Arginine Vasotocin Preprohormone Is Expressed in Surprising Regions of the Teleost Forebrain

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Nonapeptides play a fundamental role in the regulation of social behavior, among numerous other functions. In particular, arginine vasopressin and its non-mammalian homolog, arginine vasotocin (AVT), have been implicated in regulating affiliative, reproductive, and aggressive behavior in many vertebrate species. Where these nonapeptides are synthesized in the brain has been studied extensively in most vertebrate lineages. While several hypothalamic and forebrain populations of vasopressinergic neurons have been described in amniotes, the consensus suggests that the expression of AVT in the brain of teleost fish is limited to the hypothalamus, specifically the preoptic area (POA) and the anterior tuberal nucleus (putative homolog of the mammalian ventromedial hypothalamus). However, as most studies in teleosts have focused on the POA, there may be an ascertainment bias. Here, we revisit the distribution of AVT preprohormone mRNA across the dorsal and ventral telencephalon of a highly social African cichlid fish. We first use *in situ* hybridization to map the distribution of AVT preprohormone mRNA across the telencephalon. We then use quantitative real-time polymerase chain reaction to assay AVT expression in the dorsomedial telencephalon, the putative homolog of the mammalian basolateral amygdala. We find evidence for AVT preprohormone mRNA in regions previously not associated with the expression of this nonapeptide, including the putative homologs of the mammalian extended amygdala, hippocampus, striatum, and septum. In addition, AVT preprohormone mRNA expression within the basolateral amygdala homolog differs across social contexts, suggesting a possible role in behavioral regulation. We conclude that the surprising presence of AVT preprohormone mRNA within dorsal and medial telencephalic regions warrants a closer examination of possible AVT synthesis locations in teleost fish, and that these may be more similar to what is observed in mammals and birds.

Keywords: nonapeptide, arginine vasopressin, arginine vasotocin, behavior, preoptic area, amygdala, hippocampus
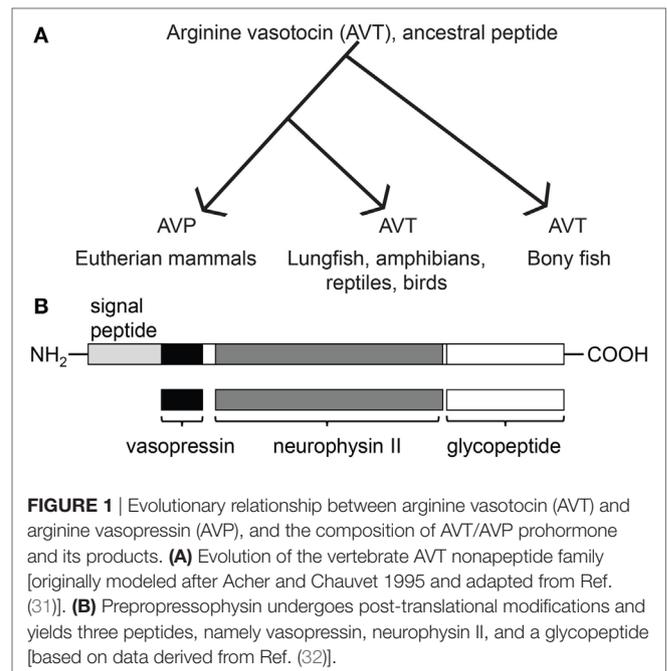
INTRODUCTION

A fundamental aspect of studying animal physiology and behavior is understanding the pathways and mechanisms by which they are regulated. Many studies have focused on understanding how certain neurochemicals, such as neurotransmitters or neuromodulators, influence behavior. One such family of neurochemicals, a class of nine amino acid molecules known as nonapeptides, is

of particular interest. Nonapeptides are highly conserved across vertebrates and play crucial roles in numerous physiological functions and behaviors (1). Their exact effects vary widely between species for reasons that are not fully clear, making them the subject of studies spanning taxa, sexes, social contexts, brain regions, and scientific fields.

One of the nonapeptides, arginine vasopressin (AVP; also known as antidiuretic hormone, ADH), is of particular interest in the study of social behavior across animals. AVP is a highly conserved nonapeptide that has a wide range of modulatory effects across vertebrates (2). Most vertebrate classes possess the ancestral nine amino acid peptide form, arginine vasotocin (AVT; AVP has a phenylalanine substitution of isoleucine in position 3) (3). Originally identified for its role in osmoregulation, cardiovascular function, and stress hormone release (4–6), AVP/T has also been shown to play a key role in modulating social behavior such as courtship and aggressive behavior in fish (7–9), amphibians (10–12), birds (13–16), and in mammals (17–19). AVP/T has also been shown to modulate territoriality and space use [reviewed in Ref. (20)] and alternative reproductive phenotypes in teleost fish (21–29). These effects are mediated by sex, social context, and the neural expression of the nonapeptide and its receptors (2, 3).

