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Putative Isotocin Distributions in Sonic Fish: Relationship to Vasotocin and Vocal-Acoustic Circuitry

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Abstract

Recent neurophysiological evidence in the plainfin midshipman fish (*Porichthys notatus*) demonstrates that isotocin (IT) and arginine vasotocin (AVT) modulate fictive vocalizations divergently between three reproductive morphs. In order to provide an anatomical framework for the modulation of vocalization by IT, and to foster comparisons with the distributions of the IT homologues mesotocin (MT) and oxytocin (OT) in other vertebrate groups, we here describe putative IT distributions in the midshipman and the closely related gulf toadfish, *Opsanus beta*. Double-label fluorescent histochemistry was used for IT and AVT (using antibodies for MT, OT and the mammalian AVT homologue, arginine vasopressin, AVP). MT/OT-like immunoreactive (MT/OT-lir) cell groups are found in the anterior parvocellular, posterior parvocellular and magnocellular preoptic nuclei. MT/OT-lir fibers and putative terminals densely innervate the ventral telencephalon and numerous areas in the hypothalamus and brainstem. These distributions include all sites of vocal-acoustic integration recently identified for the forebrain and midbrain, as well as diencephalic components of the ascending auditory pathway. Results were qualitatively comparable across morphs, species and seasons. In contrast to the widespread distribution of MT/OT-lir, AVP-lir somata, fibers and putative terminals are almost completely restricted to vocal-acoustic regions. These data parallel earlier descriptions of AVT-ir in these species, although the present methods reveal a previously undescribed, seasonally variable AVP-lir cell group in the anterior tuberal hypothalamus, a vocally active site and a component of the ascending auditory pathway. These findings provide anatomical support for the role of IT and AVT in the modulation of vocal behavior at multiple levels of the central vocal-acoustic circuitry.

Keywords

preoptic area; reproduction; oxytocin; vasopressin; mesotocin; evolution

Introduction

Neuropeptides of the vasotocin family influence behavior across a broad range of vertebrate groups. However, whereas the behavioral functions of neuropeptides in the vasopressin lineage of this family (arginine vasotocin, AVT, and arginine vasopressin, AVP; found in non-mammals and mammals, respectively) are increasingly well known (reviews: Foran and Bass, 1999; Insel and Young, 2000; Bass and Grober, 2001; Goodson and Bass, 2001), those of the oxytocin lineage (isotocin, IT, mesotocin, MT and oxytocin, OT) remain far less explored (for

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reviews of OT functions, see Witt, 1995; Young, 1999; Insel and Young, 2000). In fact, given that virtually no behavioral data are available on the evolutionary precursors of OT (which are IT, found in fish, and MT, found in lungfish, marsupials and non-mammalian tetrapods) and that OT is involved in a variety of mammal-specific functions (e.g., lactation and parturition), OT functions are often regarded as uniquely mammalian.

Although some physiological functions of OT are indeed clearly unique to mammals, we hypothesize that social behavior functions of OT must nonetheless be evolutionarily derived from similar functions of IT and MT in the ancestors of modern mammals (see Moore, 1992). This hypothesis is supported by the fact that AVT and AVP modulate similar behavioral processes in multiple vertebrate classes (e.g., communication, aggression and sexual behavior; Goodson and Bass, 2001), and that some of these functions are very similar to the functions of OT (Witt, 1995; Insel and Young, 2000). Parsimony thus suggests that peptides of the vasotocin family influenced social behavior prior to the gene duplication event that led to the expression of both AVT and IT (see Acher, 1972), and that some of these functions were conserved in the OT lineage. Indeed, peptides which are structurally similar to those of the vasotocin family serve reproductive functions in invertebrates as well (Moore, 1992; Fujino et al., 1999), thus social/sexual behavior functions of this family may have arisen exceptionally early in animal evolution.

Recent data demonstrating vocal-motor modulation by IT in the plainfin midshipman fish (Goodson and Bass, 2000a) are of particular interest in this context, as they represent a starting point from which behavioral comparisons may be made of oxytocin and non-mammalian oxytocin homologues. In female midshipman, local administration of IT inhibits vocal-motor responses elicited by anterior hypothalamic stimulation and this effect is reversed by an OT antagonist. AVT and an AVP antagonist are relatively ineffective. This modulatory pattern is shared by type II males, which sneak/satellite spawn and are female-typical in multiple aspects of morphology and behavior, but the pattern is reversed in type I males that vocally court, defend nests and exhibit parental care (for a review of midshipman behavioral biology, see Bass, 1996). These data provide good support for our hypothesis that behavioral functions of OT may be evolutionarily derived from those of AVT in stem vertebrates, as peptidergic modulation of vocal processes in the midshipman (Goodson and Bass, 2000a, 2000b) is paralleled by similar findings for AVT/AVP in multiple vertebrate groups and for OT in mammals (reviews: Insel and Young, 2000; Goodson and Bass, 2001). Thus, peptidergic modulation of processes related to communication (e.g., motivation or sensorimotor integration) likely antedated the divergence of the vasopressin and oxytocin lineages.

It should be noted that peptidergic influences on vocalization cannot be homologous as *vocal* functions of IT and OT (Goodson and Bass, 2001), given the independent evolution of vocalization in teleost fish and mammals (Bass, 1989; Bass and Baker, 1991). Nonetheless, anatomical data strongly suggest that vocal-motor systems in all vocal taxa are derived from more generalized and homologous behavioral circuits (following the basic pattern of preoptic area/anterior hypothalamus→periaqueductal gray/dorsal tegmentum→medullar motoneuron pools; see Fig. 1 and Bass and Baker, 1997; Goodson and Bass, 2002) and these circuits are characterized by an AVT/AVP fiber innervation in all gnathostome vertebrates (review: Goodson and Bass, 2001). Similar fiber distributions are found for MT and OT systems (e.g., Moore, 1992), and limited IT data are likewise consistent (van den Dungen et al., 1982; Batten et al., 1990). Thus, these circuits provide a conserved anatomical framework for the influence of neuropeptides on a diversity of behavioral processes.

