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Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*☆

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ABSTRACT

Nonapeptides and their receptors have important functions in mediating social behavior across vertebrates. Where these nonapeptides are synthesized in the brain has been studied extensively in most vertebrate lineages, yet we know relatively little about the neural distribution of nonapeptide receptors outside of mammals. As nonapeptides play influential roles in behavioral regulation in all vertebrates, including teleost fish, we mapped the distributions of the receptors for arginine vasotocin (AVT; homolog of arginine vasopressin) and isotocin (IST; homolog of oxytocin/mesotocin) throughout the forebrain of *Astatotilapia burtoni*, an African cichlid fish with behavioral phenotypes that are plastic and reversible based on the immediate social environment. We characterized the distribution of the AVT V1a2 receptor (V1aR) and the IST receptor (ITR) using both immunohistochemistry for protein detection and *in situ* hybridization for mRNA detection, as well as AVT and IST using immunohistochemistry. Expression of the neuropeptide receptors was widely distributed throughout the fore- and midbrain, including the proposed teleost homologs of the mammalian amygdala complex, striatum, hypothalamus, and ventral tegmental area. We conclude that although the location of nonapeptide synthesis is restricted compared to tetrapod vertebrates, the distribution of nonapeptide receptors is highly conserved across taxa. Our results significantly extend our knowledge of where nonapeptides act in the brains of teleosts to mediate social transitions and behavior.

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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; Dlg, granular 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, preglomerular 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 nucleus 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.

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1. Introduction

Neuropeptide regulation of social behavior is ubiquitous across vertebrate taxa and can vary by sex, social context, and the neural expression of their respective receptors. Arginine vasotocin (AVT; the non-mammalian homolog of arginine vasopressin) and isotocin (IST; present as oxytocin in mammals and mesotocin in birds, reptiles, and amphibians) are neuropeptides that are nine amino acids in length (hence nonapeptides) and highly conserved throughout vertebrate evolution, consistently influencing aggressive and reproductive behavior, although their specific effects vary widely (for a review, see Goodson, 2008). Across diverse taxa, the nonapeptides are consistently found in the preoptic area (POA) and the anterior hypothalamus (AH), suggesting that these cell populations are ancient in the vertebrate lineage. Although the neural distribution of AVT and IST expression and their homologous nonapeptides has been extensively described across vertebrate classes (for a review, see Goodson and Bass, 2001), much less is known about the distribution of their receptors, especially in non-mammalian vertebrates.

The relative expression of nonapeptide receptors across brain regions is exceptionally diverse. While there appears to be only one OXY receptor, many AVP receptors have been described in tetrapods, and it appears that the ancestral gnathostome lineage comprised four distinct subtypes (V1a, V1b, V2a, V2b; Hasunuma et al., 2007; Ocampo Daza et al., 2012). The V1b receptor mediates the effects of AVP on adrenocorticotropic hormone (ACTH) in the pituitary (Jurkevich et al., 2005; Tanoue et al., 2004), whereas the V2a receptor (previously known as the V2 receptor) regulates water retention in the kidney *via* aquaporins (Hayashi et al., 1994). The V1a and V2b subtypes, on the other hand, are widely distributed throughout the brain (Leung et al., 2011). The V1a has been shown to regulate sex and species differences in many social behaviors in mammals, birds, amphibians, and fish (Insel et al., 1994; Semsar et al., 2001; Goodson and Wang, 2008; Baeyens and Cornett, 2006; Hasunuma et al., 2007).

Surprisingly little is known about the expression and distribution of these receptor genes in the brains of teleost fish. In a recent analysis in the Amargosa pupfish, *Cyprinodon nevadensis amargosae*, Lema (2010) isolated mRNA sequences for three AVT receptor subtypes and identified them by their mRNA tissue distribution and amino acid homologies as V1a1, V1a2 and V2 receptors. Using PCR, this study showed that the two distinct forms of the V1a subtype are expressed in the forebrain, midbrain, cerebellum, and hindbrain. Kline (2010), working on the rock hind grouper, *Epinephelus adscensionis*, also used PCR in gross dissections of the brain and found that the V1a2 subtype is more widely distributed in the brain compared with the V1a1 subtype. Furthermore the expression of the V1a2 subtype is more closely associated with sex and reproductive state in the rock hind. These authors then used both *in situ* hybridization and immunohistochemistry to describe the distribution of the V1a2 subtype throughout the rock hind brain (Kline et al., 2011). However, rockhind and pupfish represent only a fraction of the diversity found in an ancient clade such as teleosts, and to this date published accounts of ITR brain distribution are lacking for this vertebrate group. Thus, it is high time to examine these systems in other teleosts, such as cichlids, which are by far the most species-rich vertebrate clade (Kocher, 2004).

The African cichlid fish *Astatotilapia burtoni* has become an important model system in social neuroscience due to its extensive suite of complex social behaviors and inducible phenotypic plasticity (Hofmann, 2003; Robinson et al., 2008). Male *A. burtoni* can be either socially dominant or subordinate, and this phenotype is reversible based on social environment. Dominant males display stereotypical patterns of aggression, coloration, and reproductive behavior, while subordinate males are non-reproductive and submissive, behaviorally and morphologically resembling females (Hofmann, 2003). It is known that preoptic expression levels of AVT differ between dominant and subordinate males (Greenwood et al., 2008) and that expression is largely limited to three nuclei of the POA (gigantocellular, magnocellular, and parvocellular), with a small amount of expression in the anterior tuberal nucleus of the hypothalamus. Interestingly, males showed opposite patterns of differential expression in two of the three POA nuclei, with dominant males having higher AVT expression in the gigantocellular nucleus and subordinate males in the parvocellular nucleus. The physiological functions that are modulated by each nucleus have been investigated, and all three nuclei have projections to the pituitary (Greenwood et al., 2008). The relationship between each nucleus and social behavior is not clear although studies in goldfish have suggested that projections from the parvocellular nucleus to the hindbrain may modulate social approach (Thompson and Walton, 2009).

The role of IST has not yet been investigated in *A. burtoni*, but work in other teleosts suggests that this neuropeptide is also largely expressed in POA cell populations (Buchholz et al., 1995; Hur et al., 2011). Very little is known, however, about the distribution of the IST receptor throughout the brains of teleosts (Hausmann et al., 1995). By examining the neural distribution of mRNA and protein for the AVT and IST receptors, we can significantly increase our understanding of how these nonapeptides modulate may phenotypic plasticity in *A. burtoni*.

The main aim of this study was to describe the distribution of the AVT and IST nonapeptide systems throughout the forebrain of a teleost with plastic behavioral phenotypes in order to gain insight into where nonapeptides may modulate behavioral phenotypes. We found that the nonapeptide receptors are expressed in brain regions important for the regulation of social behavior and evaluation of stimulus salience in the African cichlid fish, *A. burtoni*.

2. Methods

2.1. Animals

Astatotilapia burtoni from a wild-caught stock population were kept in aquaria under conditions mimicking their natural environment as in Munchrath and Hofmann (2010). The animals chosen for this study were dominant and subordinate males as described by Fernald (1976), who had been in their respective social states for at least four weeks. Dominant males were identified as aggressively defending a territory within the tank, courting females, and displaying bright color with an eye bar. Subordinate males were identified by absence of a territory, schooling with the females, fleeing from territorial males, and lack of bright body coloration and eye bar. Adult, non-brooding females that varied in reproductive state were also included as described below. All work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

We used the neuroanatomical nomenclature for *A. burtoni* as in Munchrath and Hofmann (2010) and O'Connell et al. (2011).