AVP/T is synthesized in magnocellular neurons of the hypothalamus in animals and is produced from prohormones that also encode a carrier protein, neurophysin. There are two types of neurophysin: the prohormone proxyphysin that is hydrolyzed to oxytocin and neurophysin I, and the prohormone propressophysin that is hydrolyzed to vasopressin and neurophysin II, in addition to a short glycopeptide (**Figure 1**). Studies previously done in mammals have shown that these distinct neurophysins may be essential for the implementation of hormonal activity (30). The axon terminals of these hypothalamic neurons extend to the neurohypophysis, where the secretions of these neurosecretory

FIGURE 1 | Evolutionary relationship between arginine vasotocin (AVT) and arginine vasopressin (AVP), and the composition of AVT/AVP prohormone and its products. **(A)** Evolution of the vertebrate AVT nonapeptide family [originally modeled after Acher and Chauvet 1995 and adapted from Ref. (31)]. **(B)** Prepropressophysin undergoes post-translational modifications and yields three peptides, namely vasopressin, neurophysin II, and a glycopeptide [based on data derived from Ref. (32)].

cells are picked up by the circulatory system and transported to target organs.

In the brain, AVP/T exerts its effects in particular regions by binding to distinct receptors. The expression of these receptors differs across tissues and by function (33, 34). For example, the AVP/T receptor subtype, V1a, has been shown to regulate sex and species differences in many social behaviors in mammals, birds, amphibians, and fish (24, 35–37). For teleosts in particular, AVT receptors consist of one V2-type and two V1a types (V1a1 and V1a2) (38–40). The distributions of these receptors are widespread throughout the brain and are found in regions of interest for social regulation, such as the olfactory bulb (OB), telencephalic areas, POA, hypothalamus, midbrain sensory regions, and hindbrain regions important for social approach responses (41, 42).

AVP/T cell bodies are found in the preoptic area-anterior hypothalamus (POA-AH) complex, an integration center that also regulates numerous physiological and hormonal processes through the pituitary gland (16, 23, 43–48). AVP/T peptides are produced by populations of magnocellular and parvocellular neurons within this POA-AH complex. In amniotes, these magnocellular neurons are found in the supraoptic nucleus (SON) of the hypothalamus, while parvocellular neuron populations are found in the paraventricular nucleus (PVN) of the hypothalamus (3, 47). In fish and amphibians, AVT in these magnocellular and parvocellular neuronal populations are found in the POA and AH. These cell groups project to the neurohypophysis, where AVP/T exerts a wide range of peripheral effects (31). Previous studies have used immunohistochemical (IHC) techniques to label immunoreactivity of AVP/T protein product, or *in situ* hybridization (ISH) to label AVP/T preprohormone mRNA across the brain. **Table 1** provides a summary of the brain regions where AVP/T has been

Abbreviations: AC, anterior commissure; An, anterior thalamic nucleus; aTn, anterior tuberal nucleus; Cn, central nucleus of the inferior lobe; CP, central posterior thalamic nucleus; CV, cerebellar valvula; D, dorsal (pallial) part of the telencephalon; Dc, central part of D; Dc-2, subdivision of Dc; Dd, dorsal part of D; DH, dorsal hypothalamus; Dl, lateral part of D; Dld, dorsal region of Dl; Dlv, ventral region of Dl; Dlvv, ventral zone of Dlv; Dm, medial part of D; Dm-1,2,3, subdivisions of Dm; Dm2c, caudal part of Dm-2; Dn, diffuse nucleus of the inferior lobe; Dp, posterior part of D; Dx, unassigned part of D; E, entopeduncular nucleus; GR, corpus glomerulosum pars rotunda; H, habenula; HC, horizontal commissure; IL, inferior lobe; LHn, lateral hypothalamic nucleus; LPGn, lateral preglomerular nucleus; LR, lateral recess; LT, longitudinal torus; LZ, zona limitans of the diencephalon; MB, mammillary body; mPGn, medial preglomerular nucleus; nLT, nucleus of the lateral torus; nMLF, nucleus of the medial longitudinal fascicle; OB, olfactory bulb; OPT, optic tract; OT, optic tectum; P, pituitary; PAG, periaqueductal gray; PGCn, preglomerula commissural nucleus; PN, prethalamic nucleus; POA, preoptic area; PPd, dorsal periventricular pretectal nucleus; PPr, rostral periventricular pretectal nucleus; pTGN, preglomerular tertiary gustatory nucleus; pTn, posterior tuberal nucleus; PVO, paraventricular organ; ST, semicircular torus; TPp, periventricular nucleus of the posterior tuberculum; V, ventral (subpallial) division of the telencephalon; Vc, central part of V; Vd, dorsal part of V; Vdc, caudal region of Vd; Vdr, rostral region of Vd; VH, ventral hypothalamus; Vi, intermediate part of V; Vl, lateral part of V; VM, ventromedial thalamic nucleus; Vp, postcommissural nucleus of V; vPPn, ventral portion of the periventricular pretectal nucleus; Vs, supracommissural nucleus of V; Vsl, lateral region of Vs; Vsm, medial region of Vs; vTn, ventral tuberal nucleus; Vv, ventral part of V.

found, along with the technique used to map either AVP/T protein product or label AVP/T preprohormone mRNA in the respective studies. In general, amniotes have similar patterns of AVT expression throughout the forebrain. In teleosts, however, AVT-containing neurons have been shown to be localized to the POA region.

TABLE 1 | Presence of forebrain arginine vasotocin/arginine vasopressin across vertebrates.