In teleost fish, isotocin-immunoreactive (IT-ir) neurons in the preoptic area are known to project to regions of the diencephalon and the midbrain tectum and tegmentum (van den Dungen et al., 1982; Batten et al., 1990; Holmqvist and Ekström, 1991; see Discussion), but

available descriptions are not extensive and do not allow a direct comparison with the vocal-acoustic/general behavioral circuits now identified for vocal teleosts (Goodson and Bass, 2002). The present experiments were therefore conducted to describe the distribution of IT in two closely-related vocal teleosts (order Batrachoidiformes, family Batrachoididae), the plainfin midshipman (*Porichthys notatus*) and the gulf toadfish (*Opsanus beta*), with special attention being paid to vocal-acoustic circuits and the ascending auditory pathway. IT distributions were additionally mapped in relation to AVT, thus allowing direct comparisons of their potential sites of action.

Methods

Subjects

A total of sixteen plainfin midshipman and two gulf toadfish were used in the present experiments. Fourteen of the midshipman (eight type I males, three type II males and three females) were collected from intertidal field sites near Tomales Bay, California during the summer nesting season. These subjects were held in running seawater tanks at the University of California Bodega Marine Laboratory prior to perfusion (1-3 days following capture). In addition, one female and one type I male were netted in deep water (~90 m) off the northern California coast during the winter. These fish were perfused upon capture. Gulf toadfish were purchased from a commercial supplier (Gulf Specimen, Panacea, FL) and were maintained in artificial seawater tanks at room temperature prior to sacrifice. Care and experimental procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committees of Cornell University and the University of California.

Immunocytochemistry (ICC)

Subjects were anesthetized by immersion in 0.2% benzocaine (Sigma Chemical Co., St. Louis, MO) and perfused with ice-cold teleost Ringer's solution followed by 4% paraformaldehyde. Brains were removed, post-fixed for 1 h and transferred to 0.1M phosphate buffer (PB; pH 7.2) for shipment to the University of California, San Diego. Following overnight incubation of brains in 30% sucrose, 50 μ m sections were cut on a sliding microtome and collected into PB.

Free-floating tissue sections were processed using OT or VA-10 MT antiserum raised in rabbit and an AVP antibody raised in guinea pig (OT and AVP antisera were purchased from Bachem Immunochemicals, Torrance, CA; VA-10 MT antiserum was a gift of Dr. Harold Gainer, National Institutes of Health). All sections were processed for MT or OT and were counterlabeled with DAPI nuclear stain; alternate MT/OT series were either double-labeled for AVP or were incubated with a fluorescent Nissl stain (NeuroTrace 530/615; Molecular Probes, Eugene, OR). Antibody specificities were determined in alternate series of type I male brains by preadsorption with 5, 50 or 100 μ M IT or AVT.

ICC was performed as follows: 20 min in 0.1M phosphate buffered saline (PBS; pH 7.4); 1 hr in PBS + 5.0% bovine serum albumin (BSA); 16-18 h in primary antiserum diluted 1:1000 in PBS + BSA + 0.3% triton-X + 0.01% sodium azide (performed at 4°C); two 20 min rinses in PBS; 2 h in anti-rabbit secondary conjugated to Alexa Fluor 488 (green; Molecular Probes) and either a red fluorescent Nissl stain (NeuroTrace 530/615; Molecular Probes) or an anti-guinea pig secondary conjugated to Alexa Fluor 594 (red). Sections were extensively washed in PBS and then transferred to PB prior to mounting. Sections were mounted on slides and coverslipped with Vectashield containing DAPI nuclear stain (Vector Labs, Burlingame, CA).

Cell counts

As presented in the Results, clear seasonal or morph variation was noted only for the AVP-lir cell group in the anterior tuberal nucleus of the hypothalamus, which varied by season. The

number of AVP-lir labelled somata was therefore quantified in this region by counting somata in midshipman. Only somata with a distinct perimeter and at least one neurite were measured. Given that AVP immunostaining was conducted on alternate 40 μm series, individual somata were not counted twice (soma size of these cells is $\sim 12\text{-}14\ \mu\text{m}$).

Photomicroscopy

Photomicrographs were generated using a Zeiss Axioscop microscope and an Optronics Magnafire digital camera linked to a dual-processor Macintosh G4 computer. For each fluorophore, monochrome images were captured and contrast enhanced in Photoshop 5.5 for Macintosh prior to digital color merging and balancing. No re-touching of tissue flaws was performed. For the presentation of some material, all red and green peptide label was color merged in the camera software, selected and excised from the black background in Photoshop, and then superimposed on the DAPI background. This layered color montaging allowed greater color contrast against the intense DAPI label. This procedure was employed for three of the 16 photos here presented (Figs. 2B-C; 5B). All other photos are of data which was fully color merged within the camera software.

Nomenclature

The nomenclature of Braford and Northcutt (1983) was adopted for descriptions of the preoptic area and diencephalon. Other works based on this nomenclature were additionally consulted (e.g., Striedter 1990, 1991). Nomenclature of vocal-acoustic structures is consistent with that used for previous descriptions of vocal-acoustic pathways and neuropeptide distributions in the midshipman (Bass et al., 1994, 2000, 2001; Goodson and Bass, 2000b, 2002).

Results

Overview

Several recent publications extensively delineate the vocal-acoustic circuitry, central AVT pathways and cytoarchitecture of the midshipman and toadfish brains (Foran and Bass, 1998; Bass et al., 2000, 2001; Goodson and Bass, 2000b, 2002). The series of drawings and low-power photomicrographs presented in Figures 2-5 were thus selected to illustrate putative IT distributions in relation to those recently described circuits. Figure 1 (adapted from Goodson and Bass, 2002) indicates the approximate level of the drawings and photographs and provides a schematic overview of the pattern of connectivity of forebrain and midbrain vocal-acoustic complexes (fVAC and mVAC, respectively).

