

AN ABSTRACT OF THE THESIS OF

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Title: Identification and Characterization of Vasotocin and Mesotocin
Peptides and Receptors

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The neurohypophysial peptide system is involved in modulating a variety of physiological, neurological, and behavioral responses in vertebrates. The principal forms of these peptides in non-mammalian tetrapods are vasotocin (VT) and mesotocin (MT). The studies described in this thesis used pharmacological, molecular, and biochemical techniques, along with phylogenetic analyses, to identify and characterize the mRNA sequences encoding the neurohypophysial peptide precursor proteins and their receptors in urodele amphibians.

The cDNAs encoding preproVT and preproMT were amplified by PCR from the brains of two salamander species; the rough-skinned newt, *Taricha granulosa*, and the red-legged salamander, *Plethodon shermani*. The neurohypophysial peptides encoded by the identified *Taricha* cDNAs were VT and MT; the *Plethodon* cDNAs encoded VT and a novel MT-like

peptide, [Val⁴]-MT. Phylogenetic analyses grouped both the *Taricha* and *Plethodon* preproVT and preproMT-like sequences with previously identified tetrapod preproVT-like and preproMT-like sequences, respectively. Additional analysis of the preproneurohypophysial sequences indicated that gene conversion (non-homologous crossing over of DNA sequences) appears to have occurred more frequently in mammals than in other tetrapod classes.

The cDNAs encoding the VT receptor (VTR) and MT receptor (MTR) were amplified from the brain of *T. granulosa* by PCR. Sequence identity, and phylogenetic analysis, indicated that the *Taricha* MTR and VTR were most similar to MTR/OTRs and V_{1a}-like VTRs, respectively. Distribution of PCR amplicons specific to the *Taricha* MTR and VTR matched previously reported tissue distributions of MTRs and VTRs in other vertebrates in every tissue but kidney, from which the *Taricha* primers were unable to amplify a cDNA product. Binding experiments of transiently expressed *Taricha* MTR indicated two binding states, and allowed the determination of ligand binding affinities for this receptor. Inositol phosphate accumulation assays demonstrated that the expressed *Taricha* MTR and VTR cDNA produced functional receptors, and allowed calculation of ligand potencies of activation and inhibition. Surprisingly, an antagonist frequently used in behavioral experiments to specifically block VTR activity, inhibited inositol phosphate accumulation in cells transfected with either the *Taricha* MTR or VTR.

In conclusion, these studies report the first identified cDNA sequences encoding the preproVT, preproMT, MTR, and V_{1a}-like VTR proteins from urodele amphibians.

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**Identification and Characterization of Vasotocin and Mesotocin Peptides
and Receptors**

by

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Brian T. Searcy, Author

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These manuscripts were greatly enhanced by the editing of Dr. Frank L. Moore and C. Samuel Bradford. Eliza Walthers is responsible for the amplification and sequencing of the neurohypophysial hormone cDNAs from *Plethodon shermani*. Bethany Heppner helped with the amplification of the *Taricha* prepromesotocin gene; and Dr. Richmond R. Thompson helped with amplification of the *Taricha* preprovasotocin and vasotocin receptor cDNAs. Dr. Joanne Chu provided the β -actin primers used in the rtPCR experiments included in Chapter 3.

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**Identification and Characterization of Vasotocin and
Mesotocin Peptides and Receptors**

Chapter 1

General Introduction to the Neurohypophysial Peptides and Receptors

Neurohypophysial peptides have been shown to modulate a variety of physiological, neurological, and behavioral actions in vertebrates. The name of this system is derived from the neurohypophysis (posterior pituitary), the primary site from which these peptides are released as hormones into the peripheral circulatory system. In most vertebrates, there are two primary types of neurohypophysial peptide; a mesotocin (MT)-like, and a vasotocin (VT)-like peptide.

When released as hormones, the neurohypophysial peptides are synthesized both in magnocellular and parvocellular neurons of the hypothalamus and then released directly into the circulatory system from nerve terminals in the posterior pituitary and median eminence, respectively. In addition, neurohypophysial peptides are known to be synthesized at other sites in the brain (Barberis and Tribollet 1996; Moore and Lowry 1998; Smeets and Gonzalez 2001) where they act as neurotransmitters and/or neuromodulators in synapses between neurons.

Physiological Roles of the Neurohypophysial System

The neurohypophysial peptides were originally discovered because of their effects on physiological processes. Vasopressin (VP), thought to be the ortholog of amphibian VT (Acher 1980; Urano et al. 1992; Acher et al. 1995), was initially studied for its ability to rapidly increase blood pressure in mammals. Additional physiological responses attributed to VP include the regulation of hydromineral balance, vascular tone, glucose metabolism, and adrenocorticotropin (ACTH) release. Similar responses

have been attributed to VT in amphibians. VT has been found to act both as an antidiuretic (Schmidt-Nielsen and Forster 1954; Eggena et al. 1968; Shoemaker and Nagy 1977; Warburg 1995)) and as a modulator of ACTH release (Larcher et al. 1989; Larcher et al. 1992).

Oxytocin (OT) is thought to be the mammalian ortholog of amphibian MT (Acher 1980; Urano et al. 1992; Acher et al. 1995). OT was first studied because of its ability to induce uterine contractions (review (den Hertog et al. 2001)). In mammals, known physiological actions of OT include stimulation of milk let-down, luteolysis, and enhancement of uterine contractility during parturition. In amphibians, the only proposed physiological action of MT is as a diuretic, antagonizing the actions of VT on water balance (Pang and Sawyer 1978; Warburg 1995).

Behavioral Modulation by the Neurohypophysial Peptide System

Neurohypophysial peptides are thought to directly affect behavior, acting as neurotransmitters and/or neuromodulators in the brain (Barberis and Tribollet 1996; Moore and Lowry 1998; Smeets and Gonzalez 2001). In mammals, OT and VP have been found to influence a variety of behaviors. As a general rule, OT influences behaviors in females; whereas, VP influences behaviors in males (Young 1999).

In mammals, VP has been found to affect aggression (Ferris and Delville 1994; Ferris et al. 1997; Young et al. 1997), scent-marking behaviors (Ferris et al. 1984), parental care (Bult et al. 1992; Bamshad et al. 1994; Wang et al. 1994), pair-bonding by males (Winslow et al. 1993),

mating behaviors (Albers and Rawls 1989), memory and learning (Alescio-Lautier et al. 2000; Winslow and Insel 2004), and other social behaviors. Similar to the effects of VP, VT has been found to enhance aggression in birds (Goodson 1998; Goodson 1998), and vocalization in birds (Maney et al. 1997; Goodson 1998), fish (Goodson and Bass 2000), and frogs (Penna et al. 1992; Boyd 1994; Marler et al. 1995; Propper and Dixon 1997; Semsar et al. 1998). Additional behavioral effects of VT that have been demonstrated in amphibians include enhancing male courtship (Moore and Miller 1983; Toyoda et al. 2003), female receptivity (Diakow 1978; Boyd 1992), and egg-laying behaviors (Moore et al. 1992).