Class	Brain regions	Species	Study	Methods
Fish	Diencephalon: Preoptic area	*Anguilla anguilla*	Olivereau et al. (49)	IHC
		Astatotilapia burtoni	Greenwood et al. (21)	*In situ* hybridization (ISH)
		Carassius auratus	Reaves and Hayward (50)	
		Halichoeres trimaculatus	Hur et al. (51)	qPCR
		Oncorhynchus keta	Ota et al. (52)	ISH, IHC
		Oncorhynchus masou	Ota et al. (28, 53)	ISH, IHC
		Oncorynchus mykiss	Gilchriest et al. (54)	ISH
		Poecilia latipinna	Batten et al. (55)	IHC
		Protopterus aethiopicus	Goossens et al. (56)	IHC
		Prochthys notatus	Goodson and Bass (22, 23)	IHC
		Salmo gairdneri	van den Dungen et al. (57)	IHC
		Scyliorhinus caniculus	Vallarino et al. (58)	IHC
		Thalassoma bifasciatum	Godwin et al. (59)	ISH
		Xiphophorus maculatus	Schreibman and Halpern (60)	IHC
Amphibians	Pallial telencephalon Subpallial telencephalon	*Pleurodeles waltlii*	Gonzalez and Smeets (61, 62)	IHC
		Rana catesbeiana	Boyd et al. (63); Gonzalez and Smeets (61, 62); Mathieson (64)	IHC
		Rana ridibunda	Gonzalez and Smeets (61, 62)	IHC
		Rana sylvatica	Mathieson (64)	IHC
		Taricha granulosa	Lowry et al. (65); Lowry et al. (45)	ISH, IHC
		Xenopus laevis	Gonzalez and Smeets (61, 62)	IHC
	Diencephalon: BNST and POA	*Bufo japonicus*	Jokura and Urano (66)	IHC
		Pseudemys scripta	Smeets et al. (67)	IHC
		Rana catesbeiana	Boyd et al. (63)	IHC
		Taricha granulosa	Lowry et al. (45)	ISH, IHC
		Typhlonectes compressicauda	Gonzales and Smeets (68)	IHC
		Typhlonectes natans	Hilscher-Conklin et al. (69)	IHC
		Xenopus laevis	Gonzalez and Smeets (61, 62)	IHC
Reptiles	Subpallial telencephalon	*Anolis carolinensis*	Propper et al. (70)	IHC
		Pseudemys scripta elegans	Smeets et al. (71)	IHC
		Python regius	Smeets et al. (71)	IHC
		Gekko gecko	Stoll and Voorn (72); Thepen et al. (73)	IHC
	Diencephalon: POA, thalamic regions	*Anolis carolinensis*	Propper et al. (70)	IHC
		Gekko gecko	Stoll and Voorn (72); Thepen et al. (73)	IHC
		Lacerta muralis	Bons (74)	IHC
		Mauremys caspica	Fernandez-Llebrez et al. (75)	IHC
		Natrix maura	Fernandez-Llebrez et al. (75)	IHC
		Pseudemys scripta elegans	Smeets et al. (71)	IHC
		Python regius	Smeets et al. (71); Smeets et al. (67)	IHC
Birds	Subpallial telencephalon	*Coturnix japonica*	Aste et al. (76)	ISH
		Gallus domesticus	Aste et al. (76); Jurkevich et al. (77)	ISH, IHC
		Junco hyemalis	Panzica et al. (78)	IHC
		Serinus canaria	Kiss et al. (79)	IHC
		Taeniopygia guttata	Voorhuis and de Kloet (80)	IHC
	Diencephalon: POA, thalamic regions	*Columba livia*	Berk et al. (81)	IHC
		Coturnix japonica	Bons (82); Panzica et al. (83)	IHC
		Serinus canaria	Kiss et al. (79)	IHC
		Taeniopygia guttata	Voorhuis and de Kloet (80)	IHC

(Continued)

TABLE 1 | Continued

Class	Brain regions	Species	Study	Methods
Mammals	Subpallial telencephalon	*Felis catus*	Caverson et al. (84)	
		Macaca fascicularis	Caffe et al. (85)	IHC
		Mesocricetus auratus	Dubois-Dauphin et al. (86)	IHC
		Mus musculus	Castel and Morris (87)	IHC
		Rattus norvegicus	Rhodes et al. (88); DeVries et al. (89); van Leeuwen et al. (90); Urban et al. (91); Wang et al. (92); Planas et al. (93)	IHC, ISH
		Sus scrofa	van Eerdenburg et al. (94)	IHC
	Diencephalon: POA, hypothalamic regions	*Cavia porcetella*	Dubois-Dauphin et al. (86)	IHC
		Felis catus	Caverson et al. (84)	
		Jaculus orientalis	Lakhdar-Ghazal et al. (95)	IHC
		Macaca fascicularis	Caffe et al. (85)	IHC
		Meriones unguiculatus	Wu and Shen (96)	IHC
		Mus musculus	Castel and Morris (87)	IHC
		Rattus norvegicus	Rhodes et al. (88); DeVries et al. (89); Dobie et al. (97); Miller et al. (98); Miller et al. (99); Brot et al. (100); Szot and Dorsa (101); Szot and Dorsa (102)	IHC, ISH

Tetrapod vertebrates exhibit additional anatomical characteristics that remain largely conserved. AVP is produced in neurons of the bed nucleus of the stria terminalis and the medial amygdala, and projections extend to the lateral septum, nucleus accumbens, amygdala, and periaqueductal gray (PAG) (47, 103, 104). These circuits are particularly important for social behavior, such as mate affiliation, nest defense, and parental care of offspring (92, 105–107). Putative teleost homologs of these regions also contain AVT fiber innervation, though these fibers are generally thought to originate in the POA (22, 55). AVP/T fibers are located throughout the brain in jawed vertebrates, likely conserved for at least 500 million years, including the POA, anterior and lateral hypothalamic areas, midbrain tegmentum, PAG, isthmal structures (i.e., locus coeruleus), and viscerosensory areas of the caudal medulla (3).