2.2. Cloning of the A. burtoni ITR cDNA

The *A. burtoni* V1a receptor gene sequence was already available in GenBank (accession number AF517936.1). We used nested degenerate primers designed for *Xiphophorus* to initially clone ITR, which gave us a large portion of the highly conserved transmembrane region. (See Table 1 for primer details.) We then used nested 3′ RACE to extend our sequence into 3′ UTR, resulting in a final fragment of 751 bp (GenBank accession number: GQ288467.2). To confirm the identity of the sequences, a nearest neighbor tree was assembled in MEGA with pairwise deletion and bootstrap values from 1000 replicates (Fig. 1).

Table 1
Primers for cloning ITR in *A. burtoni*.

Nested PCR (outer)	F-5′ AGTACCTGCAGGTGGTGGGNATGTTYGC
Nested PCR (outer)	R-5′ GCAGCAGGAGTTCAGGCAGSCNARNARCAT
Nested PCR (inner)	F-5′ CGGTGCATGGCCATCTGBCARCCNYT
Nested PCR (inner)	R-5′ CATCTGCACGAAGAAGAAGGGNGTCCARCA
3′ RACE	Outer-5′ GACTGCTGGGGCGACTTCGTGAAACC
3′ RACE	Inner-5′ CGGGAGCAGTGCATAAACCTGACGCCTA

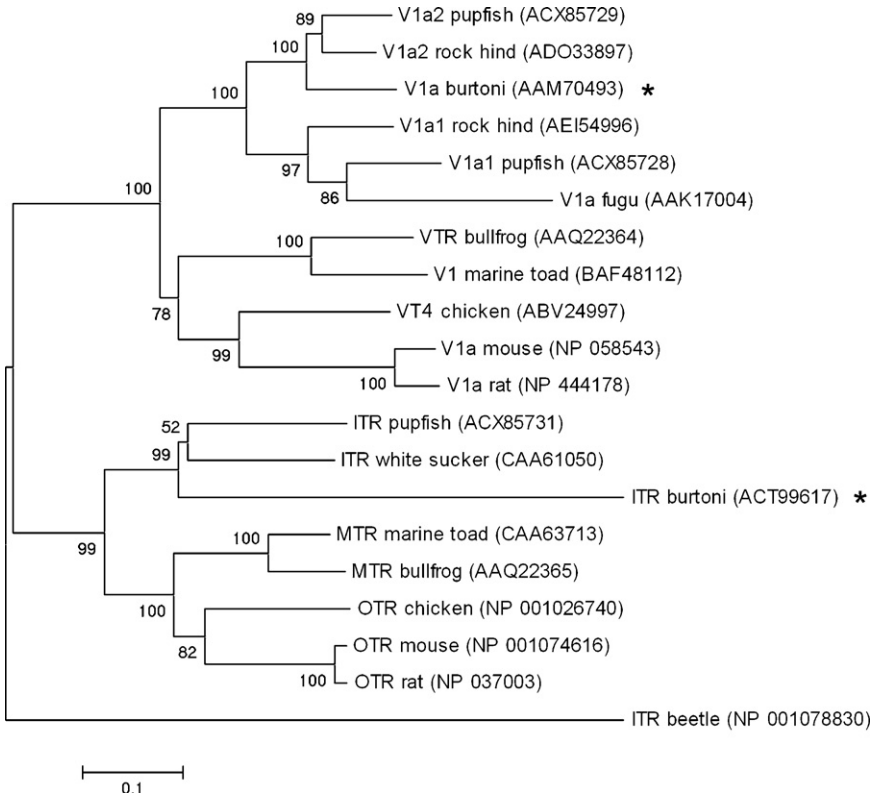

Fig. 1. Comparison of *A. burtoni* V1a and ITR receptors with orthologous sequences from other vertebrates. A neighbor-joining tree based on the alignment of amino acid sequences for AVT/AVP and IST/OT/MT receptors across many vertebrates shows that each *A. burtoni* nonapeptide receptor sequence robustly groups with the respective gene paralog.

2.3. In situ hybridization (ISH)

Dominant ($n = 3$) and subordinate ($n = 3$) males and females ($n = 3$) were killed by rapid cervical dissection and their brains were rapidly dissected, fresh frozen in OCT Compound (Tissue-Tek, Torrance, CA) on a block of dry ice, and stored at −80 °C. Brains were then sectioned in four series on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) that were stored at −80 °C for at least six weeks until processing for ISH as in Munchrath and Hofmann (2010) and O'Connell et al. (2011). Due to regions of high sequence similarity in the coding regions, probes for receptors were designed to exclude the transmembrane region (see Table 2 for primer sequences). The template used to make the V1aR probe was 142 bp in length, and the ITR probe was 158 bp in length. Experimental slides were exposed to anti-sense fluorescein-labeled probe, whereas control slides were incubated with sense fluorescein-labeled probe. After the overnight hybridization, slides were processed for detection of mRNA by non-radioactive, non-fluorescent detection. Sections were washed repeatedly in 0.2 × SSC at 65 °C and equilibrated in 150 mM NaCl/100 mM Tris (pH 7.5) at room temperature before incubation in 1:1000 anti-fluorescein–alkaline phosphatase Fab fragments (Roche, Indianapolis, IN) 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, Indianapolis, IN) 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, Itasca, IL).

Table 2
Primers for *in situ* hybridization probes. Forward (F) and reverse (R) primers for V1aR and ITR probes are written 5′ to 3′.

Probe	Primer
V1aR	F-5′ GACAGTAGCCTCCGCAGAAC R-5′ TTAACAGGGAAGGGTGTTCG
ITR	F-5′ GGCATCTGTTCCAGGATCTTA R-5′ TGTGATGCTCCTCTGACTGC

2.4. Immunohistochemistry (IHC)

Dominant ($n = 6$) and subordinate ($n = 6$) males were killed, and their brains were rapidly dissected and incubated in 4% paraformaldehyde in 1X PBS, pH 7.4 at 4 °C overnight. Brains were then washed in 1X PBS and cryoprotected in 30% sucrose in 1X PBS overnight at 4 °C before embedding in OCT Compound (Tissue-Tek, Torrance, CA), and then stored at −80 °C. Brains were then sectioned in four series on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) that were stored at −80 °C until processing for IHC as in Munchrath and Hofmann (2010). Sections were incubated in primary antibody (V1a 1:500, ITR 1:500, see Table 3 for antibody details) in PBS with 2% normal goat serum and 0.3% Triton-X at room temperature overnight.

Sections were rinsed, incubated for 2 h in a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), rinsed again and, after treatment with the ABC peroxidase staining kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions, immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA). Sections were then dehydrated and cover-slipped with Permount (Fisher Scientific, Itasca, IL). For control sections, all procedures were the same except that primary antibody was omitted.

2.5. Verification of OTR antibody specificity

We used a commercial OTR antibody (MBL International, Woburn, MA, Cat No. LS-A246) whose antigenic sequence (Table 3) comprises amino acids 244–259 in the 3rd intracellular loop of the human OTR (Genbank accession number NP_000907.2) and corresponds to the respective sequence of the *A. burtoni* ITR gene. To determine whether the OTR antibody would bind specifically to the cichlid antigens, we extracted protein from *A. burtoni* whole brain using a Mammalian Cell Lysis kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Whole brain protein extract was run on an SDS-PAGE gel and then was transferred onto a nitrocellulose membrane overnight. The membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20 in 1X Tris-buffered saline [TBS]) for 30 min and then incubated in primary antibody (1:2000 OTR in 1X TBS and 2% NaN_3) for 1 h. After incubation, the membrane was washed five times for 3 min each in wash buffer and then incubated in goat-anti-rabbit HRP-conjugated antibody (Santa Cruz, CA) in blocking solution for 30 min. After washing five times for three min each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western, Millipore, Billerica, MA) and exposed to film for 10 min. Using

Table 3
Antibody information.