In mammals, behavioral effects attributed to OT include maternal behaviors (Pedersen and Prange 1979; Kendrick et al. 1987; McCarthy 1990), sexual receptivity (Caldwell et al. 1986; McCarthy et al. 1994), memory and learning (Kinsley et al. 1999; Tomizawa et al. 2003), and pair-bond formation (Williams et al. 1994). No behaviors have been found that are definitively attributed to MT in any species. MT administered to zebra finches (*Taeniopygia guttata*) does not appear to affect social behaviors (Goodson et al. 2004). Although OT injection causes increases in locomotion and decreases in feeding in chickens (Jonaidi et al. 2003), because of the cross-reactivity of neurohypophysial peptides and their receptors, the identity of the receptor system activated in these experiments remains to be determined (Jonaidi et al. 2003). In the plainfin midshipman (*Porichthys notatus*), isotocin (IT), a potential OT and MT

ortholog (Acher 1980; Urano et al. 1992; Acher et al. 1995), has been found to increase calling behaviors in females and type II males (sneaker males) (Goodson and Bass 2000). Because both OT and IT have been found to modulate behaviors, it is likely that MT will also be found to affect specific, as yet unidentified, behaviors in amphibians (and other non-mammalian tetrapods).

Neurohypophysial Peptide Structure

In mammals, the only vertebrate class in which the genomic sequence and chromosomal locations of neurohypophysial peptide genes have been characterized, the OT and VP genes are located proximally, on opposite strands of the same chromosome (Ivell and Richter 1984; Sausville et al. 1985; Hara et al. 1990). Following transcription, the neurohypophysial peptides are each initially translated as part of a larger prepropeptide. This larger protein is then cleaved by proteolysis into smaller subunits releasing the active neurohypophysial peptide (review (Burbach et al. 2001)). The preproneurohypophysial peptides (preproNHP) contain the following three to four segments: a) a signal peptide, b) the neurohypophysial peptide (*i.e.* VT, MT, etc.), c) neurophysin, d) and in the case of VT and VP, copeptin, a short glycoprotein. In mammals, copeptin is cleaved from the neurophysin moiety, but in amphibians and birds they apparently remain together, creating a "big" neurophysin (Michel et al. 1987; Michel et al. 1990). Neurophysins are thought to help with proper trafficking and packaging of

the peptides into neurosecretory granules (Chaiken et al. 1983; Breslow and Burman 1990).

The functionally active neurohypophysial peptides are predominantly nine amino acids in length, consisting of a six amino acid ring and a three amino acid side chain. The exceptions to this rule are the hydrins, ten to twelve amino acid proteins (thought to be extended forms of VT (Acher et al. 1997)) found only in frogs (Rouille et al. 1989; Acher et al. 1997). In all neurohypophysial peptides a disulfide-bond is formed between two Cys residues, which are located at amino acid positions one and six, causing these peptides to fold into a ring-like structure. The VT-like family of peptides all have a basic amino acids at position 8 (an Arg residue in the case of VT), and the MT-like peptides all have a neutral amino acid (an Ile residue in the case of MT) at position 8.

Evolution of the Neurohypophysial Peptides

The neurohypophysial peptides are structurally and functionally conserved throughout vertebrate evolution (Acher 1980; Urano et al. 1992; Acher et al. 1995). In addition, homologs of these peptides have been found in several invertebrate species. In invertebrates, these peptides are thought to be involved in modulating actions similar to those attributed to neurohypophysial peptides in vertebrates (Reich 1992; van Kesteren et al. 1992; Oumi et al. 1994; Fujino et al. 1999; Satake et al. 1999). In agnathans (jawless fishes), extant ancestors of one of the first derived vertebrate classes, only a single neurohypophysial peptide has been

identified, specifically VT (Heierhorst et al. 1992; Suzuki et al. 1995).

Whereas VT has been found in all non-mammalian gnathostomes, mammals predominantly synthesize the VT-like peptide VP (VP = [Phe³]-VT).

In addition to a VT-like peptide, gnathostomes also have MT-like peptides (Acher et al. 1999). In elasmobranches, seven MT-like peptides have been identified (Acher et al. 1999; Hyodo et al. 2004). Bony fishes (osteichthys) all have isotocin (IT, IT = [Ser⁴]-MT) (Acher 1980; Urano et al. 1992; Acher 1996), except for lungfish which have MT (Hyodo et al. 1997). The majority of tetrapods, including amphibians, reptiles, birds (Acher et al. 1995), and some marsupials (Chauvet et al. 1981; Parry et al. 2000), all express MT. Other marsupial species have been reported to have both MT and OT (OT = [Leu⁸]-MT) (Chauvet et al. 1984; Rouille et al. 1988); whereas in eutherian mammals, OT is the dominant MT-like neurohypophysial peptide.

Two hypotheses have been proposed to explain the evolution of the preproNHP genes in vertebrates. One hypothesis, based on variations in the nine amino acid neurohypophysial peptide sequences, proposes that throughout gnathostome evolution two distinct neurohypophysial peptide lineages exist, the VT-like peptides and the MT-like peptides (Acher 1980; Acher et al. 1995). This hypothesis further proposes that these two lineages are derived from a common ancestral preproVT gene that

underwent gene duplication early in the evolution of cartilaginous fishes (Acher 1980; Acher et al. 1995).

The second hypothesis proposed to explain the evolution of the neurohypophysial peptides (Hyodo et al. 1991; Suzuki et al. 1992; Urano et al. 1992) is based on the calculated DNA substitution rates of full-length preproNHP cDNA sequences. This hypothesis proposes that independent gene duplication events gave rise to the teleost preproNHP genes and the tetrapod preproNHP genes (Hyodo et al. 1991; Suzuki et al. 1992; Urano et al. 1992). Because this second hypothesis is based on differences in the much longer cDNA sequences, it would be expected to be more accurate at describing the evolution of the neurohypophysial peptide genes. Despite using the full-length cDNA sequences, this analysis was unable to describe the evolution of the tetrapod preproNHP genes with any confidence. Specifically, based on this analysis, it remains unclear whether mammalian preproVP and preproOT genes arose from an additional gene duplication derived from the amphibian preproVT gene, or whether the mammalian preproVP and preproOT are orthologs of the amphibian preproVT and preproMT genes, respectively (Hyodo et al. 1991; Urano et al. 1992).

Neurohypophysial Peptide Receptor Evolution

The neurohypophysial peptide receptors have been predominantly studied in mammals; investigations of these receptors from other vertebrate classes are less extensive. Because of the limited studies in

non-mammals, the evolution of this receptor system is not well defined. Within mammals there have been four types of neurohypophysial peptide receptors characterized; a single oxytocin receptor (OTR), and three vasopressin receptor subtypes. The vasopressin receptor subtypes are V_{1a} receptor ($V_{1a}R$), V_{1b} receptor ($V_{1b}R$), and V_2 receptor (V_2R) (also called the $V1R$, $V3R$ and $V2R$ vasopressin receptors, respectively (Thibonnier et al. 1998)). These neurohypophysial peptide receptors are found in different sites within the body and regulate different functions.