In the teleost POA, the magnocellular and gigantocellular AVT neuron populations are hypothesized to be homologous to the supraoptic nucleus in tetrapods based on colocalization with corticotropin-releasing hormone-producing neurons and expression of the Nurr1 receptor, while the parvocellular cell group is the putative homolog of the PVN of the mammalian POA (47, 49, 108, 109). AVT appears to be limited to the POA (1). Weaker expression also appears in the anterior tuberal nucleus of the hypothalamus [aTn; (21, 23)], the putative teleost homolog of the mammalian ventromedial hypothalamus [VMH; (110, 111)]. As in tetrapods, AVT is found in the parvocellular, magnocellular, and gigantocellular neuron groups, which are distinguished by soma size and location, with gigantocellular populations being found most caudally. These AVT neurons have been shown to project to the posterior pituitary through the preoptico-hypophysial tract as well as various regions in the ventral telencephalon and ventral thalamus (23, 112). Overall, the expression of AVT preprohormone mRNA and peptide seems to be fairly conserved across vertebrates. There might be an ascertainment bias as most studies only report on the POA and/or used IHC methods to map AVT-positive neurons, which may not be sensitive enough

to detect low levels of peptide expression in other brain regions [but see Ref. (21, 51, 59, 113)].

Importantly, AVP/T has been shown to be socially regulated [see Ref. (3, 20) for reviews]. For example, non-monogamous male Montane voles have fewer V1a receptors in the ventral pallidum compared to monogamous Prairie voles, and the induction of these receptors in the Montane voles *via* viral vector gene transfer yields pair bonding behavior similar to Prairie voles (114). White-throated male sparrows (*Zonotrichia albicollis*) have more AVT expression in the medial portion of the BNST and in a subdivision of the caudal lateral septum compared to tan-striped male sparrows. This neural AVT expression is associated with aggression, since white-striped males defend their territories more vigorously and intrude into other territories more often than their tan-striped male counterparts (115). Research in teleosts suggests that AVT preprohormone mRNA levels might be more reliable indicators of social status than the number or size of AVT-positive neurons (as determined by immunohistochemistry). In Burton's Mouthbrooder cichlid, *Astatotilapia burtoni*, socially dominant males exhibit higher levels of AVT expression than subordinate males in gigantocellular nucleus of the preoptic area, whereas the inverse was found in the parvocellular preoptic nucleus (21). The number or size of AVT-immune-reactive (ir) neurons was, however, not correlated with behavior (126). Similarly, in the sex-changing Bluehead wrasse, *Thalassoma bifasciatum*, preoptic AVT mRNA levels predicts male behavior robustly, while AVT-ir neuron size does not (59). These examples illustrate the role AVP/T plays in modulating social behavior across species, and how these effects are not just sex- and context-specific but also brain region-specific.

The majority of studies that examine the expression and distribution of either AVT preprohormone mRNA or the AVT peptide in teleost fish have primarily focused on the POA. These studies utilize quantitative real-time polymerase chain reaction (qPCR), immunohistochemistry, immunocytochemistry, or radioactive ISH to quantify mRNA and/or protein expression (for more

information regarding these methods see **Table 2**). In the present study, we revisit the neural distribution of AVT nonapeptide expression, in particular expanding on the existing knowledge of its mRNA distribution within the forebrain of a highly social cichlid fish. We first used ISH to examine whether the AVT prepro-hormone mRNA is expressed in pallial and subpallial regions of the telencephalon of *A. burtoni*. In a second experiment, we used qPCR to ask whether AVT preprohormone mRNA expression in pallial area Dm, the putative homolog of the mammalian basolateral amygdala, is modulated by social context. We provide evidence of AVT preprohormone mRNA expression in forebrain regions never previously reported to contain nonapeptides in teleost fish. Furthermore, our results suggest that AVT preprohormone mRNA expression in the putative homolog of the mammalian basolateral amygdala can be regulated by social context.

MATERIALS AND METHODS

Study 1: AVT Distribution in the Cichlid Forebrain

Animals

The African cichlid fish, *Astatotilapia burtoni* (Burton's Mouthbrooder), has become an important model system for the study of social neuroscience. Males of this species can be one of two phenotypes—dominant or subordinate—and this reversible phenotype depends on the immediate social context. Dominant males are highly territorial, aggressive, and reproductively active while subordinate males are non-reproductive and non-territorial. *A. burtoni* descended from a wild-caught stock population were kept in aquaria under naturalistic environmental conditions and stable naturalistic communities as previously described (116). The animals used for mapping the distribution of AVT with ISH were the same as those used in a previous study (42). All work was carried out in compliance with the Institutional Animal Care and Use Committee at the University of Texas at Austin.