1°	Antigen	Supplier	Source	IHC dilution	Type
V1a2R	V1a2: IKYKKRKSTAGAANK	Custom-made[a]	Synthetic fragment	1:500	Polyclonal
ITR	OTR: PEGAAAGDGGRVALAR	MBL	Human	1:500	Polyclonal

[a] See Kline et al. (2011) for details.

the OTR antibody, one band was visualized at the predicted size of 45 kDa, putatively representing cichlid ITR (Fig. 2A). To predict the protein size for ITR, we used the full ITR amino acid sequence of the Amargosa pupfish (*C. nevadensis amargosae*; Genbank accession number ACY07774) and the Science Gateway protein molecular weight prediction tool at http://www.sciencegateway.org/tools/proteinmw.htm.

2.6. Verification of V1a2 antibody specificity

The V1a2 antibody was raised in rabbit against a 15 amino acid sequence corresponding to the 3rd intracellular loop of the rock hind V1a2 receptor (Kline et al., 2011). To test the specificity of this antibody in the cichlid brain, we performed a western blot analysis. Protein was extracted from one male and one female brain using the Qproteome Mammalian Protein Prep Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Extracted protein (15 μg) was re-suspended in 1X reducing loading buffer (Pierce, Rockford, IL) and boiled for 10 min then loaded and run on a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel in duplicate, followed by overnight transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were washed three times for 5 min with PBS-T (20 mM phosphate base, 150 mM NaCl, 0.1% Tween-20, pH 7.6) and immersed in blocking buffer (5% normal goat serum and 0.5% porcine gelatin in PBS-T) for 1 h at room temperature. Membranes were rinsed in PBS-T and incubated overnight at 4 °C with AVTr antibody or antibody pre-absorbed overnight with 1 μg of antigen peptide to 1 μl antibody at a final dilution of 1:1000 in PBS-T. Following primary antibody incubation, membranes were washed three times for 5 min with PBS-T and incubated with a secondary goat anti-rabbit antibody linked to horseradish peroxidase (AbCam, Cambridge, MA) at a final concentration of 1:5000 in PBS-T with 5% nonfat milk for 2 h at room temperature. Membranes were washed three times for 5 min with PBS-T, and immunolabelled band(s) were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL) and ECL hyperfilm (Amersham, Piscataway, NJ). This analysis revealed a single band of ~45 kDa, which corresponds to the expected size of the V1a2 protein (Fig. 2B). Results for the male and female samples were identical, though only the male band is shown. Additional controls included a preabsorption of the antibody with the antigen peptide prior to immunohistochemistry, which blocked all signal.

2.7. Photomicroscopy

Brightfield optics were used to visualize immunohistochemical and *in situ* staining throughout the brain at low (5×) and high magnification (10×). Photographs were taken with a digital camera (AxioCam MRc, Carl Zeiss AG, Germany) attached to a Zeiss AxioImager.A1 AX10 microscope (Carl Zeiss AG, Germany) using the AxioVision (Carl Zeiss AG, Germany) image acquisition and processing software. Images were compiled and brightness- and contrast-enhanced in Adobe Photoshop CS3.

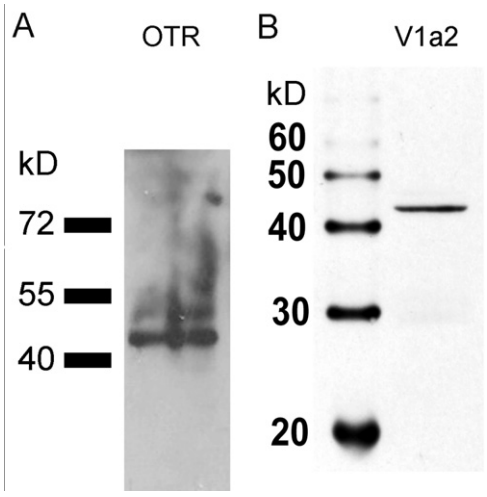

Fig. 2. Confirmation of antibody specificity. Western blot analysis of the oxytocin receptor (OTR, A) and V1a2 receptor antibody (B) against *A. burtoni* whole brain protein extract confirms that the antibodies are recognizing protein of the predicted size. Ladder units are in kDa.

3. Results

In the following, we present a distribution map along with photomicrographs of representative brain areas for the arginine vasotocin (AVT) V1a receptor (V1aR) and the isotocin receptor (ITR). For each representative section of the map, the nomenclature is displayed on the left side while the receptor distribution is presented on the right side. The degree of shading qualitatively represents the density of mRNA expression in that region. The density of dots representing protein indicates qualitatively the density of cells positive for the protein of interest. The general patterns shown here are representative of both dominant and subordinate males for IHC and dominant males, subordinate males, and females for ISH (notwithstanding possible quantitative differences, which we do not investigate here). Overall, the mRNA detection *via in situ* hybridization and protein immunohistochemistry staining for nonapeptide receptors showed high concordance. Control slides that either omitted antibody for immunohistochemistry or hybridized with sense probes for *in situ* hybridization showed no specific signal.

Robust expression of V1aR and ITR protein and mRNA is seen throughout the telencephalon, diencephalon, and mesencephalic structures of *A. burtoni*. In general, V1aR and ITR show similar patterns of mRNA expression and consistently overlap with protein immunoreactivity.

3.1. Telencephalon

Strong signal for V1aR and ITR protein and mRNA is found in discrete parts of the dorsal and ventral telencephalon (Fig. 3). There is robust expression of receptor mRNA and protein in the granule cell layer of the olfactory bulb (OB, Fig. 3A). Relatively fewer cells are immunoreactive to V1aR and ITR in the glomeruli region, although we did not observe receptor mRNA in this region. In the dorsal telencephalon, there are cells expressing V1aR and ITR including the central, dorsal, lateral, medial, and posterior parts (Dc, Dd, Dl, Dm, and Dp, respectively; Figs. 3 and 4). Subdivisions within these regions with heavy staining of both receptors are the granular part and the ventral part of Dl (Dlg and Dlv). V1aR immunoreactivity is nearly absent in the dorsal part of Dl (Dld), Dc-2, and Dm2r while there are more cells positive for ITR protein in these regions. The same is true for mRNA, with the exception of the dorsal region of Dc-2, where both V1aR and ITR are well represented. There are two distinct cell groups in Dc that are positive for V1aR and ITR (Fig. 4). Overall in the dorsal telencephalon, ITR mRNA is more widely distributed than V1aR.