In invertebrates, two neurohypophysial receptor subtypes have been identified in a species of snail (van Kesteren et al. 1996), which is interesting because this snail apparently has only a single neurohypophysial peptide, conopressin (CP) (van Kesteren et al. 1992). Similar to VT and VP, CP contains a basic amino acid residue at position 8 (van Kesteren et al. 1992). One of the two identified snail CP receptors exhibited high selectivity for peptides with a polar amino acid residue at position eight, whereas the other snail CP receptor was not (van Kesteren et al. 1996); this sensitivity to amino acid polarity at position 8 conforms to the findings with the mammalian VP and OT receptors, respectively. Although this finding of neurohypophysial receptor selectivity in snails suggests that the ancestral forms of OTR and the VPRs were each already present in invertebrates but because there is no evidence supporting multiple neurohypophysial peptide receptor types in cyclostomes, this finding may just be a coincidence.

In non-mammalian vertebrates only a limited number of neurohypophysial peptide receptors have been characterized. Two neurohypophysial peptide receptor types have been identified in teleosts, a V1a-like VTR (Mahlmann et al. 1994; Warne 2001) and an OTR-like isotocin receptor (ITR) (Hausmann et al. 1995). In amphibians (limited to three anuran species) there has been a single mesotocin receptor (MTR) type (Akhundova et al. 1996; Kohno et al. 2003; Acharjee et al. 2004) and two vasotocin receptor (VTR) subtypes identified; a V1a-like VTR (Mahlmann et al. 1994; Acharjee et al. 2004), and a V2-like VTR (Kohno et al. 2003). No neurohypophysial peptide receptors have been identified and reported from reptiles, but in birds two VTR subtypes have been identified (a V2-like VTR (VT1R (Tan et al. 2000)), and a V1b-like VTR (VT2R (Cornett et al. 2003)). In order to further characterize the evolution of this receptor family, more sequences, from a diverse array of vertebrate classes need to be identified.

Tissue Distribution of the Neurohypophysial Peptide Receptors

The distribution of the neurohypophysial peptide receptors reflects the physiological functions that each receptor is involved in modulating. In mammals VP and OT receptors have been found to be expressed at a number of sites throughout the body (review (Thibonnier et al. 1998)). In brief, the V_{1a}R is expressed in the liver, vascular smooth muscle cells, and testis. The V_{1b}R is expressed in the corticotroph cell of the pituitary, the pancreas and the adrenal medulla. The V₂R is expressed in the medullary

portion of the kidney. The OTR is expressed in the uterus, mammary gland, ovary, kidney, and lactotroph cells (located in the adenohypophysis). All four receptor subtypes have been found in the brain (Thibonnier et al. 1998; Gimpl and Fahrenholz 2001). In amphibians, the distribution of the neurohypophysial peptide receptors have only been characterized in a few species, but between these species there is a lot of variation (Akhundova et al. 1996; Kohno et al. 2003; Acharjee et al. 2004). In order to better define conserved patterns of distribution for these receptor types, the distribution patterns of each type needs to be identified in additional non-mammalian species.

Biochemistry of the Neurohypophysial Peptide Receptors

The MT/VT receptors belong to the super family of G-protein coupled receptors (Barberis and Tribollet 1996). These receptors activate their signal in target cells through a second messenger system that is initially tied to the activation of G-proteins. Following ligand binding to the receptor, guanosine triphosphate (GTP) replaces guanosine diphosphate (GDP) on the G-protein, causing the G-protein to dissociate from the receptor and affect downstream second-messenger components, either enhancing or inhibiting these pathways. There are many G-protein subtypes that carry out a variety of functions. A few examples of neurohypophysial peptide receptor relevant G proteins are: G_s , which activates adenylyl cyclase (AC); G_i which inhibits AC; and $G_{q/11}$ which

activates phospholipase C. Following activation of these enzymes further responses in the target cell are carried out via a variety of different pathways.

In mammals, the $V_{1a}R$, $V_{1b}R$ and the OTR are linked primarily to the $G_{q/11}$ proteins, although the $V_{1a}R$ and $V_{1b}R$ s have also been found to associate with G_i proteins (Phaneuf et al. 1996; Thibonnier et al. 1998). Similarly, the *Bufo marinus*, *Rana catesbeiana* and *Hyla japonica* MTRs all initiate cellular response consistent with being associated with $G_{q/11}$ (Akhundova et al. 1996; Kohno et al. 2003; Acharjee et al. 2004). In contrast to the other receptors in this family, the V2R- vasopressin receptors are associated primarily with the G_s subtype of G-protein (Thibonnier et al. 1998).

Pharmacology of the Neurohypophysial Peptide Receptors

Each receptor type in the MT/VT receptor family has a distinct pharmacological profile. Because these receptors are so closely related, there is considerable cross-reactivity between each of the different peptides and the receptors. For example, both OT and VP can bind to, and activate the mammalian OTR (Thibonnier et al. 1998). The ligand with the highest affinity for a given receptor typically confers the name to that receptor; the receptors with the highest affinity for VP are called the VP receptors. The pharmacological profile for many of the receptor types has been determined both by expression cloning and through binding

studies *in vivo*. The *in vivo* studies are more difficult to conduct because of the potential for a heterogeneous mix of receptor types, but these studies are more likely to reflect the true physiological state of the receptors. Although neurohypophysial peptide receptors have been well characterized in mammals, few of these receptors have been studied in other vertebrates. Because comparative receptor pharmacologies are useful both in determining sequence-pharmacology relationships, and for comparative behavioral and physiological studies, additional non-mammalian receptors should be characterized.

Vasotocin, Mesotocin and *Taricha granulosa*

A large body of research has been conducted using *Taricha granulosa*, the rough-skinned newt, as a model organism for VT-induced behaviors. *Taricha* is an exceptional model for the study of these behaviors because of the robustness and stereotypical nature of responses to specific stimuli. Both systemic (Moore and Zoeller 1979; Moore and Miller 1983) and centrally administered VT (Moore and Miller 1983) stimulates reproductive behavior in males. In addition, VT has been found to enhance egg-laying behaviors in female newts (Moore et al. 1992), and increase appetitive behaviors in male newts in response to visual and pheromonal stimuli (Thompson and Moore 2000). Although the distribution of both the neurohypophysial peptides (Hollis et al., in press; Lowry et al. 1997; Moore et al. 2000) and receptors (as determined by *in vitro* receptor-autoradiography) (Tripp and Moore 1988) have been

characterized in the newt brain, little is known about the sequence identity encoding any of these proteins.

Specific Aims

This thesis has seven specific aims: 1) identify the cDNAs encoding the proneurohypophysial peptide sequences from the rough-skinned newt, *Taricha granulosa*, and the red-legged salamander, *Plethodon shermani*; 2) describe the phylogeny of the proneurohypophysial peptide genes in tetrapods; 3) identify the full length cDNA sequences encoding the neurohypophysial peptide receptors from *Taricha*; 4) determine the phylogeny of the vertebrate neurohypophysial peptide receptors; 5) describe the tissue distribution of these receptors in the newt; 6) characterize ligand binding affinities for the newt neurohypophysial peptide receptors; and 7) demonstrate the functional activity of the *Taricha* neurohypophysial peptide receptors and describe ligand potencies through inositol phosphate accumulation assays.