In Situ Hybridization

Brains from dominant and subordinate males and females were rapidly dissected and fresh frozen in OCT compound (Tissue-Tek, USA) on dry ice, and stored at −80°C. Brains were subsequently sectioned and stored until processing for ISH as previously described (116). Due to regions of high sequence similarity in the coding regions between neuropeptides and receptors used

in the original study (42), the probe for AVT was designed to identify the 3′ untranslated region. The template used to make the AVT probe was 378 bp in length (21). Experimental slides were exposed to anti-sense fluorescein-labeled probe, whereas control slides were incubated with sense fluorescein-labeled probe (**Figure 2**). After the overnight hybridization, slides were processed for detection of mRNA by non-radioactive, non-fluorescent detection. Sections were washed in a series of 0.2x SSC washes at 65°C and equilibrated in 150 mM NaCl/100 mM Tris (pH 7.5) at room temperature before incubation in 1:1,000 anti-fluorescein-alkaline phosphatase Fab fragments (Roche) in 0.05% Tween 20/PBS for 2 h at room temperature. Sections were then washed in 150 mM NaCl/100 mM Tris (pH 7.5). Chromogenic product was formed using BM Purple (Roche) at room temperature until desired darkness was achieved and was terminated simultaneously for all slides within a gene group. Slides were then washed, dehydrated in an ethanol series ending in xylene, and cover-slipped with Permount (Fisher Scientific). These slides were previously used in Ref. (42) to examine the distribution of AVT and isotocin receptor in *A. burtoni*.

Microscopy

Micrographs were captured and processed as previously detailed (42). Brightfield optics were used to visualize staining throughout the brain at low (5×) and high magnification (10×). Photographs were taken with a digital camera (AxioCam MRc, Zeiss) attached to a Zeiss AxioImager.A1 AX10 microscope using the AxioVision (Zeiss) image acquisition and processing software. Images were compiled and brightness-enhanced in Adobe Photoshop.

Study 2: AVT Expression Variation in Dm in Socially Relevant Contexts

Animals

A. burtoni descended from a wild-caught stock population were kept in stable naturalistic communities, as described (117) until they were transferred into the experimental conditions. These animals were the same as those used in a previous study (118). All work was carried out in compliance with the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Behavior

Animals were placed in experimental tanks which had one territorial male and two non-reproductive females [as described in

TABLE 2 | Differences between methodological techniques.

| Technique	How does it work?	What is measured and visualized?	Advantages of each method
Quantitative real-time polymerase chain reaction (qPCR)	Binds cDNA (complementary DNA, after reverse transcription of mRNA) with a light-emitting molecule	Amplified cDNA	Quantitative
In situ hybridization (ISH)	Binds nucleic acid strands complementary to the mRNA of interest which is labeled with a chromophore or radioisotope	mRNA, fluorophore, or silver grains	Spatial resolution
Immunohistochemistry (IHC)	Uses an antibody that specifically binds a protein of interest for visualization in sectioned tissues, these antibodies are visible under fluorescence or brightfield microscopy when bound to a fluorophore or chromophore	Protein, cells or fibers	Spatial resolution
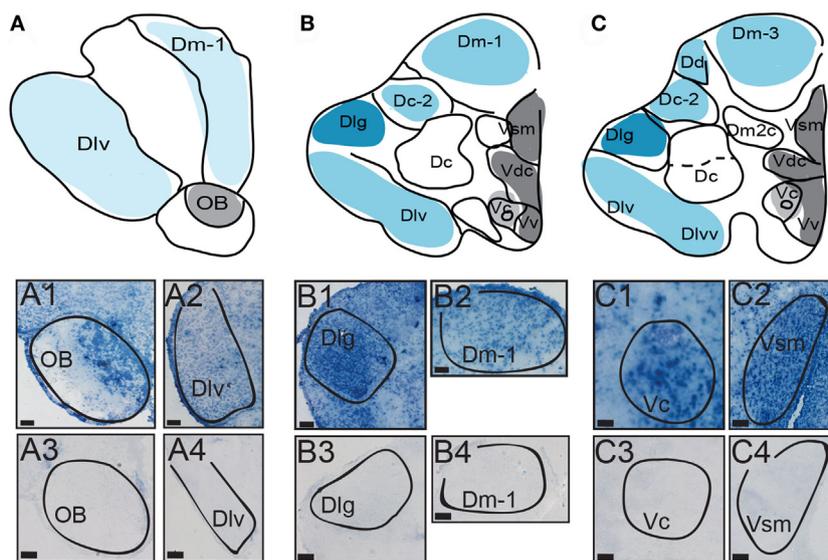
FIGURE 2 | Distribution of AVT preprohormone mRNA in the telencephalon. **(A–C)** The first row represents a template marked with the distribution of AVT preprohormone mRNA. mRNA is shown as shading on the representative template, and the degree of shading corresponds to the qualitative density of expression. Micrographs show AVT preprohormone mRNA in the olfactory bulb (OB; A1), in the ventrolateral part of D (Dlv; A2), the granular region of D (Dlg; B1), a subregion of the medial part of D (Dm-1; B2), the central part of V (Vc; C1), and in the medial part of Vs (Vsm, C2). The sense controls show a lack of AVT preprohormone mRNA signal in the OB (A3), Dlv (A4), Dlg (B3), Dm-1 (B4), Vc (C3), and Vsm (C4). All scale bars are shown at 20 µm.