Within the ventral telencephalon, there is staining of both neuropeptide receptor-immunoreactive cells within the ventral, central, dorsal, lateral, postcommissural and supracommissural parts (Vv, Vc, Vd, Vl, Vp, and Vs, respectively; Figs. 3 and 4). Similar

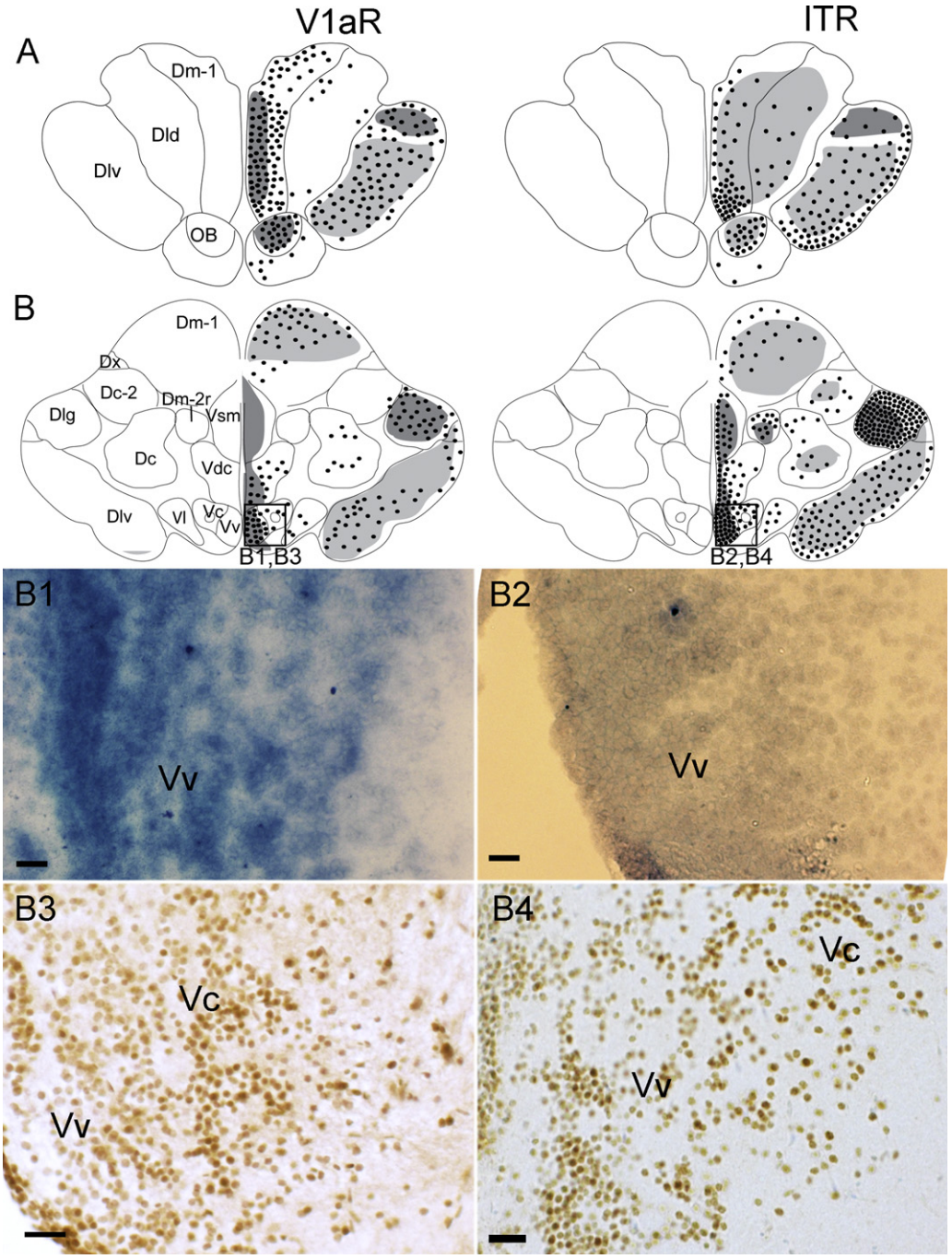

Fig. 3. Distribution of nonapeptide receptors in the rostral telencephalon of *A. burtoni*. The first column of representative sections of rostral telencephalon (A and B) depict the distribution of the V1a receptor (V1aR, left side) while the second column depicts the distribution of the isotocin receptor (ITR, right side). mRNA is shown as shading while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the qualitative density of expression, while the density of dots indicating protein corresponds to the qualitative density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (B1 and B2) and in the bottom row show V1aR and ITR protein in the ventral and central part of the ventral telencephalon (B3 and B4). All scale bars are shown at 20 μm.

to the dorsal telencephalon, ITR immunoreactive cells are more abundant than V1aR immunoreactive cells. V1aR immunoreactive cells in the medial region of Vs (Vsm) are found in more caudal areas of this region. Both receptor mRNAs are widely distributed throughout Vsm, but ITR protein is not present in the lateral region of Vs (Vsl, Fig. 4). Finally, both V1aR and ITR protein and mRNA expression of both receptors are present in the entopeduncular nucleus (E). The preoptic area (POA) has very heavy staining of V1aR and ITR protein and mRNA. The teleost POA has three cell populations that play distinct roles in modulating behavior (Greenwood et al., 2008): parvocellular, magnocellular, and gigantocellular neurons, and the V1aR and ITR proteins and mRNA are present in each of these cell types.

3.2. Diencephalon

The pattern of both V1aR and ITR expression shows extensive overlap, similar to patterns seen in the telencephalon, although the diencephalic patterns of both receptors are more diffuse than those seen in the telencephalon (Fig. 5). Caudal to the POA, V1aR and ITR protein and mRNA are found in the habenula (H). mRNA and protein for the receptors are also found in the ventromedial