This thesis is composed of two research chapters, written in manuscript format, followed by a general summary chapter. Chapter 2 presents the full-length cDNA sequences encoding the prepromesotocin and preprovasotocin sequences, amplified from both *T. granulosa* and *P. shermani*, and also includes a phylogenetic analysis of tetrapod proneurohypophysial peptide deduced amino acid sequences. In Chapter 3 the full length cDNA sequences encoding the *T. granulosa* mesotocin and vasotocin receptors are reported. Chapter 3 also

describes the tissue distribution and binding pharmacology, and functional responses of the newt neurohypophysial receptors, in addition to an analysis of vertebrate neurohypophysial peptide receptor evolution.

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Chapter 2

Identification and Analyses of cDNA Sequences for Vasotocin and Mesotocin from Two Urodele Amphibians: Evolutionary Relationships and Gene Conversion

Abstract

This paper reports the cDNA sequences that encode preprovasotocin (preproVT) and prepromesotocin (preproMT) in the rough-skinned newt, *Taricha granulosa*, and red-legged salamander, *Plethodon shermani*. These are the first urodele amphibian sequences from the preproneurohypophysial peptide (preproNHP) family to be identified. These preproNHP sequences were amplified from brain cDNA by 5' and 3' RACE PCR. In *T. granulosa*, cDNA sequences were identified that encode preproVT and preproMT. In *P. shermani*, the identified cDNA sequences encode preproVT and a previously unidentified mesotocin-like precursor, prepro-[Val⁴]-MT. The deduced amino acid sequence of each of the four preproNHPs conforms to the conserved organization of other preproNHPs. In mammals, the central regions of the preproNHP genes are hypothesized to undergo gene conversion (non-homologous crossing over). A phylogenetic analysis of the tetrapod preproNHP sequences, excluding the region of gene conversion, grouped preproVT and preproVP together in one cluster, and preproMT and preprooxytocin (preproOT) in another. To examine the occurrence of gene conversion in tetrapods we calculated the sequence identity between the preproVT-like and preproMT-like sequences for both the gene conversion region, and the non-gene conversion regions, within each individual species; these levels of identity were then compared between tetrapods. The level of sequence identity for the gene conversion region

in mammals was higher than that of other tetrapods. We suggest that this finding indicates a lower frequency of gene conversion among non-mammalian tetrapods than mammals.

Introduction

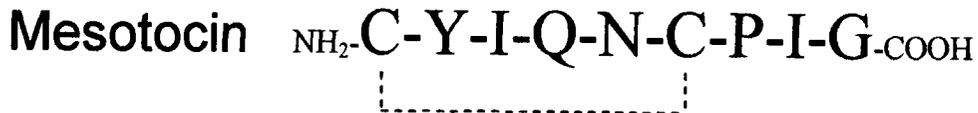
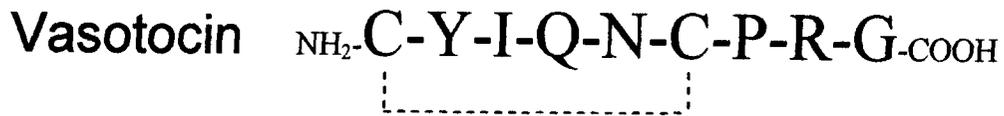
Amphibians are known to express two neurohypophysial peptides, vasotocin (VT) and mesotocin (MT), thought to be orthologs of mammalian vasopressin (VP) and oxytocin (OT), respectively (Acher 1980; Urano, Hyodo et al. 1992; Acher et al. 1995). Neurohypophysial peptides are nonapeptides (Fig 2.1A) released in response to a variety of behaviorally and physiologically relevant stimuli. These peptides function both as hormones and as neuromodulators. The neurohypophysial peptides released as hormones are produced in magnocellular and parvocellular cells of the hypothalamus and are secreted into the circulatory system at the posterior pituitary (neurohypophysis/pars nervosa) and median eminence. Other regions of the brain also express neurohypophysial peptides (review (Moore and Lowry 1998; Smeets and Gonzalez 2001)) that are released at synapses between neurons.

Acting as neuromodulators, these peptides are thought to modify behaviors (for review (Young 1999; Insel and Young 2000; Goodson and Bass 2001; Rose and Moore 2002)), memory and learning (Kinsley et al. 1999; Alescio-Lautier et al. 2000; Tomizawa et al. 2003; Winslow and

Insel 2004), and thermoregulation (Wilkinson and Kasting 1987; Wilkinson and Kasting 1987; Kluger 1991; Pittman and Wilkinson 1992).

In amphibians, VT has been found to influence water balance ((Schmidt-Nielsen and Forster 1954; Eggena et al. 1968; Shoemaker and Nagy 1977; Pang and Sawyer 1978; Warburg 1995), (Acher et al. 1997)), thermoregulation (Bicego-Nahas et al. 2000), and behaviors. Some of these behaviors include male courtship (Moore and Miller 1983; Toyoda et al. 2003), female receptivity (Diakow 1978; Boyd 1992), egg-laying (Moore et al. 1992) and frog vocalization (Penna et al. 1992; Boyd 1994; Marler et al. 1995; Propper and Dixon 1997; Semsar et al. 1998). Currently MT has no specific known function in any tetrapod. In birds, MT administered to zebra finches (*Taeniopygia guttata*) does not appear to affect social behaviors (Goodson et al. 2004). In chickens (*Gallus gallus*), intracerebroventricular injection of oxytocin (OT) causes increases in locomotion and decreases in feeding (Jonaidi et al. 2003), but due to the cross reactivity of neurohypophysial peptides and their receptors, the identity of the receptor system activated in these experiments remains to be determined (Jonaidi et al. 2003).

A.



B.

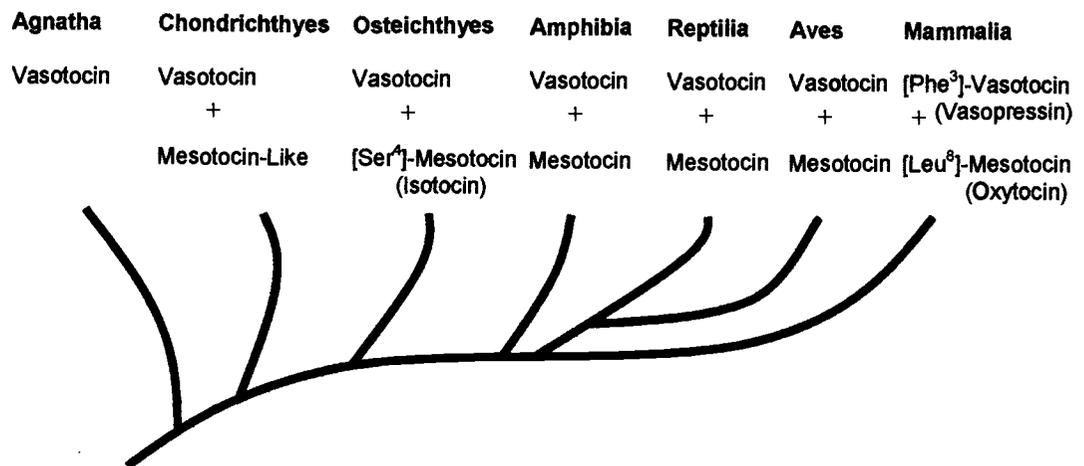


Fig 2.1 Structure and evolution of the vertebrate neurohypophysial hormones. A. The amino acid structure of the dominant neurohypophysial hormones found in non-mammalian tetrapods. The dashed line indicates the formation of a disulfide bond between the Cys residues at position 1 and 6 of each of these hormones. B. A diagram of the dominant form of the neurohypophysial hormones found in each vertebrate class above a evolutionary tree showing the divergence of each of these vertebrate classes (adapted from (Campbell et al. 1999)).

