained at natural osmotic conditions (2.1 ppt). Elevated salinity (34 ppt) caused a reduction in hypothalamic *pro-VT* mRNA levels at both the 5 h (Welch ANOVA, $F_{2,18.323} = 3.37$, $p = 0.057$) and 20 h ($F_{2,21.679} = 4.67$, $p = 0.021$) sampling times (Fig. 7). Transcripts for VT receptors were also altered, with *V1a1* mRNA levels increasing by 50% in the hypothalamus

of fish transferred to 34 ppt salinity, relative to control (2.1 ppt) fish (Fig. 8A). This increase in *V1a1* mRNA levels was observed at both the 5 h ($F_{2,27} = 6.492$, $p = 0.005$) and 20 h ($F_{2,34} = 11.194$, $p = 0.0002$) sampling times. *V1a2* mRNA levels also showed a significant increase in pupfish experiencing 34 ppt salinity conditions, although this increase was observed only at the 20 h sampling time ($F_{2,34} = 11.386$, $p = 0.0002$) (Fig. 8B). Transcript abundance for the V2 receptor showed a distinctly different pattern of change, with a significant elevation in V2 mRNA levels in pupfish exposed to 17 ppt conditions, but a decline in V2 mRNAs under the 34 ppt conditions 5 h after commencing the osmotic challenge ($F_{2,27} = 10.456$, $p = 0.0004$) (Fig. 8C). These changes in V2 transcript abundance were more consistent at 20 h of exposure, when fish in both the 17 ppt and 34 ppt treatments displayed reduced hypothalamic V2 mRNA levels ($F_{2,34} = 15.708$, $p < 0.0001$). Salinity also altered the abundance of transcripts encoding the ITR receptor, although these changes were not observed until 20 h after exposure to elevated salinity, when *ITR* mRNAs were significantly reduced in the hypothalamus of pupfish from the 17 ppt treatment, but elevated in fish from the 34 ppt exposure group ($F_{2,34} = 19.634$, $p < 0.0001$) (Fig. 8D).

Acute hypersalinity challenge also affected levels of mRNAs encoding two of the VT receptors – as well as the ITR receptor – in the gills, although these changes were distinct from those observed in hypothalamic tissues. In the gills, mRNA levels for the *V1a2* receptor increased in pupfish 2- to 2.5-fold within 5 h of exposure to 17 ppt and 34 ppt salinities, and remained elevated at these levels for at least 20 h in fish exposed to the 34 ppt conditions (Fig. 9B). Transcripts for the *V1a1* receptor declined 40% in the gill within 5 h of transfer to 17 ppt water ($F_{2,27} = 6.204$, $p = 0.006$), but not 34 ppt

Fig. 8. Regulation of *V1a1*, *V1a2*, *V2* and *ITR* mRNA levels in the Amargosa pupfish hypothalamus following acute salinity challenge. Results are expressed as normalized fold transcript abundance with respect to *EF-1 α* mRNA levels from the same sample, with the mean values for each control group normalized to a value of 1.0. Values are shown as mean $\pm$ SEM, and sample sizes (*n*) for each group are denoted on the graph in A. Values significantly different from the control of the same treatment time (Dunnett's tests) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 9. Acute salinity elevation regulates mRNAs encoding the *V1a1* and *V1a2* receptors for vasotocin, and the IT receptor in gills of juvenile Amargosa pupfish. Transcripts for the V2 receptor were unaltered by exposure to elevated salinity. Values are shown as mean $\pm$ SEM, and sample sizes (*n*) for each group are denoted on the graph in A. Values significantly different from the control of the same treatment time (Dunnett's tests) are indicated as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

water; however, this decline in *V1a1* mRNA levels was not seen at the 20 h sampling time (Fig. 9A). Transcripts for the IT receptor also decreased in abundance by approximately 40%, although this decline was not apparent until 20 h of exposure to elevated salinity, and was only statistically significant in pupfish from the 34 ppt treatment ($F_{2,34} = 3.968$, $p = 0.028$) (Fig. 9D). V2 mRNAs in the gills were unaffected by elevated salinity (Fig. 9C).