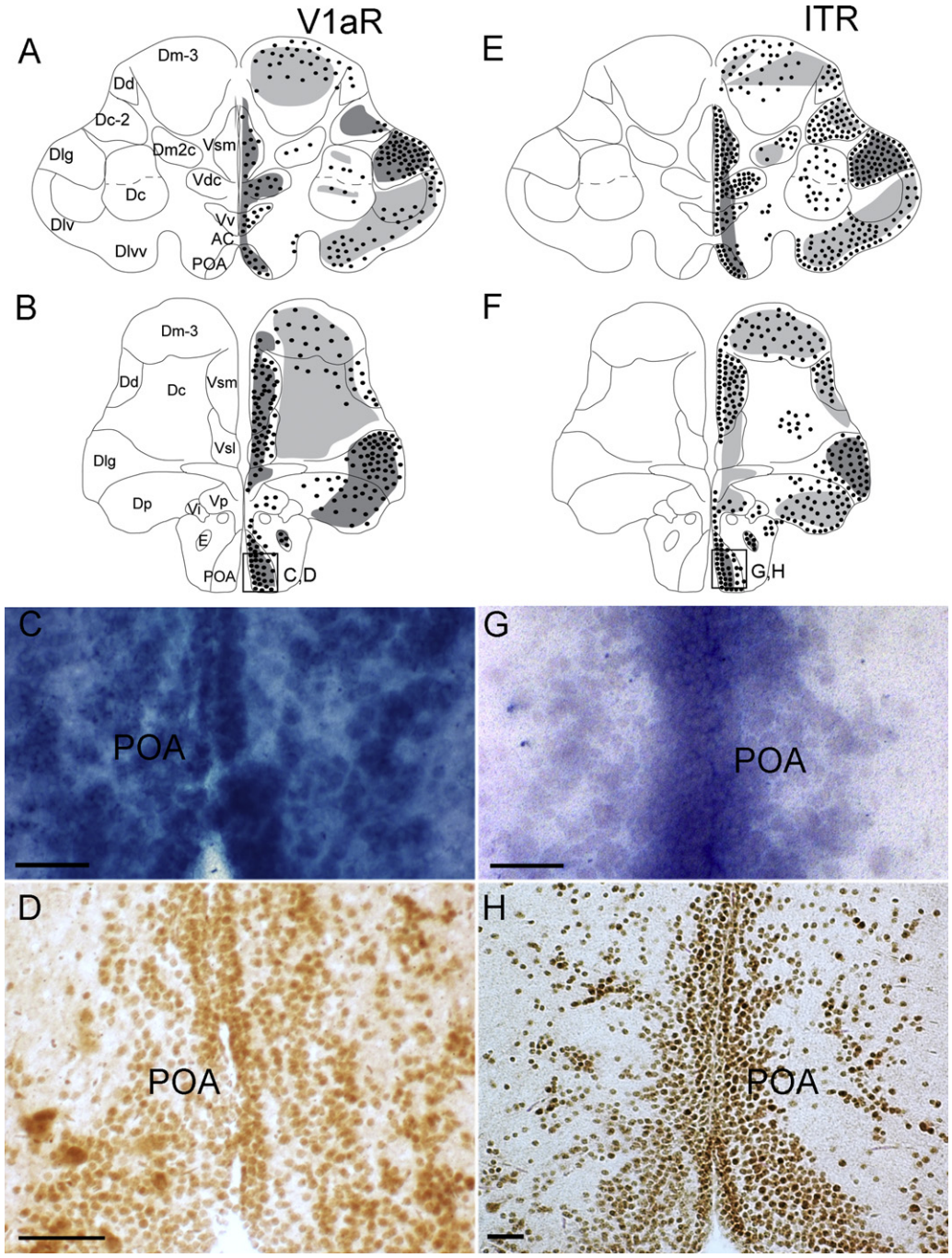

Fig. 4. Distribution of nonapeptide receptors in the caudal telencephalon of *A. burtoni*. The first column of representative sections of caudal telencephalon (A and B) show the distribution of the V1a receptor (V1aR, right side) while the second column (E and F) depicts the distribution of the isotocin receptor (ITR, right side). mRNA is shown as shading while cells positive for protein are shown as dots. The degree of shading for mRNA corresponds to the qualitative density of expression, while the density of dots indicating protein corresponds to the qualitative density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (C and G) and in the bottom row show V1aR and ITR protein in the preoptic area (POA, D and H). All scale bars are 50 μm.

thalamic nucleus (VM). Several periventricular pretectal nuclei also contain V1aR and ITR mRNA and protein including the rostral, dorsal, and ventral regions (PPr, PPd, and vPPn, respectively), with the exception of PPd, which contains only protein. Within the prethalamic nucleus (PN), which lies ventrolateral to VM, we found ITR protein and mRNA but no signal for V1aR. Both V1aR and ITR protein and mRNA are abundant in the ventral tuberal region of the anterior ventral hypothalamic nuclei (vTn; Fig. 5). Both receptors are also found in the anterior tuberal region, but only mRNA for V1aR is present (aTn; Fig. 5). Protein for both receptors is found in several periventricular hypothalamic regions including the ventral hypothalamus (VH), lateral hypothalamic nucleus (LHn), and the dorsal hypothalamus (DH). mRNA for both receptors is also found in VH, LHn, and DH, but mostly in the more caudal portions. Lateral to these regions, V1aR and ITR protein are found within the inferior lobe including the central (Cn) nucleus; mRNA and protein for both receptors are also found in the diffuse nuclei (Dn) (Fig. 6). V1aR and ITR protein and mRNA are also found in the periventricular nucleus of the posterior tuberculum (TPp; Fig. 6B), posterior tuberal nucleus (pTn; Fig. 7) and the thalamic region, central posterior

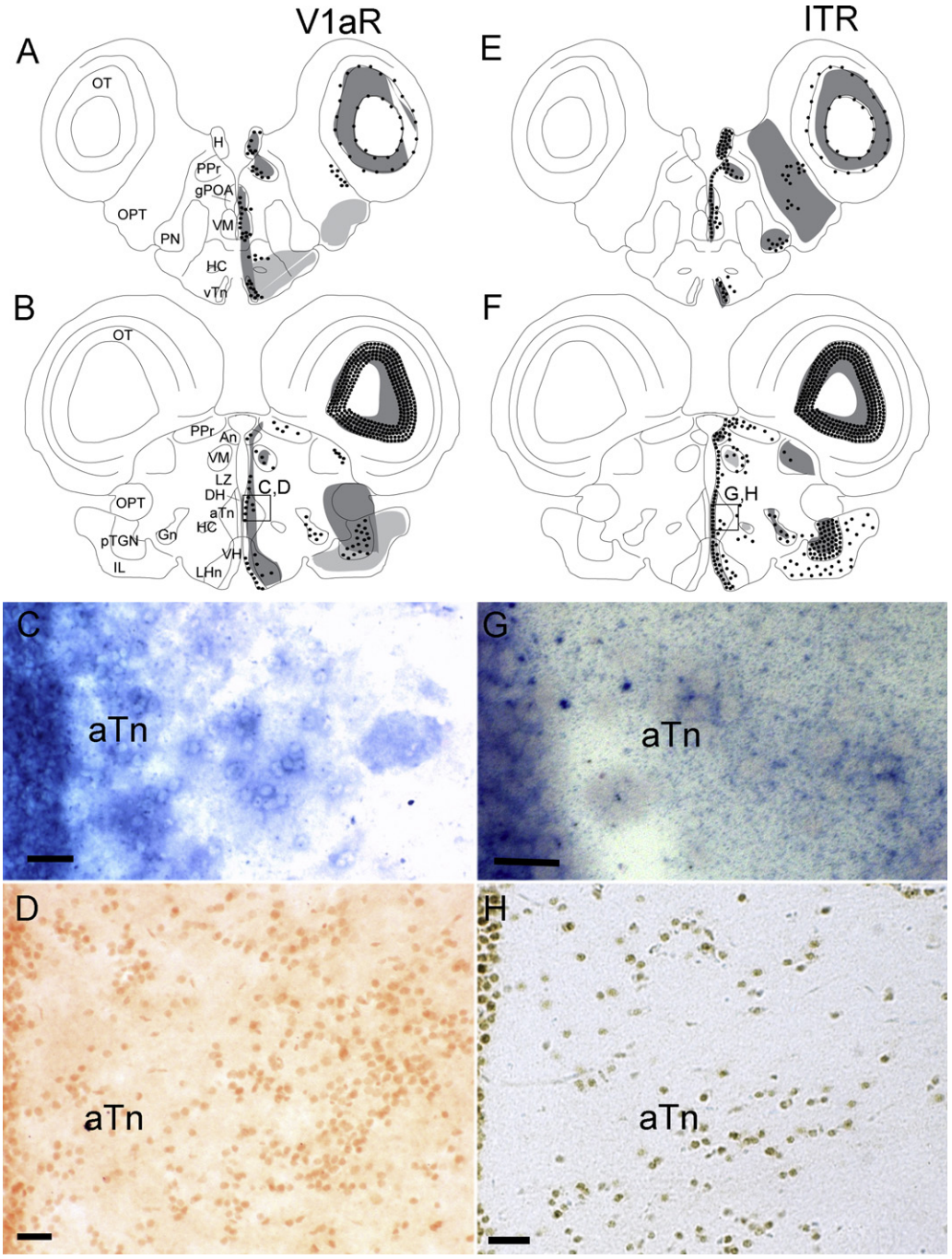

Fig. 5. Distribution of nonapeptide systems in the rostral diencephalon of *A. burtoni*. The first column of representative sections of rostral diencephalon (A and B) show the distribution of the V1a receptor (V1aR, right side) while the second column (E and F) depicts the distribution of the isotocin receptor (ITR, right side). Receptor mRNA is shown as shading while cells positive for nonapeptide or receptor protein are shown as dots. The degree of shading for mRNA corresponds to the qualitative density of expression, while the density of dots indicating protein corresponds to the qualitative density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (C and G) and in the bottom row show V1aR and ITR protein in the anterior tuberal nucleus (aTn, D and H). All scale bars are 20 μm.