MT and VT are each initially translated as part of larger prepropeptides that are cleaved by proteolysis into smaller subunits (review (Burbach et al. 2001)). The preproneurohypophysial peptides (preproNHP) consist of the following three to four components: a) a signal peptide, b) the neurohypophysial peptide (*i.e.* VT, MT, etc.), c) neurophysin, d) and in the case of VT and VP, copeptin, a short glycoprotein. In mammals, copeptin is cleaved from the neurophysin moiety, but in previously studied amphibians and birds it apparently remains linked, creating a “big” neurophysin (Michel et al. 1987; Michel et al. 1990). Neurophysins are thought to help with proper trafficking and packaging of the hormones into neurosecretory granules (Chaiken et al. 1983; Breslow and Burman 1990).

Two separate hypotheses have been proposed that describe the evolution of the preproNHP genes. One hypothesis, based on variations in the nine amino acid neurohypophysial peptide sequences, proposes that two distinct neurohypophysial hormone lineages exist in gnathostomes, the VT-like peptides and the MT-like peptides. The two neurohypophysial peptide lineages are hypothesized to be derived from a gene duplication of an ancestral preproNHP gene early in the evolution of cartilaginous fishes (Fig 2.1B)(Acher 1980) (Acher et al. 1995). An alternative hypothesis, based on calculated DNA substitution rates of full-length preproNHP cDNA sequences, proposes that separate gene duplication events gave rise to the teleost preproisotocin and preproVT

genes and the tetrapod preproMT-like and preproVT-like genes (Hyodo et al. 1991; Suzuki et al. 1992; Urano et al. 1992). Based on the substitution rate analysis, it remains unclear whether the mammalian preproVP and preproOT genes arose from additional gene duplication events, or if the mammalian preproVP and preproOT are direct descendents of the amphibian preproVT and preproMT genes, respectively (Hyodo et al. 1991; Urano et al. 1992).

We report here the cDNA sequences encoding the neurohypophysial hormone precursors of two amphibians, the rough-skinned newt (*Taricha granulosa*) and the red-legged salamander (*Plethodon shermani*). These are the first reported neurohypophysial hormone sequences from caudate amphibians (salamanders). Both *Taricha* and *Plethodon* are used as models for studying neurohypophysial hormone effects on behavior. Each cDNA sequence, and its associated deduced amino acid sequence, is analyzed and discussed. To further clarify the phylogeny of the tetrapod neurohypophysial hormones, these sequences are also used as a part of a phylogenetic analysis. In addition, we report evidence for a novel MT-like peptide, [Val⁴]-mesotocin, expressed as a native hormone in *P. shermani*.

Materials and Methods

Animals.

Adult rough-skinned newts (*Taricha granulosa*) were captured in Benton and Lincoln Co., Oregon, maintained in an environmentally controlled room (14 °C, 12 L/12D) for about 24 h. The newts were held in large tanks containing dechlorinated water and fed earthworms upon arrival in their storage tank. The newts were cryoanesthetized and then sacrificed by rapid decapitation. Brains were quickly removed and snap-frozen in liquid nitrogen and then stored at -80 °C until proceeding with RNA preparation.

Preparation of RNA.

Total RNA was extracted from *T. granulosa* brains using a guanidinium thiocyanate based kit, Totally RNA (Ambion Inc., Austin, TX), according to the protocol recommended by the manufacturer.

Polymerase Chain Reaction (PCR) Amplification from cDNA.

Taricha and *Plethodon* brain total RNA was reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Corp. Carlsbad, CA) following the protocol recommended by the manufacturer. Degenerate primers were designed to be located in regions of high sequence identity based on an alignment of multiple neurohypophysial hormone cDNA sequences obtained from the Entrez Nucleotide Database. The successful primers for the preproVT cDNA were: VTsense; 5'-TCA TCA GCC TGC TAC ATC CAG-3' and

VTantisense: 5'-CCR TCC ATY TGN GTC ATR TT-3', yielding a predicted product of 500 bp. Successful primers for the preproMT cDNA were; MTsense: 5'-AAY TGY CCC ATH GGM GGN AAR MG-3' and MTantisense: 5'-GGS AGR WAR TTY TCY TCC TGG CAV CT-3', yielding a predicted product of 184 bp. Degenerate primers (0.5 μ M) were used in conjunction with cDNA template (50 ng) in 20 μ l PCRs under the following reaction conditions: 94 °C for 3 min; 4 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s; final extension at 72 °C for 6 min; hold at 4 °C. PCR products were either purified using a Qiagen PCR Pure Kit following the recommended protocol of the manufacturer and then sequenced at the Central Services Laboratory (CSL) at Oregon State University (OSU); or ligated into pCR4-TOPO vector (Invitrogen Corp.), transformed into TOP10 cells, the plasmid purified by alkaline lysis using the Qiaprep kit (Qiagen, Valencia, CA) and then sequenced at the CSL. The sequences of the 5' and 3' ends of the VT and MT preproNHP cDNA were obtained by rapid amplification of cDNA ends (RACE) PCR using a FirstChoice RLM-RACE PCR kit (Ambion Inc.) following the protocol recommended by the manufacturer. All sequences were amplified using the following reaction conditions: 94 °C for 3 min.; 4 cycles of 94 °C for 45 s., 60 °C for 45 s., 72 °C for 90 s.; 35 cycles of 94 °C for 30 s., 60 °C for 30 s., 72 °C for 60 s.; final extension at 72 °C for 6 min.; hold at 4 °C. PCR products were then sequenced as above. Contiguous cDNA sequences for each preproNHP

were determined by alignment of overlapping cDNA sequences. All four sequences was amplified and sequenced independently three to five times.

Phylogenetic Analysis.

Amino acid sequences were downloaded from Entrez-Protein (NCBI) and aligned using ClustalX (Thompson et al. 1997). The alignments were then manually adjusted prior to phylogenetic analysis to achieve optimal alignment. Phylogenetic trees were constructed by Bayesian inference with a Markov-chain Monte Carlo (MCMC) sampling method using MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003). The parameters were set so the program used the Blossum (Henikoff and Henikoff 1992) amino acid-substitution model with a gamma distribution of among-site rate variation and all amino acid substitution rates permitted to be different. One out of every 100 trees was sampled for 500,000 generations. The first 1000 trees were discarded as burn-in. The 50% consensus tree was computed by MrBayes using the last 4000 sampled trees. In each analysis we ran four simultaneous MCMC chains.