4. Discussion

In the present study, three full-length cDNAs encoding putative VT receptors were identified and sequenced from a teleost, the Amargosa pupfish. In addition, a single cDNA encoding an IT receptor was isolated and sequenced from the same species. Phylogenetic comparisons of amino acid sequences for these pupfish VT receptors with other vertebrate neurophyophyseal hormone receptors revealed that two of the three pupfish VT receptors belong to the V1a-type clade of VP/VT receptors (*V1a1* and *V1a2*), while the third cDNA (*V2*) shares greatest amino acid structural similarity with V2-type receptors from mammals and amphibians. To the author's knowledge, this is the first evidence for two distinct V1a-type VT/VP receptor cDNAs in a teleost, as well as the first identification of a V2-type receptor transcript in a ray-finned fish.

Examination of the deduced amino acid sequences for these receptors reveals homology at several amino acid sites identified previously as important for receptor interaction with VT/IT (Hausmann et al., 1996; Archajee et al., 2004; Ślusarz and Gieidoń, 2006; Cho et al., 2007). All three putative VT receptor cDNAs contain Gln residues in the TM II helix (amino acid 106, as illustrated in the *V1a1* deduced protein sequence presented in Fig. 3) and TM III (*V1a1* amino acid 129) domains, which generate a hydrophilic cleft in the receptor for VT/IT binding. Similarly, a Lys residue within TM III (*V1a1* amino acid 126 in Fig. 3), Thr in TM V (*V1a1* amino acid

219), and Phe and Gln residues in TM VI (*V1a1* amino acids 303 and 306, respectively) are conserved across all three VT cDNAs. These four residues produce a lipophilic pocket in the receptor for VT binding (Hausmann et al., 1996). Several other amino acid residues also suggested to be important for VT/IT binding, including a Thr in TM V (*V1a1* amino acid 216 in Fig. 3), are also conserved in the putative VT/IT receptor cDNAs identified in pupfish. Although additional biochemical characterization of the three putative VT receptors is still needed, the conservation of these key residues across the three pupfish VT receptors suggests that these cDNAs may encode functional VT receptors.

4.1. Identification of two pupfish V1a-type VT receptor cDNA paralogs

Sequence comparison of nucleotide and amino acid structures for the two V1a-type VT receptors (*V1a1* and *V1a2*) from Amargosa pupfish suggests that these receptors are likely paralogs that evolved through a gene duplication event. Genome duplications have occurred repeatedly in many teleost fishes including those of the order Cyprinodontidae, and these duplications, whether occurring at the whole or partial genome level, led Cyprinodontid fishes to have multiple copies of many endocrine-related genes present only singly in mammals (Robinson-Rechavi et al., 2001; Larhammar et al., 2009). Further support for this hypothesis comes from the identification of partial cDNA sequences for a homologous pair of V1a-type receptors in the closely related Atlantic pupfish (*Cyprinodon variegatus*) (*V1a1*, Genbank accession no. GU120189; *V1a2*, GU120190) (S.C. Lema, current study, results not shown). Whether two V1a-type receptors have evolved more broadly in teleosts remains to be determined, but given the broad patterns of gene duplications seen in fish (Larhammar et al., 2009), and the presence of two gene sequences for homologous V1a-type receptors

in the Tetraodontid fish *Takifugu rubripes* (GenBank accession nos. AY027886 and AY027887), it seems likely that similar V1a-type receptor paralogs may be found in many teleost taxa.