thalamic nucleus (CP; Fig. 6A). Both neuropeptide receptors are also found within the medial preglomerular and glomerular nuclei (mPGn and Gn, respectively). Both receptor mRNAs are present in the mPGn and Gn. V1aR and ITR expression and protein are also present within the lateral torus (nLT). Both protein and mRNA of V1aR and ITR are present within the semicircular torus (ST, Fig. 7A) and the periaqueductal gray (PAG; Fig. 7A). In the caudal diencephalon, the preglomerular commissural nucleus (PGCn) and the mammillary body (MB) also contain both V1aR and ITR protein and mRNA (Fig. 7B).

4. Discussion

We report that nonapeptide receptors are widely distributed throughout the brain of *A. burtoni*, providing an important foundation for understanding how nonapeptides modulate phenotypic plasticity in this highly social cichlid. While cells producing these neuropeptides are localized exclusively to the POA in most teleost fish, nonapeptide-positive fiber distributions are moderately distributed throughout the forebrain. Expression and synthesis of the respective receptors, V1aR and ITR, are

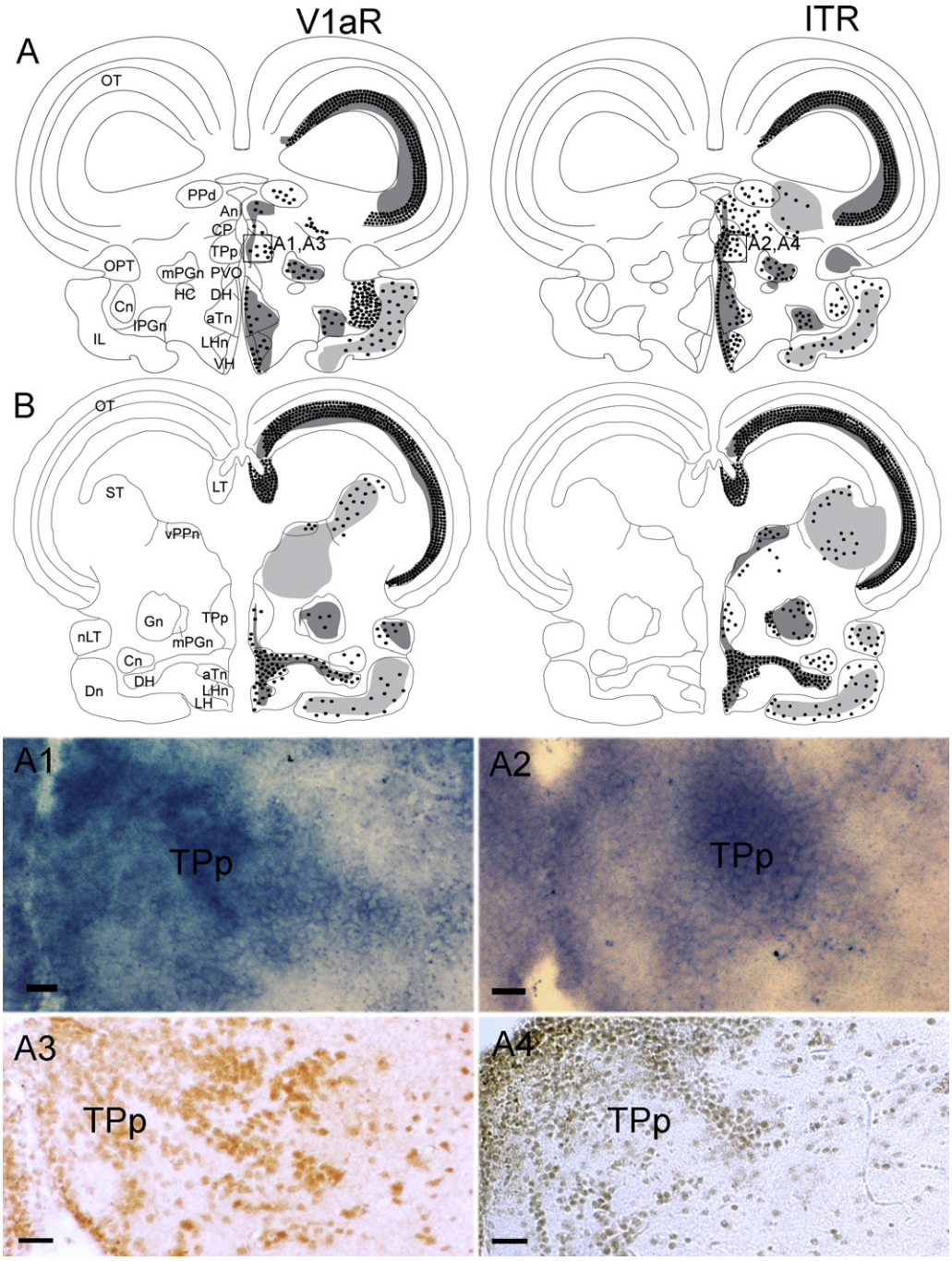

Fig. 6. Distribution of nonapeptide receptors in the diencephalon of *A. burtoni*. The first column of representative sections of the diencephalon (A and B) shows the distribution of the V1a receptor (V1aR, left side) while the second column depicts the distribution of the isotocin receptor (ITR, right side). Receptor mRNA is shown as shading, while cells positive for receptor protein are shown as dots. The degree of shading for mRNA corresponds to the qualitative density of expression, while the density of dots indicating protein corresponds to the qualitative density of cells positive for immunoreactivity. Micrographs in the top row show V1aR and ITR mRNA (A1 and A2) and in the bottom row show V1aR and ITR protein in the posterior tuberculum (TPp, A3 and A4). All scale bars are 20 μm.

distributed widely throughout the telencephalon and diencephalon, providing candidate areas for neuropeptidergic regulation of social behavior in teleost fish.

There was extensive overlap between mRNA expression and protein for the receptors, although Dc and PPd contained V1aR-ir and ITR cells but little to no mRNA expression. Cells in the IL were also immunoreactive for ITR but did not indicate mRNA expression. Finding discrepancies between protein immunoreactivity and mRNA expression in receptor distribution was not surprising, as receptor protein may be located on dendrites far from the cell body where the mRNA is located.

