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Research report

Neuroanatomical distribution of vasotocin and mesotocin in two urodele amphibians (*Plethodon shermani* and *Taricha granulosa*) based on in situ hybridization histochemistry

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Abstract

Previous research suggests that considerable species-specific variation exists in the neuroanatomical distributions of arginine vasotocin (AVT) and mesotocin (MST), non-mammalian homologues of vasopressin and oxytocin. An earlier study in rough-skinned newts (*Taricha granulosa*) indicated that the neuroanatomical distribution of cells labeled for AVT-immunoreactivity (ir) was greater in this urodele amphibian than in any other species. It was unknown whether the widespread distribution of AVT-ir is unique to *T. granulosa* or a feature common among salamanders. Using in situ hybridization (ISH) histochemistry and gene-specific riboprobes, the current study labeled AVT and MST mRNA in *T. granulosa* and the red-legged salamander (*Plethodon shermani*). In *T. granulosa*, AVT ISH-labeled cells were found to be widespread and localized in brain areas including the dorsal and medial pallium, lateral and medial septum, bed nucleus of the stria terminalis, amygdala, preoptic area, ventral hypothalamus, nucleus isthmus, tectum mesencephali, inferior colliculus, and hindbrain. In *P. shermani*, the distribution of AVT ISH-labeled neurons matched that of *T. granulosa*, except in the lateral septum, ventral hypothalamus, and inferior colliculus, but did however include labeled cell bodies in the lateral pallium. The distribution of MST ISH-labeled cells was more restricted than AVT ISH labeling and was limited to regions of the preoptic area and ventral thalamus, which is consistent with the limited distribution of MST/OXY in other vertebrates. These findings support the conclusion that urodele amphibians possess a well-developed vasotocin system, perhaps more extensive than other vertebrate taxa.

Theme: Neurotransmitters, modulators, transporters, receptors
Topic: Peptides: anatomy and physiology

Keywords: Amphibian; Vasotocin; Mesotocin; Vasopressin; Oxytocin

1. Introduction

Arginine vasotocin (AVT) and mesotocin (MST) are structurally similar nonapeptides, differing from each other by only two amino acid residues and both belonging to the neurohypophysial peptide family. AVT has been found in many species of non-mammalian vertebrates from cyclostomes to birds [2,4,20,39,45,53,54], and is the ancestral orthologous peptide of arginine vasopressin (AVP) found in mammals [3]. MST has been found in lung-fishes, amphibians, reptiles, birds, and marsupials, and is the ancestral peptide for oxytocin (OXY) in eutherian mammalians [4].

Neurohypophysial peptides were first identified as hormones secreted from nerve terminals in the pars

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nervosa and as having a variety of endocrine functions. AVT and AVP regulate hydromineral balance, vascular tone, and glucose metabolism; whereas, OXY controls smooth muscle contractions associated with parturition, lactation, and sexual arousal [1,22,41]. Less is known about the endocrine functions of MST; although MST has been shown to play a role in parturition in a marsupial mammal [10]. Neurohypophysial peptides also act centrally as neurotransmitters and neuromodulators, and appear to regulate a wide variety of brain functions and behaviors [7,26,40,50,58].

Consistent with the diverse central and peripheral functions attributed to neurohypophysial peptides, neuroanatomical studies reveal complex and, perhaps, species-specific neuroanatomical patterns of distribution. Immunocytochemical (ICC) and/or in situ hybridization (ISH) studies have found that, in most non-mammalian species, cell bodies labeled for AVT and MST are localized in the anterior hypothalamus/preoptic areas that are homologous to the AVP- and OXY-containing cell bodies in the paraventricular and supraoptic nuclei of mammals [12,15,19,28,29,49,51,60,63,64]. In addition to these conserved populations of cells, neurohypophysial peptide-containing cells reportedly occur in a variety of other sites in the brain [26,30,51,57]. But it is unknown whether these variations in neurohypophysial distribution within the brain reflect differences in techniques, species differences, sexual dimorphism, or other factors.