Results

Preprovasotocin Sequences.

Both the 727 base pair (bp) *Taricha* (Fig 2.2A) and the 771 base pair *Plethodon* (Fig 2.2B) full-length VT precursor cDNA sequences possess start and stop codons that border a 161 deduced amino acid (aa) protein, preproVT. Like other previously identified preproNHP sequences in the VT/VP category, each of the preproVT sequences is composed of four regions: a short hydrophobic signal peptide (19 aa), the neurohypophysial peptide (9 aa), neurophysin-II (93 aa), and a C-terminal glycosylated copeptin (37 aa) (Fig 2.2A, Fig 2.2B).

Two of the *Taricha* and *Plethodon* full-length cDNA sequences contain regions that encode for the nine amino acid neurohypophysial peptide VT. The VT and neurophysin-II portions of each preproVT are separated from each other by a Gly-Lys-Arg sequence conserved in all preproNHP sequences (Fig 2.3). Within the neurophysin-II sequence, there are fourteen conserved Cys residues, as found in all other neurophysin sequences (Fig 2.3). At the C-terminal end of neurophysin II, each preproVT sequence includes a conserved Arg residue found in mammalian preproVP sequences. The deduced copeptin sequences from *Plethodon* and *Taricha* each contain an N-linked glycosylation site and a hydrophobic core, features conserved in all other copeptin proteins. Unlike previously identified copeptin sequences, a partial substitution of

2.2A *Taricha granulosa* Preproneurohypophysial Peptides

34

Taricha Vasotocin cDNA: AAAAGTAACGAAGCTAACAGGAAGCACAGCAGCAGACCCCCCTCGCCTCAGAG
Taricha Mesotocin cDNA: AAAACGCTGATTTTGAACGAAGAGCGTGCCGAGA

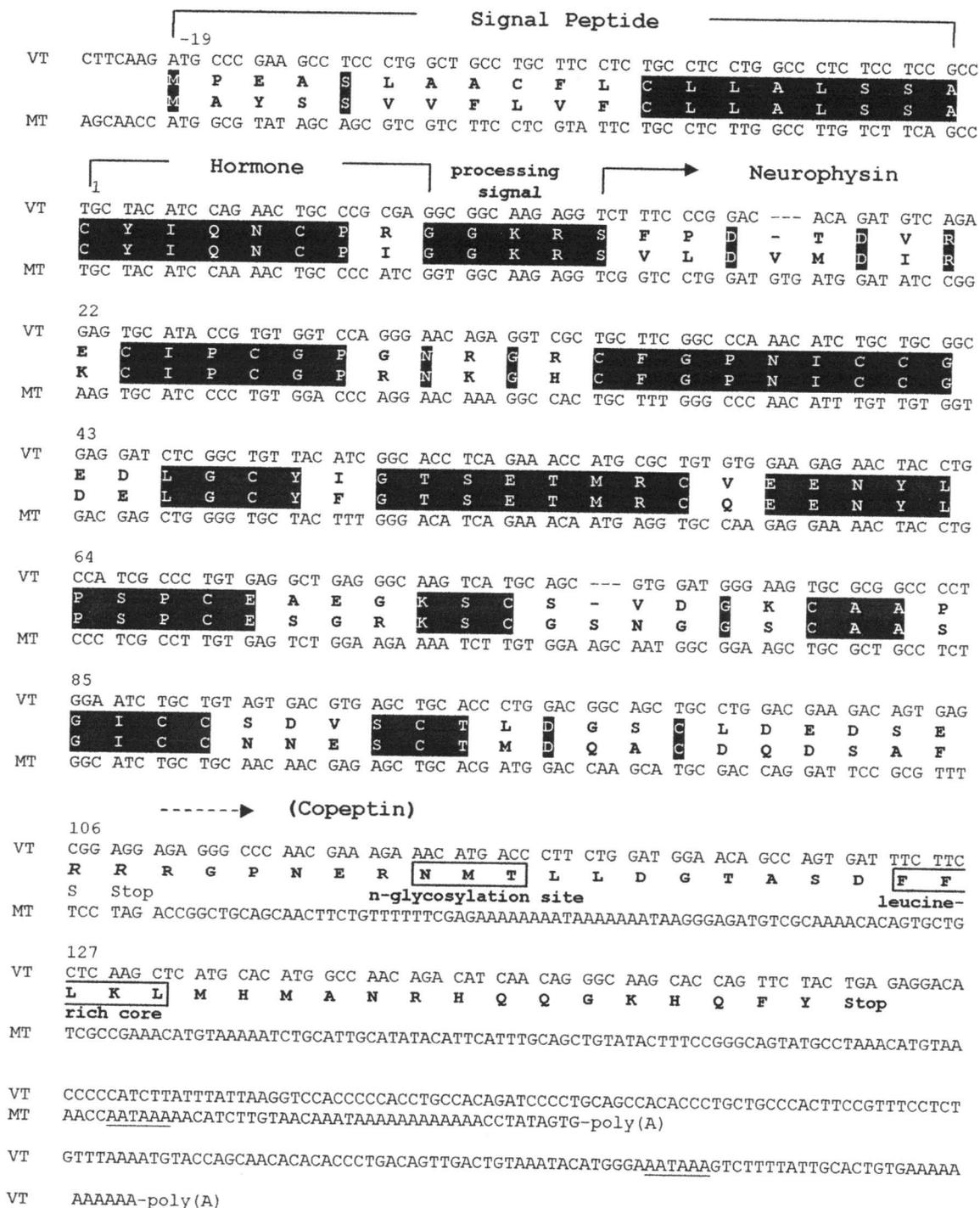


Fig 2.2 *Taricha granulosa* and *Plethodon shermani* preprovasotocin and prepromesotocin cDNA and deduced amino acid sequences.

The amino acid sequences are numbered so that Cys¹ of both mesotocin and vasotocin is defined as amino acid one for each complete proVT sequence. Conserved sequences are in white. The features of each neurohypophysial hormone are labeled above the relevant region of each sequence. The N-linked glycosylation site and leucine-rich core are boxed, and the potential neurophysin-copeptin cleavage site is in bold. A) *T. granulosa*, B) *P. shermani*.

Leu by Phe has occurred in the hydrophobic core of copeptin for each of these caudate amphibians (Fig 2.3).

At the nucleic acid level, neither the *Taricha* nor the *Plethodon* VT precursor sequence includes a translational start site that conforms exactly to Kozak's rule (Kozak 1981), but both sequences have a single in-frame start codon that matches the translational start point of most other neurohypophysial peptides (Fig 2.3). Both preproVT cDNA sequences have a poly(A) tail preceded by a polyadenylation signal (AATAAA), although in *Taricha* the VT cDNA polyadenylation signal is slightly truncated (AATAAC).

Prepromesotocin Sequences.