As summarized in Figure 1, descending vocal pathways arise from a distributed set of structures in the fVAC that project to vocal-acoustic structures of the mVAC and rostral hindbrain, which in turn give rise to distributed projections to the vocal pattern generator of the caudal medulla (Bass and Baker, 1990; Bass et al., 1994; Goodson and Bass, 2002). All major components of this network receive direct projections from the auditory division of the torus semicircularis (nucleus centralis) and/or from the major thalamic target of the auditory torus, the central posterior nucleus (Bass et al., 2000, 2001; Goodson and Bass, 2002). The connectivity of the vocal acoustic complexes provides a framework for our earlier (Goodson and Bass, 2000b) and current studies of the distribution of neuropeptides in sonic batrachoidid fish.

As detailed below, mesotocin- and oxytocin-like immunoreactive (MT/OT-lir) structures are distributed widely throughout the brain, including the general vicinity of the medullar vocal pattern generator (Figs. 2Q-S; 5D-E) and all components of the fVAC and mVAC (Figs. 2C-M; 3C-G; 4A-D; 5A-C). MT/OT-lir fibers also innervate diencephalic components of the ascending auditory pathway and telencephalic regions connected to these diencephalic regions (Figs. 2B-G; 3A, D-F; 4B-C).

Specificity

A variety of previous data demonstrate the specificity of the VA-10 MT antiserum, including negative staining in lamprey (which produce only AVT), radioimmunoassay, differential absorption assays using AVT- and MT-coupled beads, and preadsorption with either AVT or MT (Conway and Gainer, 1987; Hilscher-Conklin et al., 1998). Consistent with these data, preadsorption of the AVP/MT antibody cocktail with 5 μ M AVT eliminated AVP-lir but had no effect on MT/OT-lir. Conversely, preadsorption of the cocktail with 5, 50 and 100 μ M IT produced a graded elimination of MT-lir while producing no obvious reductions in AVP-lir. However, although the large majority of MT-lir fibers and cells were eliminated at 50 μ M, a modest amount of fiber labeling persisted following preadsorption with 100 μ M IT and a small number of magnocellular preoptic neurons showed very light immunoreactivity. In addition, nest-like structures which were not obviously associated with labeled processes (arrows, Fig. 4A) were scattered throughout the brain both with and without IT preadsorption. There was no topographical selectivity of this label within the areas normally labeled by the VA-10 antibody, and it was therefore not restricted to areas of overlap with AVP-lir structures.

Data generated with the OT antiserum provide good clarification of this artifactual labeling, as OT-lir was virtually eliminated by preadsorption with 50 μ M IT and was completely eliminated at 100 μ M IT. In general, both the distribution and density of OT-lir and MT-lir structures were indistinguishable. However, the nest-like structures observed in the MT material were completely absent in OT material (i.e., it was not observed in OT material with or without preadsorption).

Given that the non-specific fiber label produced with the MT antiserum constitutes only a very minor fraction of the total fiber label, and that no difference could be discerned between MT-lir and OT-lir structures throughout most of the brain, we have presented photomicrographs of material generated with both antibodies. These photographs and the textual descriptions below represent material for which there is good agreement between the MT and OT material, with the exception of the few nest-like structures visible in MT material (see Fig. 4A).

Morph, species and seasonal variation

The topographical distribution of MT/OT-lir structures was qualitatively comparable across morphs, species and seasons. While more detailed quantitative comparisons with larger numbers of subjects could potentially reveal such variation, the present data suggest that any differences can be expected to be subtle and not qualitative.

In contrast, we did detect a qualitative seasonal difference in the number of AVP-lir somata in the anterior tuberal hypothalamus of the midshipman. Winter fish exhibited numerous somata while most summer fish exhibited none or just a few (55.0 ± 1.0 vs. 2.8 ± 1.3 , respectively; $p < 0.0001$, unpaired t -test). This population of neurons was not detected in our previous studies, which were conducted on summer fish (Foran and Bass, 1998; Goodson and Bass, 2000b). Other morph differences in AVP/AVT distributions have already been described in these papers.

Distribution of MT/OT- and AVP-lir somata (putative IT and AVT)

MT/OT-lir somata are found in the magnocellular, posterior parvocellular, and anterior parvocellular nuclei of the preoptic area (PM, PPp and PPa, respectively; Figs. 2C-F; 3C-G; 4A), with the large majority of parvocellular MT/OT-lir neurons being found in the PPa. Consistent with previous descriptions of AVT-lir in the midshipman (Foran and Bass, 1998; Goodson and Bass, 2000b), AVP-lir somata are also found in the PPa, PPp and PM (Fig. 3C, E-G), as well as in the ventral tuberal hypothalamus, which contains only a very small cell group (not shown). However, the present material additionally reveals the presence of a small

number of weakly labeled AVP-lir somata in the anterior tuberal nucleus of the hypothalamus (Fig. 4C). These are particularly numerous and conspicuous in the two winter fish examined (one female and one type I male) and one summer type II male. These neurons are difficult to observe in summer fish; none were found in seven summer fish and only 1-4 very lightly labeled cells were found in the remaining seven subjects.

Distribution of MT/OT- and AVP-lir fibers and putative terminals

The distribution of AVP-lir fibers and putative terminals is in agreement with our previous descriptions of AVT-lir in the midshipman, thus the present results are only briefly summarized here. In general, AVP-lir structures in the forebrain, midbrain and isthmus are almost completely restricted to vocal-acoustic regions, including the PPa (Fig. 3A-E), the ventral and anterior tuberal regions of the hypothalamus (VT, AT; Figs. 3E-F; 4B-C), the paratoral and paralemnisal midbrain tegmentum (PTT, PL; Figs. 4D; 5A-B), and the isthmal paraventricular region (IP, Fig. 5C). Few regions caudal to the isthmus are innervated, with the exception of the area postrema, which is heavily labeled (AP, Fig. 5E).