4.1. Neuropeptide system distribution compared with other teleosts

The distribution of AVT has been extensively studied in teleosts. Most studies report AVT-producing neurons exclusively in the POA (Van den Dungen et al., 1982; Batten et al., 1990; Holmqvist and Ekström, 1991; Dewan et al., 2008), although some studies have also found AVT mRNA in tuberal nuclei of the hypothalamus (Godwin et al., 2000; Goodson and Bass, 2000a,b; Greenwood et al., 2008). On the other hand, IST has received relatively less attention. Studies describing IST distribution in teleost fish report IST immunoreactive cells exclusively in the POA (Van den Dungen

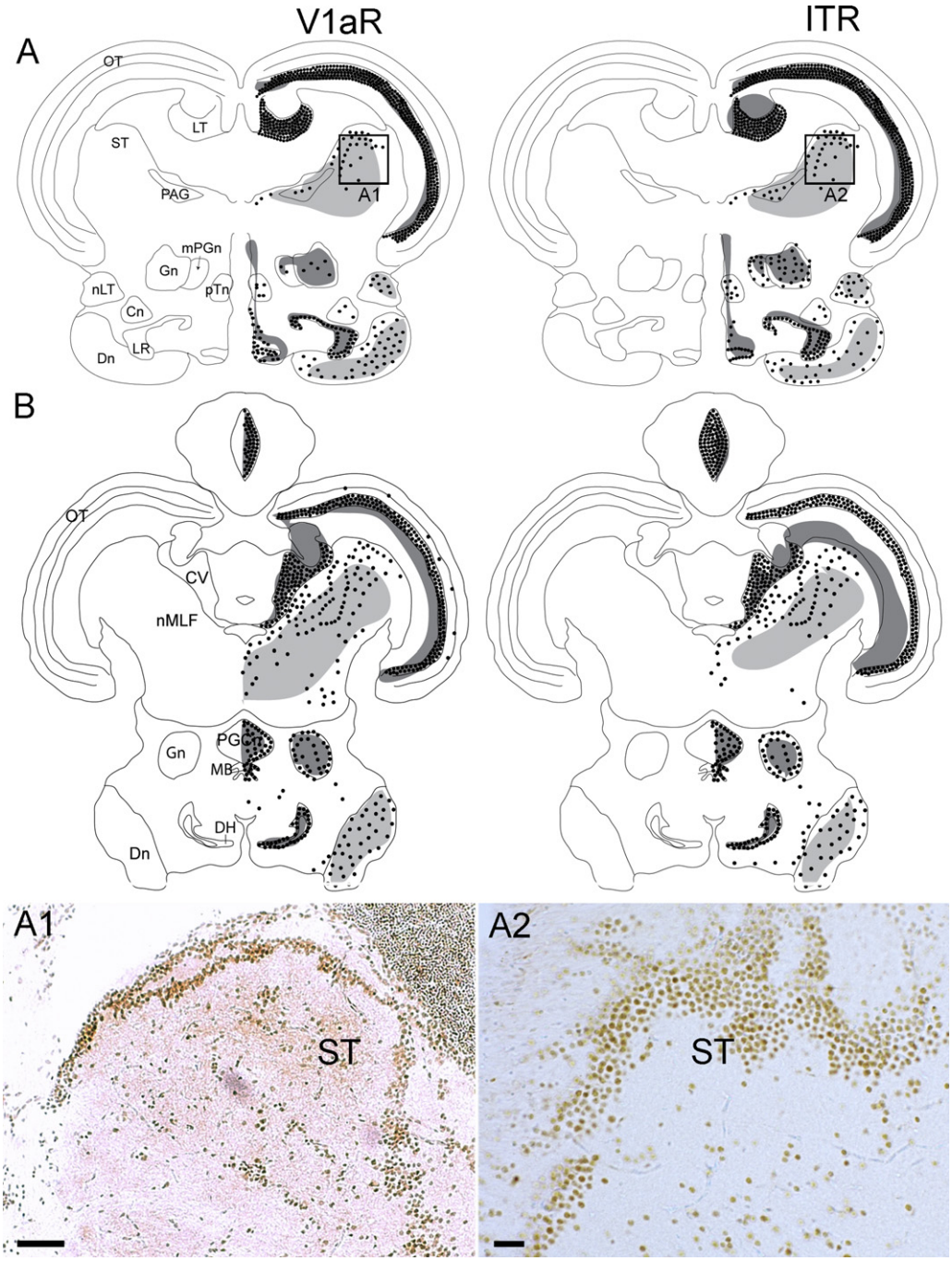

Fig. 7. Distribution of nonapeptide receptors in the caudal diencephalon and midbrain of *A. burtoni*. The first column of representative sections of the caudal diencephalon (A and B) show the distribution of the V1a receptor (V1aR, left side) while the second column (D and E) depicts the distribution of the isotocin receptor (ITR, right side). Receptor mRNA is shown as shading while cells positive for nonapeptide or receptor protein are shown as dots. The degree of shading for mRNA corresponds to the qualitative density of expression, while the density of dots indicating protein corresponds to the qualitative density of cells positive for immunoreactivity. Micrographs show V1aR and ITR protein in the torus semicircularis (ST, A1 and A2, respectively). All scale bars are 50 μm.

et al., 1982; Batten et al., 1990; Holmqvist and Ekström, 1991; Goodson et al., 2003). Although nonapeptide cells are restricted to the POA, studies in other teleosts have shown neuropeptide-immunoreactive fibers to be spread extensively throughout the brain. For example, Batten et al. (1990) found AVT-ir and IST-ir fibers throughout the majority of the brain in the green molly, and Goodson et al. (2003) found a relatively wide distribution of IST-ir fibers in the midshipman.

Compared to the extensive literature on nonapeptide distributions in teleosts, it is surprising that detailed descriptions of their receptors are almost completely lacking. Our study is the first to provide a simultaneous description of both the V1a2 and IST receptor distributions in the teleost forebrain. Lema (2010) used PCR to describe nonapeptide receptor expression in gross brain dissections and found V1a2 receptor expression in the forebrain, midbrain, cerebellum, and hindbrain of the Amargosa pupfish, a cyprinodontiform species that is only distantly related to cichlids. Recently, Kline et al. (2011) used *in situ* hybridization and immunohistochemistry (using the same custom-made antibody as in the present study) to describe the distribution of the V1a2

receptor in the rockhind grouper, *E. adscensionis*, a perciform fish such as *A. burtoni*, and found a distribution pattern almost identical to the one described here.

4.2. Functional implications for nonapeptides and their receptors in teleosts

Numerous studies have investigated the behavioral effects of AVT or IST administration in teleosts. Our description of the nonapeptide receptor distribution now provides a mechanistic framework for hypothesizing where neuropeptides may be acting in the brain to regulate social behavior in teleosts. Even though the widespread distribution of these receptors makes it difficult to predict in detail how they might regulate behavior, this information will be invaluable for testing specific hypotheses through quantitative assays of receptor abundance and/or functional manipulations.

Nonapeptide regulation of social behavior in teleosts has been most extensively studied in the AVT system. Administration of AVT or a V1a antagonist (Manning compound) in a variety of species supports the role of AVT in modulating both aggression and courtship in males, although the effect directionality appears to vary with species, social state, and context. In the bluehead wrasse and damselfish, AVT increases aggression in males (Semsar et al., 2001; Santangelo and Bass, 2006), whereas AVT inhibits aggression in the brown ghost knife fish (Bastian et al., 2001) and Amargosa River pupfish (Lema and Nevitt, 2004). AVT administration consistently increases teleost male courtship as seen in the bluehead wrasse (Semsar et al., 2001), male white perch (Salek et al., 2002), and the brown ghost knife fish (Bastian et al., 2001). Lesion and stimulation studies have identified the Vd, Vs, Vv, and POA (putative homologues of the mammalian nucleus accumbens, bed nucleus of the stria terminalis, lateral septum, and POA, respectively; O'Connell and Hofmann, 2011b) as potential neural substrates of aggression and courtship behavior in teleosts (Demski and Knigge, 1971; Macey et al., 1974; Kyle and Peter, 1982; Satou et al., 1984). V1aR is present in all of these regions in *A. burtoni*; thus, the neuropeptides may be acting at one or many of these brain regions to modulate aggression and reproduction in teleosts.