Ref. (118)]. Focal males were tested in one of three social contexts; namely (1) a Reproductive Context, in which an adjacent tank contained one gravid and two non-reproductive females, (2) a Familiar Neighbor context, in which the adjacent tank contained one size-matched territorial male and two non-reproductive females, and (3) a Neutral Stimulus context that contained three non-reproductive females. Non-reproductive females were stripped of their brood immediately before placement in each tank, ensuring that they would remain non-reproductive for the duration of the study (119). Males were killed by rapid cervical transection and brains were flash frozen in O.C.T. (Tissue-Tek; Fisher Scientific Co., Pittsburgh, PA, USA) and stored at −80°C.

Quantitative Real-time Polymerase Chain Reaction

Brains were sectioned on a cryostat in the transverse plane at 300 µm. A 300 µm diameter sample corer tool (Fine Science Tools, Foster City, CA) was used to micro-dissect the Dm-1. Two micro-dissected punches (left and right hemisphere) were taken from a single brain slice and stored in DNA/RNA Shield (Zymo Research, Irvine, CA, USA) at −80°C until processing. ZR BashingBeads (Zymo Research) were added to samples suspended in DNA/RNA Shield for tissue homogenization before RNA extraction. Proteinase K digestion was done for 2 h at 55°C to lyse tissue. Total RNA was then extracted in accordance with the protocol for the Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA, USA). RNA samples were treated with DNase (Zymo) during isolation procedure to prevent DNA contamination. The GoScript Reverse Transcription System (Promega Corporation, Madison, WI, USA) was used to reverse transcribe RNA to cDNA.

Quantitative real-time polymerase chain reaction was used to measure the mRNA levels of AVT preprohormone and the primers were designed to flank exon-exon boundaries (AVT forward: 5′-AGGCAGGAGGGAGATCCTGT; AVT reverse: 5′-CAGGCAGTCAGAGTCCACCAT. 18S forward: 5′-CCCTT CAAACCCTCTTACCC; 18S reverse: 5′-CCACCGCTAAGAGT CGTATT). Target gene expression was measured in triplicate in the ViiA™ 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using GoTaq qPCR Master Mix (Promega). Amplification efficiency for the primer pair was determined using standard curves made from serial dilutions of cDNA.

Statistical Analyses

Statistical tests were performed using R v. 3.1.0. We used the R package mcmc.qpcr to determine relative gene expression for each sample. 18S was used as a control gene, and other target genes measured within the same region were included in the normalization analysis. This package analyzes qPCR data using generalized linear mixed models based on lognormal Poisson error distribution, fitted using Markov chain Monte Carlo statistical methods (120).

RESULTS

In Situ Hybridization of AVT Preprohormone mRNA across the Pallium and Subpallium

We first describe the distribution of AVT preprohormone mRNA throughout the *A. burtoni* pallium and subpallium using ISH.

In **Figures 3** and **4**, we present a distribution maps along with photomicrographs of representative brain areas for AVT expression in the *A. burtoni* brain. For each representative section of the map, the teleost nomenclature is displayed along with the preprohormone distribution. The degree of shading represents the approximate density of mRNA expression in that brain region. Pallial regions are colored in shades of blue while subpallial regions are colored in shades of gray. The general patterns are qualitatively independent of reproductive or social status and similar in males and females. Control slides hybridized with sense probes showed no specific signal (**Figure 2**).

Robust expression of AVT preprohormone mRNA is seen throughout the *A. burtoni* pallium. AVT preprohormone mRNA is present in the central, medial and lateral parts of the pallium (Dc, Dm, and Dl, respectively, **Figure 2**). The ventral subregion of Dl (Dlv) has mild staining of AVT preprohormone mRNA (**Figure 2**, A2), while the granular part of Dl (Dlg) has darker staining (**Figure 2**, B1). AVT preprohormone mRNA is present across all subdivision of the Dm (Dm-1,2,3) but has lighter stain in the Dm-1 subdivision (**Figure 2**, B2). The Dc-2 subdivision of the Dc telencephalon also shows light staining of AVT preprohormone mRNA, which is absent from the Dc (**Figure 2B**). In general, AVT expression becomes more robust in more caudal sections of these pallial regions.

There is robust AVT expression within the OB and subpallium as well as in the granule cell layer of the OB (**Figure 2**, A1), while preprohormone mRNA is predominantly absent from the glomeruli region. Ventral, central, and supracommissural parts (Vv, Vc, Vs; **Figure 2C**) of the subpallium also show robust AVT expression. This is also present in Vv, Vd, and the subregions of the Vs (Vsm and Vsl). There is AVT expression in the Vc (**Figure 2, C1**), and expression is more robust in more caudal regions of the Vs (Vsm, **Figure 2**, C2). AVT preprohormone mRNA is widely expressed throughout the POA (**Figure 3**). There is robust expression in parvocellular populations of the POA (**Figure 3B**), as well as in the magnocellular population (**Figure 3C**). AVT preprohormone mRNA expression is also present in the gigantocellular population (**Figure 3D**).