Distinct projections from each of the MT/OT-lir cell groups cannot be readily identified, as MT/OT-lir fibers from each group mingle with the those of the others and fiber density in the diencephalon is very high (e.g., Figs. 3E-G; 4A). However, as is addressed in the Discussion, a variety of other data suggest that most or all of the central MT/OT-lir projections arise in the parvocellular nuclei.

Forebrain—Within the diencephalon, dense MT/OT-lir terminal fields are observed within all components of the forebrain vocal-acoustic complex, which is comprised of the PPa and Pp (Figs. 2C-F; 3A-E, G; 4A), the ventral tuberal region (VT; Figs. 2D-E; 3E), and the anterior tuberal nucleus (AT; Figs. 2G; 4B-C) of the hypothalamus. The anterior tuberal nucleus is also a component of the ascending auditory pathway, being a major target of projections from the auditory nucleus of the torus semicircularis; the ventral tuberal region may likewise receive a small toral projection (Bass et al., 2000, 2001; Goodson and Bass, 2002). Of the other two diencephalic targets of the auditory torus, the central posterior thalamic nucleus and the lateral preglomerular nucleus, only the central posterior nucleus is directly innervated by MT/OT-lir fibers. This innervation is modest, with most punctate structures and varicosities being limited to the diffuse division (CPd, Figs. 2G; 4B) that adjoins a more medial cell plate (CPc). Importantly, although MT/OT-lir fibers do not directly enter the lateral preglomerular nucleus, fibers and putative terminals are nonetheless very dense in the adjacent neuropil (e.g., arrow adjacent to PGI; Fig. 4B). Finally, thick varicosities and axonal swellings are observed in association with the preoptico-hypophysial tract (PHT, Figs. 3C-F), with some being so large that they were initially mistaken for cell bodies. However, these structures are clearly devoid of Nissl and/or DAPI counterstain.

Within the telencephalon, MT/OT-lir fiber distributions are largely restricted to the neuropil regions which lie along the ventrolateral aspect of the ventral division of the telencephalon. This label is often dense and putative terminals are found adjacent to all of the ventral telencephalic nuclei (dorsal, ventral, posterior and supra commissural nuclei; Vd, Vv, Vp and Vs; Figs. 2B-D; 3A-B, D). Only scattered fibers are found within most other telencephalic regions.

Fibers coursing caudally from the preoptic nuclei give off dense terminals in the neuropil region that lies between the medial preglomerular nucleus and the periventricular hypothalamus (Figs. 2G-H; 4B). Other fibers sweep dorsally around the region of the medial and lateral preglomerular nuclei and corpus glomerulosum (PGm, PGI, G; Figs. 2G-H; 4B, D). In addition to vocal-acoustic structures innervated at this approximate level (central posterior thalamic nucleus, anterior tuberal nucleus), strong innervation is also observed for the pretectum, the

periventricular nucleus of the posterior tuber (TPp) and the lateral hypothalamus (LH; Figs. 2G; 4B). Fibers coursing caudally through the tuberal hypothalamus continue to skirt the medial margin of the medial preglomerular nucleus, which occupies a more ventromedial position at its caudal extent; these fibers wrap the dorsal periventricular hypothalamus and innervate the central region of the inferior hypothalamic lobes (Hd; Figs. 2H; 4D). This innervation of the inferior lobes is limited to their rostral-most extent.

Midbrain—Descending MT/OT-lir projections to the mesencephalon are very heavy and are virtually ubiquitous in the tectum and tegmentum for their full rostrocaudal extents (Figs. 2H-M; 4D; 5A-C). Within the tectum, the majority of label is found within the stratum album centrale and the stratum griseum centrale (see Brantley and Bass, 1988 for description of tectal layers in the midshipman). Labeled fibers also occur within the tectobulbar bundle that streams through the torus en route to the tectum (not shown but see Bass et al. 2000 for topography).

Labeled fibers are particularly dense in the dorsal tegmentum (e.g., the paratoral tegmentum and along the margin of the periaqueductal gray; PTT and PAG, respectively; Figs. 2H-L; 4D; 5B). The heaviest fiber label extends from the dorsal tegmentum along the medial margin of the lateral lemniscus and into the ventral tegmentum/reticular formation (ll, RF; Figs. 2J-L; 5B). Although MT/OT-lir fibers and putative terminals are found lateral to the lemniscus as well, their density is much lower. This pattern of label corresponds quite well to the distribution of vocal-acoustic nuclei in the tegmentum, which includes the periaqueductal gray/paratoral tegmentum, paralemniscal tegmentum (PAG, PTT, PL; medial to the lateral lemniscus), the nucleus of the lateral lemniscus (nll, sparse MT/OT-lir input), the isthmal nucleus (Is), and the isthmal paraventricular cell group (IP). This distribution of MT/OT-lir fibers overlaps with that of AVP-lir fibers most extensively in the ventral tegmentum, IP and PL (Fig. 5A-C).

In contrast to the dense MT/OT-lir innervation of the tegmentum, projections to the torus semicircularis are sparse. Fibers and putative terminals are found mainly ventral to but also within a deep cell layer of the torus (see Bass et al., 2000, 2001); labeling is most dense at the caudal pole of the torus (Fig. 2M). Labeled fibers are also seen occasionally within the nucleus ventrolateralis (NV), a lateral line-recipient nucleus that lies just dorsal to the deep cell layer of the torus (arrows, Fig. 5B). Nucleus centralis, the auditory-recipient nucleus of the torus, is virtually devoid of label (for cytoarchitectural descriptions, see Bass et al., 2000, 2001; Goodson and Bass, 2002).