While the functional roles of the *V1a1* and *V1a2* receptors remains to be determined, RT-PCR revealed that the two pupfish V1a-type VT receptors have distinct mRNA expression patterns, with *V1a1* mRNAs most abundant in the midbrain, cerebellum, pituitary gland and testis, and *V1a2* transcripts common in mid-brain, cerebellum, heart and muscle. Although RT-PCR expression patterns cannot pinpoint specific roles for either receptor, the distribution patterns do suggest functional differentiation. For instance, *V1a1* transcripts were abundant in the pituitary gland of both male and female pupfish, suggesting a possible role for this receptor in regulating negative feedback on VT secretion, or possibly as a modulator of VT-induced ACTH release and activation of the HPA axis (Baker et al., 1996; Engelmann et al., 2004). Supporting this idea, Pierson et al. (1996) demonstrated in previous work that VT stimulation of pituitary ACTH release in the rainbow trout occurred via a V1-type receptor-mediated pathway. Meanwhile, in the gonads, the dissimilar transcript distribution patterns suggest that the *V1a1* and *V1a2* receptors may have distinct functions in reproduction. Recently, VT peptide was localized to the gonads of the catfish *Heteropneustes fossilis* (Singh and Joy, 2008). Gonadal VT levels in this species showed a distinct sex difference, with higher VT in the ovary than the testis, but with both tissues showing changes in VT levels over the reproductive season (Singh and Joy, 2008). Singh and Joy (2009) subsequently found that VT regulates 17 β -estradiol production by the catfish ovary, with VT either increasing or decreasing 17 β -estradiol levels depending on the stage of the reproductive cycle. The sex difference in gonadal *V1a1* transcript abundance seen here between adult male and female pupfish therefore suggests that the *V1a1* receptor may play a role in regulating sex-specific effects of VT on the gonad.

In the central nervous system, *V1a1* and *V1a2* transcripts were abundant in the midbrain of male and female pupfish, suggesting a role for these receptors in mediating neural responses to VT. Neural expression of VT has been shown to be associated with social status in several fish species (Grober et al., 2002; Larson et al., 2006), and there is evidence that neural VT expression may correlate with species- or population-level variation in sociosexual behaviors (Lema and Nevitt, 2004a; Lema, 2006; Dewan et al., 2008). Previously, V1a-type receptor antagonists (e.g., Manning compound) have been shown to induce opposing effects to exogenous VT for social behaviors including aggression and courtship (Semsar and Godwin, 2004; Santangelo and Bass, 2006; see also Semsar et al., 2001; Lema and Nevitt, 2004b). Moreover, sex change in the bluehead wrasse (*Thalassoma bifasciatum*) has been shown to be induced by primary influences of the social environment on neural VT pathways (Semsar and Godwin, 2003). Future studies that examine the roles of *V1a1* and *V1a2* receptors in mediating behavioral transitions accompanying changes in social status or gonadal sex hold great promise for providing new insights into VT's regulation of behavior.

4.2. Identification of a V2-type receptor cDNA in pupfish

Results presented here provide the first description of a V2-type receptor cDNA in teleost fish. This full-length V2 cDNA, with an open reading frame encoding a deduced protein of 497 amino acids, showed greatest amino acid identity to V2-type receptors from tetrapods (Fig. 4). Additional evidence for the presence of V2-type receptors in other teleost fish comes from partial sequences for homologous V2 cDNAs obtained from the closely related Atlantic pupfish (*Cyprinodon variegatus*) (GenBank accession no. GU120191), and from the more distantly-related Perciform

fish, *Stegastes partitus* (GenBank accession no. GU120192) (S.C. Lema, current study, results not shown).

Even though the deduced protein encoded by the pupfish V2 cDNA showed the greatest sequence similarity to V2-type receptors from mammals and amphibians, the similarity was unexpectedly low (i.e., 47% identity to the lungfish V2-type receptor) (Table 3) due to the presence of an elongated intracellular loop III region (ICL III) in the deduced V2 receptor's structure (Fig. 3). The presence of this elongated ICL III loop was confirmed by full-length PCR and sequencing of both the V2 cDNA and gene using primers designed to the 5' and 3' UTRs.