AVT Expression in the Medial Dorsal Telencephalon

Next, we use qPCR to examine whether AVT preprohormone mRNA expression in the medial dorsal telencephalon is modulated by social context. We find significant variation in AVT expression in the Dm region of the *A. burtoni* telencephalon across social contexts (**Figure 4**). Specifically, AVT expression is higher in the Familiar Neighbor context as compared to a context with a Neutral Social Stimulus ($p = 0.003$). There is no difference in AVT expression between Reproductive Opportunity context and either Familiar Neighbor or Neutral Social Control contexts.
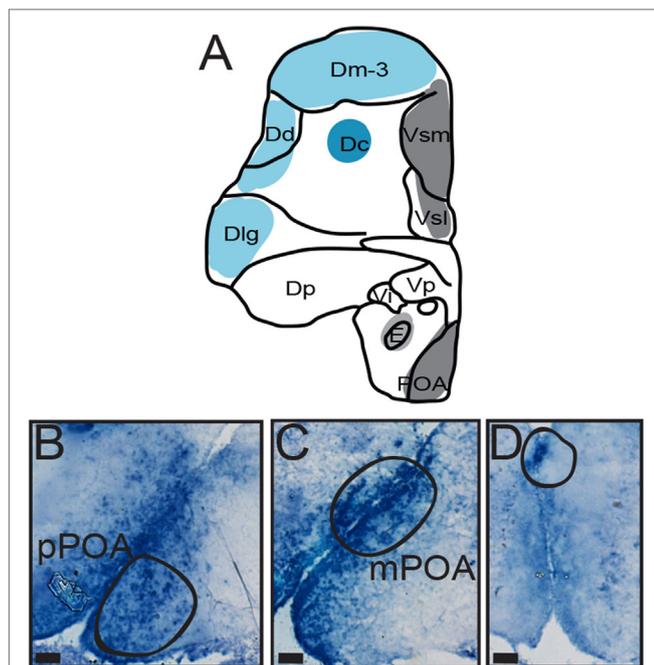

FIGURE 3 | Distribution of AVT preprohormone mRNA in the preoptic area (POA) of *A. burtoni*. **(A)** A template marked with the distribution of AVT preprohormone mRNA (shading). The degree of shading corresponds to the qualitative density of expression. **(B)** Micrograph shows AVT preprohormone mRNA in the parvocellular population of the POA.
(C) Micrograph shows AVT preprohormone mRNA in the magnocellular population of the POA. **(D)** Micrograph shows AVT preprohormone mRNA in the gigantocellular population of the POA. All scale bars are shown at 20 μm.
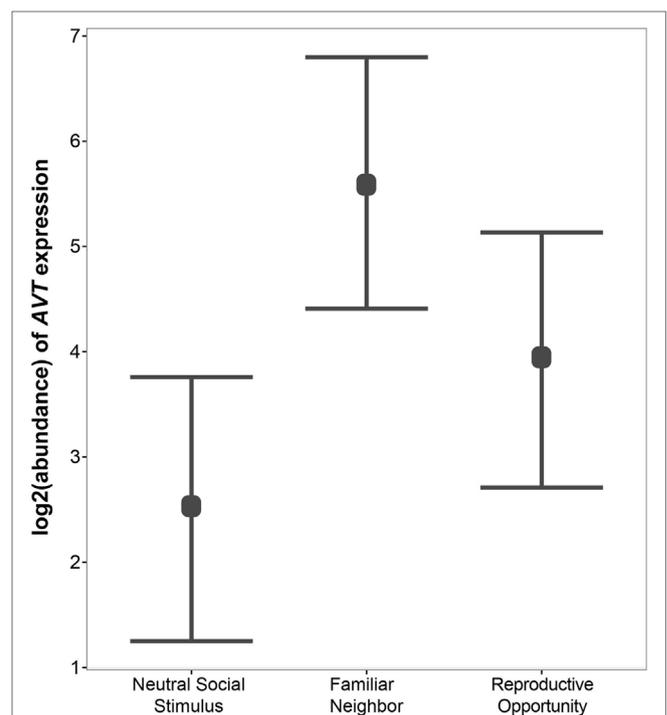

FIGURE 4 | Relative gene expression of AVT preprohormone mRNA across social contexts. AVT expression was highest in individuals engaged in Familiar Neighbor social context, and is generally higher in contexts in which the social stimulus is not neutral, such as Familiar Neighbor and Reproductive Opportunity contexts, as compared to a context with a Neutral Social Stimulus.

DISCUSSION

In the present study, we have shown that expression of AVT preprohormone mRNA in the cichlid fish *A. burtoni* is not limited to preoptic nuclei and the anterior tuberal nucleus. Rather, AVT preprohormone mRNA is expressed widely throughout pallial and subpallial regions not previously associated with the expression of the AVT nonapeptide. We have also found evidence for the social regulation of AVT expression within area Dm-1, the putative homolog of the mammalian basolateral amygdala. These surprising findings provide an important addition to our understanding of the distribution of AVT in the teleost brain and how nonapeptides modulate social behavior in cichlids.