Hindbrain—At the rostral aspect of the hindbrain, MT/OT-lir fibers innervate the hindbrain paraventricular cell group, a component of the hindbrain vocal-acoustic complex, and then sweep ventrolaterally. Thus, most MT/OT-lir fibers descending past the isthmus are restricted to the ventral aspect of the medulla (i.e., ventral to the ventricle), although scattered fibers are observed dorsally as well. Dense labeling occurs adjacent to the sensory nucleus of the trigeminal nerve (not shown but see Bass et al. 2000 for topography) and heavy label is found throughout the reticular formation and the neuropil regions of the ventral medulla. At caudal levels this includes the general vicinity of the ventral medullary nucleus (VM; Figs. 2Q; 5D), the primary afferent of the vocal pacemaker-motoneuron circuitry (Bass et al., 1994; Goodson and Bass, 2002). Although few MT/OT-lir fibers directly enter the nucleus proper, they likely overlap VM processes that extend as a dense fiber bundle ventromedially away from VM somata and cross at the midline (see Bass et al., 1994). Caudal to the ventral medullary nucleus, fibers become more widely distributed in the dorsal medulla, and a particularly dense innervation is observed for the area postrema. MT/OT-lir fiber label in the area postrema extensively overlaps with AVP-lir fibers (Fig. 5E). Finally, MT/OT-lir fibers and putative terminals are observed in a column of neurons extending ventrolaterally from the sonic motor nucleus (Figs. 2Q-R; 5E). Although this column contains vocal pacemaker neurons, these cells

are interspersed with other medullary neurons and cannot be accurately distinguished without additional anatomical and/or physiological data.

Discussion

The present experiments reveal an exceptionally widespread innervation of the brain by IT in vocal batrachoidid fish, as determined by using MT and OT antisera. No qualitative morph, species or seasonal differences were noted. While MT/OT-lir distributions in midshipman and toadfish include all identified vocal-acoustic structures of the forebrain and midbrain, they also include a wide variety of other areas that are implicated in a diversity of behavioral, sensory, autonomic, and neuroendocrine processes. We first consider the distribution of MT/OT-lir structures in relation to vocal-acoustic and related sensory circuitry in batrachoidid teleosts, and to AVT distributions which characterize the vocal-acoustic circuitry of these species. This is followed by comparisons with 1) the distributions of IT in other teleosts and with general teleost neural systems, and 2) MT and OT distributions in tetrapod vertebrates. We conclude by proposing the hypothesis that, despite the presence of many derived characters, oxytocin-like peptide systems in all gnathostome vertebrates exhibit a number of evolutionarily conserved morphological and functional characteristics.

MT/OT-lir distributions in relation to AVT, vocal-acoustic, and related sensory circuitry in batrachoidid teleosts

As outlined in Figure 1 and discussed earlier, recent experiments in the midshipman and toadfish demonstrate the presence of extensive vocal-acoustic circuitry within the forebrain, midbrain and hindbrain that provide for the integration of vocal communication processes with other social behavior and neuroendocrine processes (Goodson and Bass, 2002; also see Demski and Gerald, 1972; Fine, 1979; Bass et al., 1994; Fine and Perini, 1994). We now demonstrate that MT/OT-lir is strongly associated with all levels of this vocal-acoustic system. Of the four components of the forebrain vocal-acoustic complex, the present findings indicate that IT is produced in two, the posterior and anterior parvocellular preoptic nuclei (PPp and PPa, respectively), and suggest that IT is locally released in these sites and/or the neuropil regions immediately adjacent to them. The other two forebrain vocal-acoustic components, the ventral tuberal region and the anterior tuberal nucleus of the hypothalamus, also receive MT/OT-lir projections, as do all structures of the midbrain vocal-acoustic complex (PAG/paratotal tegmentum, paralemniscal tegmentum, isthmal nucleus, nucleus of the lateral lemniscus and the isthmal paraventricular cell group). This innervation is very strong, with the exception of the nucleus of the lateral lemniscus, which receives a very light projection. Innervation of the hindbrain vocal-acoustic complex is variable, being strong in the paraventricular cell group of the rostral hindbrain (part of a hindbrain VAC) and virtually absent in octaval components of this region. Within the vocal pattern generator, MT/OT-lir fiber and putative terminals are dense in the general vicinity of the ventral medullary nucleus and the pacemaker neuron column, although direct input cannot be established based on the present material.

Within the forebrain and midbrain, this vocal-acoustic distribution of MT/OT-lir closely matches that of AVT/AVP-lir. AVT/AVP-lir is selectively associated with the vocal-acoustic circuitry, although not exclusively so (Goodson and Bass, 2000b), and the present discovery of AVP-lir somata within the anterior tuberal nucleus of the midshipman strengthens this association. This population is seasonally variable and largely absent in summer fish. With this addition, AVT/AVP-lir cell groups have now been localized to each of the four vocal-acoustic regions of the forebrain, and only to these areas. Both AVT and IT modulate fictive vocalization elicited from the ventral tuberal region (Goodson and Bass, 2000a) and AVT additionally modulates vocal-motor processes within the paralemniscal tegmentum (Goodson and Bass,

2000b). Thus, the extensive overlap of MT/OT-lir with AVP-lir in paralemniscal tegmentum (present study) strongly suggests that IT may influence vocal-motor activity within this region.