The role of IST in mediating social behavior in teleosts is not well understood, and most work with this nonapeptide comes from studies in goldfish and the plainfin midshipman. IST administration in male goldfish induces social approach to a conspecific while AVT had the opposite effect (Thompson and Walton, 2004). Both IST and AVT inhibit vocal communication in the plainfin midshipman although IST produces this effect in females and nonterritorial males, and AVT produces this effect in territorial males (Goodson and Bass, 2000a). The vocal-acoustic circuitry that regulates these responses in the plainfin midshipman are well described (Goodson and Bass, 2000b), and we have found ITR and V1aR in each of these brain regions in *A. burtoni*, suggesting that neuropeptides can be modulating equivalent brain regions in the midshipman.

Although nonapeptides are well known for modulating affiliation in monogamous voles (Young and Wang, 2004), surprisingly little is known about the role of these neuropeptides in regulating affiliation in other vertebrates, especially teleosts with monogamous mating systems. Nonapeptide regulation of affiliation has been investigated in the monogamous convict cichlid (Oldfield and Hofmann, 2011). A general V1aR/ITR receptor antagonist inhibited affiliative behavior of males toward potential mates, although this treatment did not prevent pair-bond formation and did not disrupt affiliative behavior in an established pair-bond. Although these effects were not as striking as those seen in monogamous prairie vole males (Winslow et al., 1993), the global administration of a broad antagonist could have diluted the effects. V1aR expression in the lateral septum in male prairie voles seems to regulate both affiliation and paternal care (Liu et al., 2001), and we have found the V1aR in the homologous Vv region in *A. burtoni*, suggesting that this region may facilitate social behavior in cichlids as well.

4.3. Comparison of AVT and IST peptide and receptor distributions to tetrapods

The distribution of AVT, IST and their peptide homologues in other vertebrates are vastly different between vertebrate classes (reviewed in Moore and Lowry, 1998). Strikingly, cell bodies producing AVT or IST are restricted to the POA in teleosts, whereas tetrapods have 19 cell groups or more (Moore and Lowry, 1998), although the behavioral implications for this expansion are unknown. This remarkable neuroanatomical expansion of the neuropeptide system in the transition from water to land has been reviewed extensively (Moore and Lowry, 1998; Goodson and Bass, 2001); thus, we will focus the rest of our discussion on receptor distributions. Compared to our understanding of nonapeptide distributions in a variety of taxa, receptor distribution is not widely studied, especially in non-mammalian vertebrates. Distributions of both AVT and IST receptor mRNA have only been described for two species of birds (Leung et al., 2011) and two amphibians (Acharjee et al., 2004; Hasunuma et al., 2010), and, with the exception of the original study by Kline et al. (2011) and the present study, there have been no published studies that describe the distributions of these receptors in reptiles or teleosts.

Nonapeptide receptor distributions in every vertebrate class described thus far are very widespread throughout the fore- and midbrain. As nonapeptides play an important role in modulating social behavior across vertebrates, we focus our comparative discussion on two neural networks that regulate social behavior and/or the evaluation of stimulus salience and are conserved across mammals (O'Connell and Hofmann, 2011a,b, 2012). Many studies indicate that the "reward system" (including the midbrain dopaminergic system) is the neural network where the salience of social stimuli is evaluated (Deco and Rolls, 2005; Wickens et al., 2007). The neural substrates that regulate aggressive, sexual, and parental care behavior have been described by Newman (1999) as the "social behavior network" in mammals and has been expanded to reptiles, birds, and teleosts (Newman, 1999; Crews, 2003; Goodson, 2005; O'Connell and Hofmann, 2011b, 2012). This network includes the lateral septum, bed nucleus of the stria terminalis/medial amygdala, preoptic area, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal gray/central gray. The putative teleost homologues to these regions are Vv, Vs, POA, vTn, aTn, and PAG, respectively (Goodson, 2005; O'Connell and Hofmann, 2011b). Mammals (Tribollet et al., 1989; Beery et al., 2008; Campbell et al., 2009), birds (Leung et al., 2011), amphibians (Acharjee et al., 2004), and teleosts (present study) all express nonapeptides receptors in the six nodes of the social behavior network, which points to the important and conserved contribution of these receptors to regulating social behavior across vertebrates (O'Connell and Hofmann, 2011a, 2012). Although the brain regions involved in the dopaminergic reward system and the social behavior network are well studied in mammals and birds, descriptions of the teleost homologs are contentious (Nieuwenhuys et al., 1998; Northcutt, 2008). However, a consensus is emerging from developmental, hodological, neurochemical, and lesion studies that provide support for at least putative partial homologies for relevant areas in the teleost brain (Goodson, 2005; Northcutt, 2006, 2008; Portavella et al., 2002; Rink and Wullimann, 2001, 2002; Wullimann and Mueller, 2004; Bruce and Braford, 2009; O'Connell and Hofmann, 2011b), suggesting that these networks are ancient in the vertebrate lineage.

Nonapeptides have recently received much attention in the context of modulating the evaluation of stimulus salience in concert with the dopaminergic reward system (Young and Wang, 2004). The mesolimbic reward system consists of several fore- and midbrain regions and includes the basolateral amygdala, bed nucleus of the stria terminalis, hippocampus, lateral septum, nucleus accumbens, striatum, ventral pallidum, and ventral tegmental area. Within mammals, the prefrontal cortex is also considered part of the reward system, although we do not include it in our comparative discussion, as its evolutionary antecedents are unclear (Butler et al., 2011; but see Mueller et al., 2011). The putative teleost homologues to these regions are Dm, Vs, Dl, Vv, Vd, Vc/Vl, – no homologue for the ventral pallidum is currently known (but see Ganz et al., 2012) – and the TPp, respectively (O'Connell and Hofmann, 2011a,b). Mammals (Tribollet et al., 1989; Beery et al., 2008; Campbell et al., 2009), birds (Leung et al., 2011), amphibians (Acharjee et al., 2004), and teleosts (present study) all express the nonapeptide receptors in each of these regions, with the exception that birds do not express the mesotocin receptor in the ventral pallidum, and most mammals do not express the V1a receptor in the striatum (Insel et al., 1994; Lakhdar-Ghazal et al., 1995). This comparison suggests that the nonapeptide system may be working in concert with dopaminergic pathways to evaluate stimulus salience in other vertebrates.

An important observation to note is that quantitative variation in neuropeptide receptor expression has been linked to phenotypic diversity in social behavior in mammals (Insel and Young, 2000). Given the paucity of species for which descriptions of the nonapeptide systems exist, it would be fruitful to examine quantitative variation in receptor expression in regions that modulate social decision-making across many vertebrate species with diverse forms of sociality in order to elucidate how receptor expression covaries with the evolution of social phenotypes (O'Connell and Hofmann, 2011a, 2012).

4.4. Conclusions

We have shown that while AVT and IST nonapeptide production is restricted to the POA, the V1a and IST receptors are widely distributed throughout the forebrain of *A. burtoni*. Our work provides a functional framework on which to test the nonapeptide modulation of behavior. Furthermore, we have shown that these receptors are present in brain regions important for regulating social decision-making, and analysis across a diverse array of species in the future may help to elucidate how variation in social behavior has contributed to the rapid parallel evolution of cichlids.

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