A previous ICC study from our laboratory identified at least nineteen distinct populations of AVT-immunoreactive (ir) cells in brains of rough-skinned newts (*Taricha granulosa*) [46,51], which suggested that this amphibian might have a more widespread distribution of AVT-ir cells than other species [46,51]. Thus the questions addressed by the current study are (1) whether the same nineteen populations of AVT-ir neurons can be identified using ISH techniques to label AVT mRNA and (2) whether the widespread distribution of AVT in *T. granulosa* is unique to this species or occurs in another species of salamander, the red-legged salamander (*Plethodon shermani*). To answer these two questions and also to identify the neuroanatomical distribution of MST in both species of salamanders, the current study used ISH techniques and species-specific riboprobes. Prior neuroanatomical studies for AVT and MST in amphibians have been limited to using ICC techniques with heterologous antibodies, mainly antisera generated against mammalian AVP or OXY [6,17,23–25,36,42,46]. To our knowledge, the only ISH study of AVT and MST systems in an amphibian was our work with *T. granulosa* [46], and that study used ISH with heterologous oligonucleotide probes. That earlier ISH study was not sensitive and, mainly, was used to validate the ICC procedures. The current study reports for the first time the neuroanatomical distribution of AVT and MST ISH-labeled cells using species-specific cRNA probes in the brain of amphibians.

2. Materials and methods

2.1. Animal collection and care

Conditions of captivity for each species differed to reflect particular aspects of their respective natural environments. Adult *P. shermani* were collected in forests and near streams in Macon County, NC, during the breeding season in August, and were maintained in an environmentally-controlled room (12.7 °C, 70% humidity, 12:12 LD cycle), housed individually in plastic boxes (30.5 cm length, 15.2 cm width, 8.9 cm depth) containing moist paper towels and moss, and fed mealworms. Adult *T. granulosa* were collected from local ponds in Lincoln County, OR, during the breeding season in March and April or out of the breeding season in November, and were maintained in an environmentally-controlled room (7 °C; 12:12 LD cycle) and housed together in a large, cylindrical tank (91 cm diameter, 78 cm height) with flow-through dechlorinated water (depth about 39 cm), and fed bloodworms and earthworms.

Salamanders were anesthetized by chilling and then rapidly decapitated. The brain and rostral spinal cord were rapidly dissected (1–2 min) and embedded in Histoprep Frozen Tissue Embedding Media (Fisher Scientific, Pittsburgh, PA), frozen on dry ice, and stored at −80 °C until sectioning. Whole brains were sectioned at a thickness of 20 μm at −20 °C using a Cryostat. The sections were thaw-mounted on Superfrost Plus® positive-charged microscope slides (Shandon, Inc., Pittsburgh, PA) and stored at −80 °C until use.

Neuroanatomical analysis with *T. granulosa* used a total of 20 brains from sexually mature males (15 males in breeding condition and 5 males not in breeding condition). All *T. granulosa* were held in captivity for less than a week.

Neuroanatomical studies with *P. shermani* used a total of 30 brains collected from sexually mature males ($n = 10$) or females ($n = 20$). Female *P. shermani* were sacrificed during the breeding season, within 8 weeks of capture; whereas male *P. shermani* were sacrificed after the breeding season when secondary sexual traits were regressed [37] and after about 16 weeks in captivity. Our main objective was to identify the neuroanatomical distribution of neurons that synthesize AVT or MST by using species-specific riboprobes and ISH procedures. This study was not designed to reveal differences in AVT and MST expression in males versus females, or breeding versus non-breeding animals, in part because the ICC study with *T. granulosa* has already reported sexual and seasonal differences in AVT immunoreactivity [52].

This study was performed under the guidelines of the U.S. Public Health Service's "Guide to the Care and Use of Laboratory Animals". All procedures were approved by the Oregon State University Laboratory Animal Resource Committee.