In contrast to AVT/AVP-lir, MT/OT-lir is also found within multiple components of the ascending auditory pathway (for tracings from the batrachoidid auditory torus, see Bass et al., 2000, 2001; for tracings from the auditory diencephalon, see Goodson and Bass, 2002; for tracings and neurophysiological studies in other teleosts, see Finger, 1980; Echter, 1984; Finger and Tong, 1984; Striedter, 1991; Wong, 1997; Correa and Zupanc, 2002; Kirsch et al., 2002). While MT/OT-lir projections to the torus are very light, fibers and putative terminals are moderate to dense in two diencephalic targets of the auditory and mechanosensory torus, the anterior tuberal nucleus and the central posterior nucleus of the dorsal thalamus. A third diencephalic target of the ascending auditory pathway, the lateral preglomerular nucleus, is relatively devoid of direct MT/OT-lir innervation, but the adjacent neuropil is heavily labeled by fibers and putative terminals. Finally, the majority of telencephalic innervation by MT/OT-lir fibers closely matches the topography of projections from the central posterior nucleus of the thalamus (Goodson and Bass, 2002). This includes 1) the neuropil regions ventrolaterally adjacent to the nuclei of the ventral telencephalon, and 2) a small dorsolateral zone of the dorsomedial telencephalon (compare Figs. 2A-D and 3A-B, D to Figs. 10A-B of Goodson and Bass, 2002). These findings indicate that MT/OT-lir projections to vocal and auditory regions primarily target sites of vocal-acoustic integration. Relative to these regions, the innervation of octaval and toral sensory regions is exceptionally light, and as discussed above, direct MT/OT-lir innervation of the vocal pattern generator is not clear from the present material.

Of obvious importance here is the question of which MT/OT-lir cell groups give rise to the MT/OT-lir projections to communication-related circuitry. Although this issue cannot be decisively addressed by the present material, tract tracings of the midshipman vocal-acoustic circuitry (Goodson and Bass, 2002) strongly suggest that most or all MT/OT-lir fibers within central vocal-acoustic targets are of parvocellular preoptic origin. Thus, of the large number of biotin compound injections conducted in our tracing studies, no retrogradely labeled neurons are observed within the magnocellular preoptic nucleus, whereas both the posterior and anterior parvocellular nuclei are strongly interconnected with all vocal-acoustic centers of the forebrain and midbrain.

Comparative distributions and general functional considerations of IT-ir/MT/OT-lir in teleosts

As noted in the next section, tetrapod homologues of IT (i.e., MT and OT) exhibit highly variable distributions even within a given vertebrate class. This stands in contrast to the emerging picture of IT distributions in teleosts, which appear to be quite similar in the few species thus far examined: rainbow trout (*Salmo gairdneri*; van den Dungen et al., 1982); green molly (*Poecilia latipinna*; Batten et al., 1990); and vocal batrachoidids (present study; for considerations of the hypophysiotropic systems, also see Goossens et al., 1977; Reaves and Hayward, 1980; Maejima et al., 1994; Holmqvist and Ekström, 1995; Honma et al., 1998; Ota et al., 1999b; Ota et al., 1999a). Thus, although slight differences in neuron locations and projection topographies are found between batrachoidids and other species, these are minimal relative to the similarities. In all teleosts, IT and AVT are produced in parvocellular and magnocellular preoptic neurons (see references above), although the presence of IT and AVT perikarya in the Pp has not been previously described for non-batrachoidids.

Detailed comparisons of IT/MT/OT-lir fiber systems with other species is somewhat difficult, as descriptions available for *Poecilia* and *Salmo* are brief. However, based upon the descriptions and drawings for *Salmo* (van den Dungen et al., 1982), no major topographical differences are found - IT-ir fibers extend from the preoptic nuclei through the medial aspect of the telencephalon (exact topography not described) to enter the olfactory bulb, and dense projections are found to the posterior diencephalon, optic tectum and midbrain tegmentum in

a pattern comparable to that described here for batrachoidids. Similar to the batrachoidids, *Salmo* also exhibits a greater abundance of IT than AVT in extrahypothalamic areas. This relative abundance is reversed in *Poecilia* (Batten et al., 1990). Thus, although the descriptions for *Poecilia* suggest a general topographical similarity to *Salmo* and the batrachoidids, the distribution of IT is somewhat more sparse.

The combined data from these teleosts suggest that IT exerts very broad modulatory influences throughout the brain, including numerous sensory regions and modalities. Thus, in addition to the auditory and mechanosensory regions discussed above, putative IT fibers and terminals extensively target visual-related regions (pretectum, optic tectum, and corpus glomerulosum), viscerosensory and gustatory regions (e.g., the vagal lobes and area postrema) and ventral telencephalic regions which receive projections from the olfactory bulbs (see Meek and Nieuwenhuys, 1998 for a general discussion of these systems). In addition to these sensory regions, putative IT distributions include much of the hypothalamus, particularly the tuberal regions, suggesting that central IT systems may exert widespread influences on physiological, behavioral and sensorimotor processes.

Anatomical comparisons with the MT and OT systems of tetrapods and some speculations on their functional evolution

Within the oxytocin lineage, the most extensive comparisons have been conducted for the amphibians, with representative whole-brain descriptions being provided for multiple anuran, caecilian and urodele species (e.g., Gonzalez and Smeets, 1992a, 1992b, 1997; Hilscher-Conklin et al., 1998). These descriptions clearly demonstrate that MT distributions within the Amphibia exhibit a wide variety of species-specific characteristics. In contrast, while teleost fish do exhibit interspecific variation in the relative densities of putative AVT and IT fibers (as is found for amphibians), they do not otherwise exhibit strong variation.

When comparing the present data and various amphibian MT-ir distributions to those known for mammals (Buijs et al., 1978; De Vries and Buijs, 1983; Hermes et al., 1988; Caffé et al., 1989; Bathgate et al., 1995), reptiles (Bons, 1983; Thepen et al., 1987), and birds (Bons, 1980; Blähser, 1984), a limited number of features are found to be conserved. These include the presence of a MT/OT magnocellular hypophysiotropic system and extrahypothalamic fiber projections to limbic forebrain regions, the PAG and adjacent regions of the midbrain tegmentum, isthmal regions and the area postrema (for extensive discussion, see (Gonzalez and Smeets, 1992a, 1992b, 1997). Data for all teleosts described to date are fully consistent with this general topography (van den Dungen et al., 1982; Batten et al., 1990; present study).