In V2-type receptors (Böselt et al., 2009) and, more generally, rhodopsin-like G-coupled receptors (Probst et al., 1992), the ICL III loop of the receptor is the region showing least conservation in residue composition and length. In mammalian V2-type receptors, the ICL III loop has been shown to be critical for receptor signal transduction (Liu and Wess, 1996). The binding of VP to the mammalian V2 receptor induces transformational changes in the ICL III loop, which interacts with G-coupled protein G α S and other intracellular proteins (e.g., gC1qR) to generate an intracellular signaling cascade (Erlenbach and Wess, 1998; Bellot et al., 2009). The elongated ICL III loop of the pupfish V2 receptor might therefore indicate evolutionary divergence in the receptor's intracellular transduction mechanism.

Additional work is needed to determine whether the protein encoded by this newly identified V2 cDNA constitutes a functional receptor or represents a pseudogene or fusion gene. If this cDNA does not encode a functional receptor, the transcript might be playing a regulatory role in post-transcriptional intracellular processing of a similarly structured V2-type receptor in the Amargosa pupfish. It is interesting to note, however, that RT-PCR revealed a high abundance of the V2 transcript in the gills and kidney, which suggests a role for this receptor in maintaining hydromineral balance. Providing further support for this idea, results from the acute salinity challenge demonstrated that V2 transcripts were regulated in the hypothalamus and gill by hyperosmotic conditions (results discussed below).

Whether or not the V2-type cDNA encodes a functional receptor, its identification provides evidence that V2-type receptors evolved prior to the divergence of Actinopterygii fishes from the modern tetrapod lineage. The recent discovery and characterization of a V2-type receptor cDNA in the Sarcopterygian lungfish, *Protopterus annectens*, lends additional support for this early-origin hypothesis (Konno et al., 2009). Taken together, the presence of V2-type receptor cDNAs in these fishes indicate that V2-type VT/VP receptors evolved earlier in vertebrate evolution than previously thought, and it should be expected that similar V2-type receptor genes will be identified in other Actinopterygian fishes.

4.3. Response of pro-VT and VT/IT receptors to hyperosmotic challenge

Amargosa pupfish, and other members of the genus *Cyprinodon*, are euryhaline (Hillyard, 1981), and some taxa within the Death Valley clade of pupfishes can withstand salinities up to 105 ppt (Naiman et al., 1976). This extreme salinity tolerance has allowed *Cyprinodon* pupfishes to occupy a range of habitats – from coastal salt marshes to desert streams – which are outside the physiological scope of most other teleosts. It has not yet been examined, however, what role neurohypophyseal neuropeptides play in the regulation of hydromineral balance in pupfish.

In this study, the transfer of juvenile Amargosa pupfish from low salinity (2.1 ppt) to hypersaline (34 ppt) conditions caused a significant reduction in hypothalamic pro-VT transcript abundance. This decrease was seen in pupfish after 5 h and 20 h of exposure to 34 ppt (Fig. 7). In teleost fishes, it is well established that changes

in environmental salinity alter the secretion of VT, which then regulates hydromineral permeability at the level of the kidney, gills and intestine (reviewed by Warne, 2002). In freshwater rainbow trout (*Oncorhynchus mykiss*), hyperosmotic stress has been shown to result in increased plasma VT levels (Kulczykowska, 1997, 2001). Freshwater-acclimated medaka (*Oryzias latipes*) transferred to seawater showed reduced pituitary VT content (Haruta et al., 1991), while VT content of the pituitary has been shown to increase in seawater-acclimated medaka and flounder (*Platichthys flesus*) transferred to freshwater (Warne et al., 2000; Haruta et al., 1991).

Corresponding to these changes in pituitary and plasma VT levels following osmotic challenge, hypothalamic VT expression has also been shown to respond to altered environmental salinities. In rainbow trout, the number of VT-immunoreactive (VT-ir) neurons in the magnocellular region of the preoptic area decreased following transfer of fish from freshwater to seawater (Haruta et al., 1991). Hyodo and Urano (1991) demonstrated that *pro*-VT mRNA *in situ* hybridization signal in hypothalamic magnocellular neurons of rainbow trout declined within one day of transfer from freshwater to 80% seawater. Taken as a whole, these results indicate that acute hyperosmotic challenge increases plasma VT levels by stimulating secretion of VT peptide from the neurohypophysis, and that hyposmotic conditions have the opposite effect (reviewed by Warne, 2002). From the findings of the current study and those of previous work (Hyodo and Urano, 1991; Haruta et al., 1991), it appears that the acute changes in pituitary VT secretion are often paralleled by decreases in magnocellular *pro*-VT mRNA levels, possibly resulting from an increase in *pro*-VT mRNA translation.