The *Taricha* full-length MT precursor cDNA sequence (617 bp) contained an open reading frame that encodes a 125 deduced aa protein (Fig 2.2A), preproMT. The *Plethodon* MT precursor cDNA sequence (575 bp) contained an open reading frame encoding a 127 deduced aa protein, also preproMT (Fig 2.2B). As with other proteins in the preproMT family, these proteins contained three regions: a hydrophobic signal peptide, the neurohypophysial peptide, and neurophysin-I. The MT-like peptide encoded in the *Taricha* cDNA sequence was MT, but the *Plethodon* cDNA sequence encoded the novel protein [Val⁴]-MT. Each of the neurohypophysial peptides is separated from neurophysin-I by the same conserved Gly-Lys-Arg sequence found in the salamander preproVT

Fig 2.3 Sequence Alignment

	Signal Peptide	Hormone	PS	Neurophysin	Gene Conversion Region
VT <i>T. granulosa</i>	MPEASLAACFLCLLALSSA	CYIQNCPRG	GKR	SFPD-TDV--RE	CIPCPCGPNRGRFCFGPNICCGEDLG
VT <i>P. shermani</i>	MPEASLPACFLCLLALSSA	CYIQNCPRG	GKR	SFPD-TDV--RQ	CIPCPCGPNRGRFCFGPNICCGEEMG
VT <i>B. japonicus</i>	TAPVPACFLCLLALSSA	CYIQNCPRG	GKR	SYPD-TAV--RQ	CIPCPCGPNRGRFCFGPNICCGEDLG
VT <i>T. natans</i>	MPEALVPACFLCLLALSSA	CYIQNCPRG	GKR	SFPD-TEV--RE	CLSCGPGNRGNCFGPISICCGEEIG
VT <i>G. gallus</i>	MAEPLPLSFLCLLALSSA	CYIQNCPRG	GKR	ALGD-TAL--RQ	CLPCGPGNRGRFCFGPICCGAELG
VP <i>M. musculus</i>	MLARMLNTLSACFLSLLAFSSA	CYFQNCPRG	GKR	AISD-MEL--RQ	CLPCGPGGKGRFCFGPISICCADELG
VP <i>H. sapiens</i>	MPDTMLPACFLGLLAFSSA	CYFQNCPRG	GKR	AMSD-LEL--RQ	CLPCGPGDKGRFCFGPISICCADELG
VT <i>E. burgeri</i>	MSTMGWTLAAA-LLAISAQNSG	CYIQNCPRG	GKR	AVET--EP--RS	CAACGLGGQ--CVGFPISICCGLLG
OT <i>H. sapiens</i>	MAGPSLACLLGLLALTA	CYIQNCPLG	GKR	AAPD-LDV--RK	CLPCGPGGKGRFCFGPNICCAEELG
OT <i>M. musculus</i>	MACPSLACLLGLLALTA	CYIQNCPLG	GKR	AVLD-LDM--RK	CLPCGPGGKGRFCFGPISICCADELG
MT <i>G. gallus</i>	MFYKALTVCLLGLLALSSA	CYIQNCPIG	GKR	AVPD-MNI--RK	CLPCGPRNKGRFCFGPNICCGEELG
MT <i>T. natans</i>	MMYSTLTVMFGLLALSSA	CYIQNCPIG	GKR	SVLDVMDI--RK	CIPCPCGPRNKGRFCFGPTICCGEEIG
MT <i>B. japonicus</i>	MSYALAVTFFGWLALSSA	CYIQNCPIG	GKR	SVIDFMDV--RK	CIPCPCGPRNKGRFCFGPNICCGEELG
MT <i>P. shermani</i>	MTYSAVALCFCLLAFSSA	CYIVNCPFG	GKR	SVLDVMDLQIRK	CIPCPCGPRNKGRFCFGPNICCGDELG
MT <i>T. granulosa</i>	MAYSSVFLVFLCLLALSSA	CYIQNCPIG	GKR	SVLDVMDI--RK	CIPCPCGPRNKGRFCFGPNICCGDELG

Neurophysin Gene Conversion Region (continued)		
VT <i>T. granulosa</i>	-----CYIGTSETMRCVEENYLPSCEAEG-KSCS-VDGKCAAPGICC	SDVSCTLDGSCLEDEDSERR
VT <i>P. shermani</i>	-----CFIGTSETLHCVEENYLPSCEAGG-KPCS-VDGKCAAPGICC	SDESCTLDSSCLEDEDSERR
VT <i>B. japonicus</i>	-----CYVGTPELRLCVEETYLPSPCEAGG-KPCS-SGGRCAAPGVCC	SDDTCVVDSCLDEDSERR
VT <i>T. natans</i>	-----CYFGTSETLRCKEENYLSPCESGW-KSCS-RGGRCMSPGICC	NDESCTVDSSCLEDEDSERR
VT <i>G. gallus</i>	-----CYLGTAEATRCAEEDYMPSPCQAGG-QPCG-SDGRCAANGVCC	SADTCAMDAVCLLEGSEQA
VP <i>M. musculus</i>	-----CFVGTAEALRCQEENYLSPCQSGQ-KPCG-SGGRCAAVGICC	SDESCVAEPECHDG--FFR
VP <i>H. sapiens</i>	-----CFVGTAEALRCQEENYLSPCQSGQ-KACG-SGGRCAAFGVCC	NDESCVTEPECREV--FHR
VT <i>E. burgeri</i>	GGHGGSCIVGGPLSAPCKRENHPEPCRPGGSSCGLE-GICAAAPGICC	TDVTCSIDSTCDDVTEKAG
OT <i>H. sapiens</i>	-----CFVGTAEALRCQEENYLSPCQSGQ-KACG-SGGRCAVLGLCC	SPDGCHADPACDAEATFSQR
OT <i>M. musculus</i>	-----CFVGTAEALRCQEENYLSPCQSGQ-KPCG-SGGRCAATGICC	SPDGCRTDPACDPESAFSER
MT <i>G. gallus</i>	-----CYLGTSETLRCQEENFLPTPCESGR-KACGEDGASCAAPGICC	SSEGCVLDSNQNQEMLFA
MT <i>T. natans</i>	-----CYFGTSETLRCQEENFLSPCESGR-NPCGNSGGNCAAPGICC	NNESCTVDSACDQDPAFS
MT <i>B. japonicus</i>	-----CYFGTSETLRCQEENFLSPCESGR-KPCGNSGGNCAAPGICC	NHESCTMDPACEQDSVFS
MT <i>P. shermani</i>	-----CFVGTSETLRCQENYLSPCESGW-KPCGNSGGNCAAPGICC	SNESCTVDQCEQESFFT
MT <i>T. granulosa</i>	-----CYFGTSETMRCQEENYLSPCESGR-KSCGNSGGSCAASGICC	NNESCTMDQACDQDSAFS