Previous studies in teleost fish have reported the presence of AVT preprohormone and AVT peptide primarily in the POA and the aTn of the hypothalamus (21, 59, 121). Several studies also mapped AVT-immunoreactive fibers and found that they project extensively throughout the teleost brain, although where these fibers originate is not always obvious (55, 121). Our data expand on these studies to show the expression of AVT preprohormone mRNA in multiple regions of the dorsal, medial, central, and ventral pallium. Specifically, subpallial regions, such as the medial and lateral divisions of area Vs [putative homolog of the medial amygdala and the bed nucleus of the stria terminalis (109)] along with area Vv (putative septum homolog) and the central part of area Vd (putative striatum homolog) showed robust expression of AVT preprohormone mRNA, while pallial regions, including basolateral amygdala (area Dm) and hippocampus (area Dl), showed less but still reliably detectable abundance. Our qPCR results confirm expression of AVT in area Dm, and we show this expression to be modulated by the social context. These results suggest that AVT expression in teleosts may be more similar to AVP/T expression in birds and mammals.

If there are indeed AVT expressing neurons in the teleost pallium, why did previous authors fail to detect them? First, methodological limitations may provide an answer: all studies examining the expression and distribution of either AVT preprohormone mRNA or the AVT peptide in teleost fish to date utilize IHC, qPCR, or radioactive ISH to detect peptide and/or mRNA expression (see **Table 1**). Most do not provide information on telencephalic brain regions, instead focusing exclusively on the preoptic AVT cell populations. The few studies that investigate whether AVT preprohormone or peptide is present in the teleost telencephalon and other areas outside the preoptic nuclei and hypothalamus (21, 51), rely on either radioactive ISH or qPCR of the entire forebrain. Importantly, it is well understood that the former requires short exposure times so as to not overdevelop the signal in preoptic AVT neurons, where the preprohormone is expressed at very high levels [see, e.g., Ref. (21, 59)].

Second, it is also conceivable that AVT transcripts are transported from preoptic cell bodies to fibers (putative axons) in various telencephalic regions for local synthesis (possibly near varicosities or putative release sites). Using both ISH and PCR (122), found oxytocin preprohormone mRNA in axons and Herring bodies in the lateral and ventral hypothalamus, the median eminence, and the posterior lobe of the pituitary in rats. While it is unclear whether this can also occur in axons projecting into the telencephalon, these results nevertheless indicate that at least in the rodent oxytocin preprohormone mRNA can be transported axonally. Given that (a) oxytocin and AVP/T genes as paralogs may share a similar molecular and cellular machinery, and (b) teleosts have brain regions putatively homologous to these rodent regions (109, 117), the signal we detect in pallial regions may indeed be the consequence of axonal transport of AVP/T mRNA. Given the ISH methods used in this study, we cannot conclusively deduce if the mRNA signal resembles varicosities or puncta. Detailed tract tracing studies in combination with sensitive assays such as ISH will allow us to test this hypothesis.

Finally, another possible explanation for the distribution of AVT mRNA expression throughout the teleost telencephalon could be that we are observing preprohormone mRNA that never is translated and processed into the mature peptides AVT and/or neurophysin II. Although the enzymes processing preprohormones could be present in putative pallial AVT neurons for processing peptides others than AVT and neurophysin, any future analysis (e.g., by ISH) demonstrating that these enzymes do not co-localize in these neurons would support this idea. Alternatively, only neurophysin might be produced, for a yet to be discovered function, which can be tested once a specific antibody is available. These possible explanations notwithstanding, our results should be seen as an encouragement to examine telencephalic AVT expression in a range of teleost species.

Is telencephalic AVT of functional importance in *A. burtoni*? Interestingly, we did find significant variation in AVT preprohormone mRNA levels, albeit lowly abundant, depending on social context in area Dm-1, the putative homolog of the mammalian basolateral amygdala (109). This region is known to be important for fear conditioning in mammals, as well as being a sensory integration center that mediates emotional behavior (123, 124). Here, AVT shows increased relative expression in dominant males in the presence of a familiar neighbor, which has important implications for territory defense (125). A possible explanation for this result is that AVT expression in the Dm-1 may be modulating an individual's behavioral response to a familiar neighbor, possibly facilitating social habituation. It is important to note that we do not know baseline AVT levels in the Dm-1, and the data only represent expression in response to an intruder in a joint defense paradigm (118). Further support for a functional role of AVT expression in the basolateral amygdala homolog is provided by the finding that other candidate genes followed the same expression pattern across experimental groups that we observed with AVT expression, possibly regulated by testosterone (118).

CONCLUSION

Nonapeptides are important mediators of social behavior, such as aggression, reproduction, and paternal care, across vertebrates. Their effects are mediated by the presence of receptors and neuronal fibers found throughout the brain. AVP/AVT expression, in particular, has previously been examined across species, and it is canonically held that expression patterns in telencephalic

regions of the brain are different between tetrapods and other vertebrates. Previous work has suggested that teleost fish only express AVT cell bodies within the POA-AH complex, and send projections to other telencephalic regions. However, here we find evidence for the presence of AVT preprohormone mRNA in regions previously not associated with AVT expression, such as the dorsomedial, ventral, and central regions of the *A. burtoni* telencephalon. Based on these results, it is worthwhile to reconsider the similarity in AVT/P expression patterns between teleosts and other vertebrates.

ETHICS STATEMENT

The original research reported here was performed under guidelines established and was reviewed and approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin and in compliance with all local, state, and federal regulations.

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AUTHOR CONTRIBUTIONS

CW, HH, and LH designed the studies; LH conducted the *in situ* hybridization study; CW and JN performed the qPCR experiments; LH, MR-S, and CW performed the data analysis; MR-S and HH wrote the manuscript.

ACKNOWLEDGMENTS

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