There are a few studies, however, that suggest the response of preoptic *pro*-VT mRNAs to hypersaline challenge may vary among species or across testing conditions. For instance, flounder (*Platichthys flesus*) transferred from freshwater to seawater showed the expected increase in plasma VT and decrease in pituitary VT protein levels within 8 h, but also had elevated, rather than reduced, hypothalamic *pro*-VT mRNA levels 4 h and 8 h after transfer (Warne et al., 2005). Similarly, hypothalamic *pro*-VT mRNA levels were found to decrease in stenohaline pufferfish (*Takifugu rubripes*) one day after transfer to hyposmotic conditions (Motohashi et al., 2009). Whether the differential responses of hypothalamic *pro*-VT transcript abundance to osmotic challenge indicate species differences in the ability to acclimate to rapid salinity changes, result from variations in experimental design and testing conditions, or demonstrate differential regulation of mRNA transcriptional/translational processes is unclear. *Pro*-VT transcripts in hypothalamic parvocellular neurons of the teleost preoptic area are also known to be stress-responsive (Gilchrist et al., 2000), and inadvertent stresses associated with transfer of fish from one salinity to another might have altered hypothalamic *pro*-VT mRNA levels compared to those expected under osmotic challenge alone. In any case, magnocellular VT-ir neuron number was observed previously to be reduced in Amargosa pupfish raised in freshwater (0.4 ppt) conditions compared to more saline (3 ppt) conditions (Lema, 2006), and when combined with the changes in *pro*-VT transcript abundance observed in the present study, these findings indicate a role for hypothalamic *pro*-VT expression in regulating hydromineral balance in pupfish.

Results from the acute osmotic challenge conducted here also revealed that mRNAs encoding pupfish VT/IT receptors are differentially regulated in hypothalamic and gill tissues, suggesting that the receptors have distinct functional roles in mediating the physiological and behavioral responses to osmotic stress. Few studies have examined changes in teleost VT receptor mRNAs in response to salinity challenge. In studies using the flounder *Platichthys flesus*, chronically acclimated seawater and freshwater fish showed no difference in either gill or kidney VT receptor transcript abundance (Warne et al., 2005; Balment et al., 2006). However, transfer

of seawater-acclimated flounder to freshwater for 24 h reduced both gill and kidney VT receptor transcript abundance (Balment et al., 2006). The receptor examined in these studies with flounder was a V1a-type receptor, and the findings of Balment and coworkers (2006) are consistent with the changes in V1a2 receptor mRNA levels seen here with pupfish. This result, however, contrasts with that in olive flounder (*Paralichthys olivaceus*), where transfer of seawater-acclimated (35 psu) fish to decreased salinities for 48 h resulted in increased V1a-type receptor mRNA levels in gill (and intestine) at salinities of 4, 8.75 and 17.5 psu, and increased VT receptor mRNA in the kidney at 0 psu (An et al., 2008b).

4.4. Conclusions

Results presented here provide the first evidence for two V1a-type receptor paralogs and a V2-type receptor in an Actinopterygian fish. This research also shows that these three VT receptors exhibit distinct tissue patterns of expression and are differentially regulated in the hypothalamus and gill in response to hyperosmotic challenge. Given that VT has been found to regulate stress responses (Baker et al., 1996; Gilchrist et al., 2000) and social behaviors (for reviews, see Foran and Bass, 1999; Goodson and Bass, 2001) in teleost fishes – in addition to its well-established role in maintaining hydromineral balance – future research into the expressional regulation and function these three VT receptors promises to provide new insights into the diverse physiological and behavioral roles of VT in fish.

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