Importantly, this conserved anatomical framework within the oxytocin lineage is virtually identical to that observed for the vasopressin lineage, and comparative data suggest that the ancestral AVT distribution in stem vertebrates (prior to the expression of IT) consisted of a similar pattern (review: Goodson and Bass, 2001; also see Moore and Lowry, 1998). These observations suggest that the basic anatomical features now observed for all peptides in the vasotocin family have been conserved from the earliest AVT distributions in vertebrates (at least to 450 million years ago). Thus, we can expect to find that IT, MT, OT, AVT and AVP systems all share an involvement in a number of functions. Obvious candidates include visceromotor, arousal, motivational, and reproduction-related physiological and behavioral processes. Indeed, peptidergic involvement in all of these areas has already been documented (see Introduction and the following reviews: Moore, 1992; Witt, 1995; Insel and Young, 2000; Goodson and Bass, 2001; for additional functional considerations in teleosts, see Grober and Sunobe, 1996; Godwin et al., 2000; Bass and Grober, 2001; Semsar et al., 2001; Grober et al., 2002; Salek et al., 2002).

We hypothesize that these basic anatomical and functional characteristics have served to guide the evolution of peptide functions, with the evolution of derived processes occurring within anatomically conserved circuits which serve older, more general functions that are related in some manner to the apomorphic trait. For instance, a conserved peptidergic involvement in reproductive physiology and behavior likely set the stage for OT involvement in mammalian lactation, parturition and maternal behavior; each of these OT functions occurs within the generalized vertebrate circuits as described above (Moore, 1992; Witt, 1995; Insel and Young, 2000). In some cases, this pattern of evolution may yield exceptionally similar, but convergent, functional circuitry in distantly related groups. Thus, as recently demonstrated, the vocal-acoustic system of batrachoidid teleosts exhibits strong convergence with the vocal-acoustic systems of other taxa that have independently evolved vocalization (particularly mammals; Goodson and Bass, 2002). These circuits appear to be derived from more generalized behavioral circuits (following the basic pattern of preoptic area/anterior hypothalamus→periaqueductal gray/dorsal tegmentum→medullar motoneuron pools) that serve a variety of broader social behavior functions (e.g., sexual and agonistic behavior; VanderHorst and Holstege, 1996; Delville et al., 2000; Vanderhorst et al., 2000). As exhibited in the present material, and in the variety of studies summarized above, peptides of the vasotocin family exhibit conserved distributions within this circuitry. Therefore, it may be expected that across the vertebrate spectrum these peptides would evolve convergent roles in the modulation of various behaviors related to sexual and agonistic behavior which are themselves much more recently derived. This is in fact the case for peptide functions within both lineages of the vasotocin family. IT and OT modulate vocalization related to a variety of social contexts in monkeys (Winslow and Insel, 1991), rats (Insel and Winslow, 1991), hamsters (Floody et al., 1998), and fish (Goodson and Bass, 2000a), and influence olfactory communication as well (Winslow and Insel, 1991). A wide range of comparable data are available for peptides of the vasopressin lineage (review: Goodson and Bass, 2001). Together, these data suggest that despite the independent evolution of vocal communication in various vertebrate groups, principles of general relevance may be derived from studies of peptidergic systems in a variety of species.

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Table 1: Abbreviations

ac	anterior commissure
AP	area postrema
AT	anterior tuberal nucleus
C	cerebellum
CA	cerebral aqueduct
cc	cerebellar crest
Cg	granule cell layer of the cerebellum
Cm	molecular layer of the cerebellum
CPc	compact division of the central posterior nucleus
CPd	diffuse division of the central posterior nucleus
DF	diffuse nucleus of the hypothalamus
DL	dorsolateral telencephalon
DM	dorsomedial telencephalon
EG	eminencia granularis
fVAC	forebrain vocal-acoustic complex
G	corpus glomerulosum
H	hindbrain
Hb	habenula
Hd	dorsal periventricular hypothalamus

HoC	horizontal commissure
hVAC	hindbrain vocal-acoustic complex
HYP	hypothalamus
IL	inferior lobe of the hypothalamus
IP	isthmal paraventricular cell group
Is	isthmal nucleus
LH	lateral hypothalamus
ll	lateral lemniscus
M	midbrain
MED	nucleus medialis
MLF	medial longitudinal fasciculus
mVAC	midbrain vocal-acoustic complex
NC	nucleus centralis of the torus semicircularis
NV	nucleus ventrolateralis of the torus semicircularis
nll	nucleus of the lateral lemniscus
OB	olfactory bulb
OL	olfactory nerve
OP	optic nerve
ot	optic tract
PAG	periaqueductal gray

PGl	lateral division of nucleus preglomerulosus
PGm	medial division of nucleus preglomerulosus
PHT	preoptico-hypophysial tract
pit	pituitary
PL	paralemniscal midbrain tegmentum
PM	magnocellular preoptic nucleus
PN	pacemaker neurons
PPa	anterior parvocellular preoptic nucleus
PPp	posterior parvocellular preoptic nucleus
PTT	paratoral tegmentum
RF	reticular formation
SC	spinal cord
SMN	sonic motor nucleus
SV	saccus vasculosus
T	telencephalon
Te	midbrain tectum
TPp	periventricular nucleus of the posterior tuber
TS	torus semicircularis
v	ventricle
Vd	dorsal nucleus of the ventral telencephalon

Vg	granule layer of the valvula
VL	vagal lobe
Vm	molecular layer of the valvula
VM	ventral medullary nucleus
Vp	posterior nucleus of the ventral telencephalon
Vs	supracommissural nucleus of the ventral telencephalon
VT	ventral tuberal hypothalamus
Vv	ventral nucleus of the ventral telencephalon

Figure 1.

A schematic sagittal view of the midshipman brain showing the locations and major connections of the vocal-acoustic circuitry in the plainfin midshipman (*cross-hatching*, components of the ascending auditory pathway; *stippling*, vocal-acoustic complexes; *open ovals*, vocal pacemaker circuitry; modified from Goodson and Bass, 2002; see also Bass et al., 1994, 2000, 2001; Goodson and Bass 2000b). MT/OT-lir and AVP-lir innervation of these circuits is shown in the Figure 2 line drawings and Figure 3-5 photomicrographs corresponding to the levels indicated on the schematic. Vocal-motor activity is readily evoked from components of the forebrain and midbrain vocal-acoustic complexes (fVAC and mVAC, respectively). Auditory input gains access to the VACs first via direct eighth nerve input to rostral hindbrain nuclei (h) that are part of an hVAC. These same hindbrain auditory nuclei provide part of the auditory input to the midbrain's torus semicircularis (TS) which, in turn, projects to both the fVAC and mVAC via a dorsal thalamic nucleus, CP. CP also directs auditory information to the dorsomedial (DM) and ventral (V) telencephalon. The fVAC is the highest-order vocal center identified to date and is linked to the vocal pacemaker circuitry of the caudal medulla directly by the mVAC and indirectly via a mVAC-hVAC relay. The pacemaker circuitry includes a ventral medullary nucleus (VM) that provides the vast majority of descending input to a column of pacemaker neurons (PN) that in turn provide the sole identified input to the sonic motor nucleus (SMN). The SMN directly innervates the sonic musculature of the swimbladder.

Figure 2.

A representative series of line drawings from a type II male midshipman illustrating the distribution of mesotocin- and oxytocin-like immunoreactivity (MT/OT-lir). Parvocellular and magnocellular MT/OT-lir cell groups are indicated by filled circles (small and large, respectively; C-F). MT/OT-lir fibers and putative terminals are indicated by thin lines and dots.

Figure 3.

Distribution of mesotocin- and oxytocin-like immunoreactivity (MT/OT-lir; Alexa Fluor 488, green) in the telencephalon and rostral diencephalon of the plainfin midshipman fish. Red counterlabel in panels **D** and **E** is a fluorescent Nissl stain (NeuroTrace 530/615); other panels show MT/OT-lir in relation to arginine vasopressin-lir (AVP-lir; Alexa Fluor 594, red) with DAPI nuclear stain (blue or artificially colored purple for contrast enhancement) providing cytoarchitectural detail. Photos represent a rostral-to-caudal series through the telencephalon and show components of the forebrain vocal-acoustic complex (ventral tuberal hypothalamus, VT; anterior parvocellular preoptic nucleus, PPa; posterior parvocellular preoptic nucleus, PPa), and ventral telencephalic afferents of the forebrain vocal-acoustic nuclei (e.g., Vp, Vs,

and Vv; also targets of the auditory-recipient, central posterior nucleus of the thalamus). Arrows in **B** show the location of a small terminal field in the dorsomedial telencephalon (DM) which overlaps with projections of the central posterior nucleus of the thalamus. *A and C-E: Type II male. B and G: Type I male. F: Female. Scale bars: 200 μ m in all panels except G (100 μ m).*

Figure 4.

Distribution of mesotocin- and oxytocin-like immunoreactivity (MT/OT-lir; Alexa Fluor 488, green) in the caudal forebrain and rostral mesencephalon of the plainfin midshipman fish. Red counterlabel in panel B is a fluorescent Nissl stain (NeuroTrace 530/615); other panels show MT/OT-lir in relation to arginine vasopressin-lir (AVP-lir; Alexa Fluor 594, red) with DAPI nuclear stain (blue or artificially colored purple for contrast enhancement) providing cytoarchitectural detail. Shown are two components of the forebrain vocal-acoustic complex, the posterior parvocellular preoptic nucleus (PPp; **A**) and the anterior tuberal nucleus of the hypothalamus (AT; **B-C**) which contains a small population of weakly-labeled AVP-lir neurons. AT is also a component of the ascending auditory pathway, as are the central posterior

nuclei of the thalamus (CPc, CPd; **B**) and the lateral preglomerular nucleus (PGL; **B**). Two components of the midbrain vocal-acoustic complex are shown in panel **D** (periaqueductal gray, PAG, and the parabrachial tegmentum, PTT). Arrows show nest-like artifact produced by the MT antiserum (**A**), dense fibers and putative terminals wrapping PGL (**B**) and the deep cell layer of the torus semicircularis (**D**). *A, C and D: Type I male. B: Type II male. Scale bars: 100 μ m in A, 200 μ m in B and D, and 50 μ m in C.*

Figure 5.

Distribution of mesotocin- and oxytocin-like immunoreactivity (MT/OT-lir; Alexa Fluor 488, green) and arginine vasopressin-lir (AVP-lir; Alexa Fluor 594, red) in the caudal midbrain and medulla of the plainfin midshipman fish. Cytoarchitectural detail is provided by DAPI nuclear stain (pseudocolored purple for contrast enhancement). **A-C.** Label is particularly dense in the components of the midbrain vocal-acoustic complex (isthmal nucleus, Is; isthmal paraventricular cell group, IP; paralemniscal tegmentum, PL; and the periaqueductal gray/paratotal tegmentum; PAG/PTT; also somewhat weaker in the nucleus of the lateral lemniscus, nll). The deep cell layer of the torus semicircularis is indicated by arrows in B. *Type I male.* **D.** MT/OT-lir at the level of the ventral medullary nucleus (VM), the primary afferent of the

PN-SMN circuitry. *Type I male*. **E.** MT/OT-lir and AVP-lir at the level of the sonic motor nucleus (SMN). *Type I male*. **Scale bars:** 200 μm in all panels except A (100 μm).