	Copeptin
VT <i>T. granulosa</i>	RG--PNERNMTLLDGTASDFFLKLHMMA--NRHQQKQHFY
VT <i>P. shermani</i>	KV--SSEQNMTLLDGTASDFFLKLHMMA--NRHQQKQHFY
VT <i>B. japonicus</i>	RV--TPEQNMTQMDGSASDLLLRLHMMA--NRQQSKQHFY
VT <i>T. natans</i>	EV--SLDKNITLKDGSASDILLKLVMMA--N-QQRGKQFYS
VT <i>G. gallus</i>	EE--AAEKNLTVLDGAAGDLLLRLMHLA--NRQQGKQPGL
VP <i>M. musculus</i>	LTRAREPSNATQLDGPAPALLLRLVQLAGTRESVDSAKPRVY
VP <i>H. sapiens</i>	RARASDRSNATQLDGPAGALLLRLVQLAGAPEPFEPAPQDAY
VT <i>E. burgeri</i>	VTFSGAT

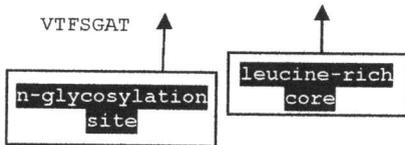


Fig 2.3 Deduced amino acid sequence alignment for tetrapod preproneurohypophysial hormones. Representative preproneurohypophysial hormone sequences (preprovasotocin (VT), prepromesotocin (MT), preprovasopressin (VP), preprooxytocin (OT)) were initially aligned with ClustalX and then manually adjusted for optimal alignment. Various components of each sequence are labeled including a box around the putative gene conversion region. The N-linked glycosylation site and leucine-rich core are highlighted. Non-*Taricha* and *Plethodon* sequences were obtained from Entrez-Protein (NCBI). Accession numbers: VT *Bufo japonicus*, P08163 ; VT *Typhlonectes natans*, AAF76847; VT *Gallus gallus*, CAA38923; VP *Mus musculus*, NP_033862; VP *Homo sapiens*, P01185; VT *Eptatretus burgeri*, BAA06668; OT *Homo sapiens*, P01178; OT *Mus musculus*, NP_035155; MT *Gallus gallus*, (Barth et al. 1997); MT *Typhlonectes natans*, AAF76848; MT *Bufo japonicus*, P08162.

sequences. All fourteen of the conserved Cys residues found in other neurophysin proteins are also conserved in each salamander's preproMT sequences.

Whereas the *Taricha* MT precursor cDNA contained a translational initiation sequence conforming to Kozak's rule (Kozak 1981), the *Plethodon* [Val⁴]-MT precursor sequence did not, although each cDNA sequence had a single in-frame start codon. Both possessed poly(A) tails preceded by polyadenylation signals (AATAAA).

Phylogenetic Analysis.

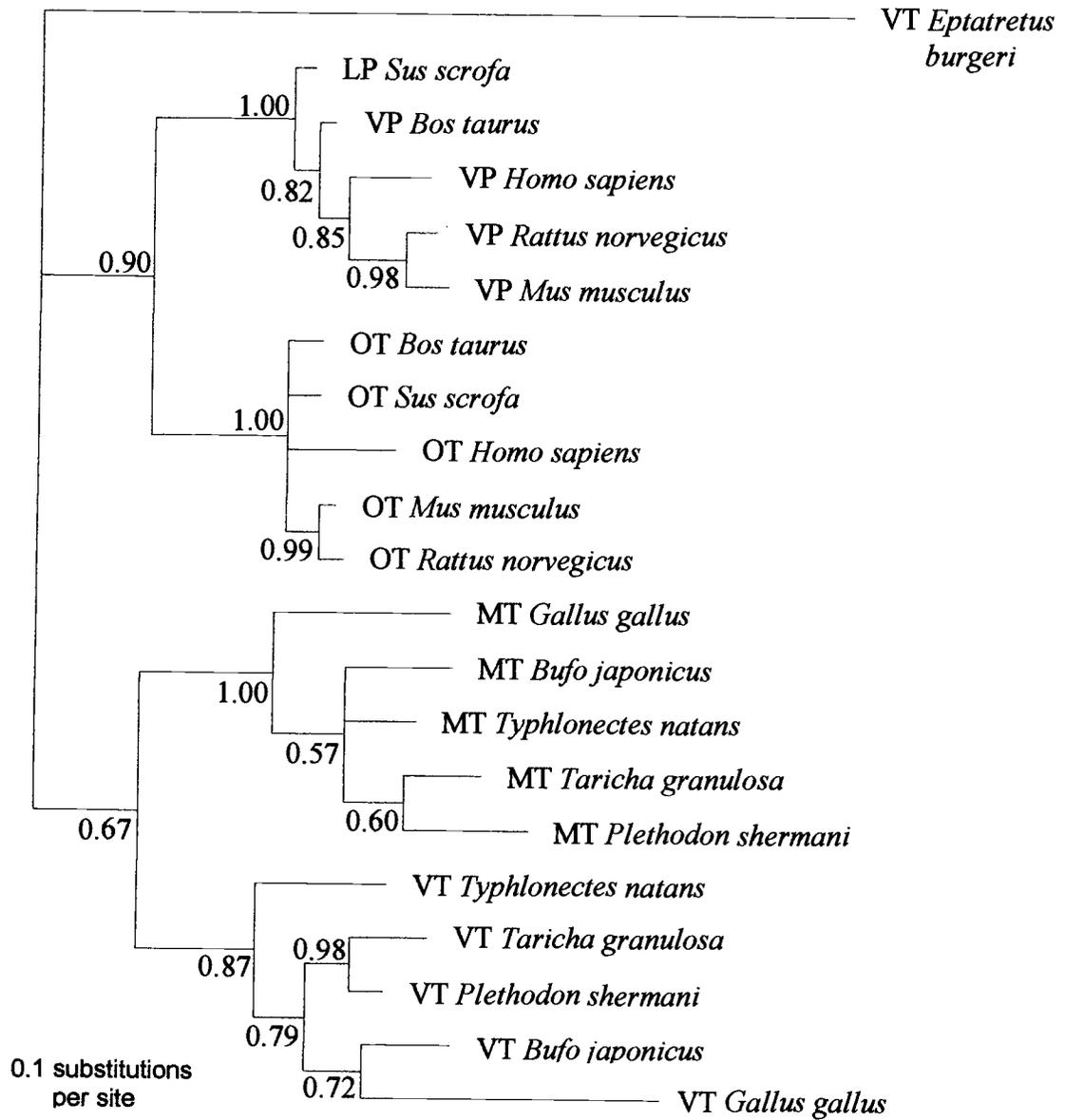
The deduced amino acid preproVT sequences from *Taricha* and *Plethodon* share highest identity with each other (90.0%). *Taricha* preproVT also shares high identity with the one other complete amphibian preproVT sequence (caecilian, 72.2%), as well as with lungfish (69.9%) and chicken (63.3%) preproVT. Identities with the preproVP, preproMT, preproIT, and fish preproVT sequences were all approximately equal (41.3 - 49.1 %). Lowest identity for the *Taricha* preproVT sequence was with hagfish preproVT (36%).

Taricha and *Plethodon* preproMT deduced amino acid sequences also shared highest identity with each other (79.5%) as well as with the other available amphibian preproMT sequence (caecilian, 80%). High identity is also shared with the mammalian preproOT, reptilian preproMT, avian preproMT, elasmobranch preproIT, and lungfish preproMT sequences (60-70% identity). Lower levels of identity occur for *Taricha*

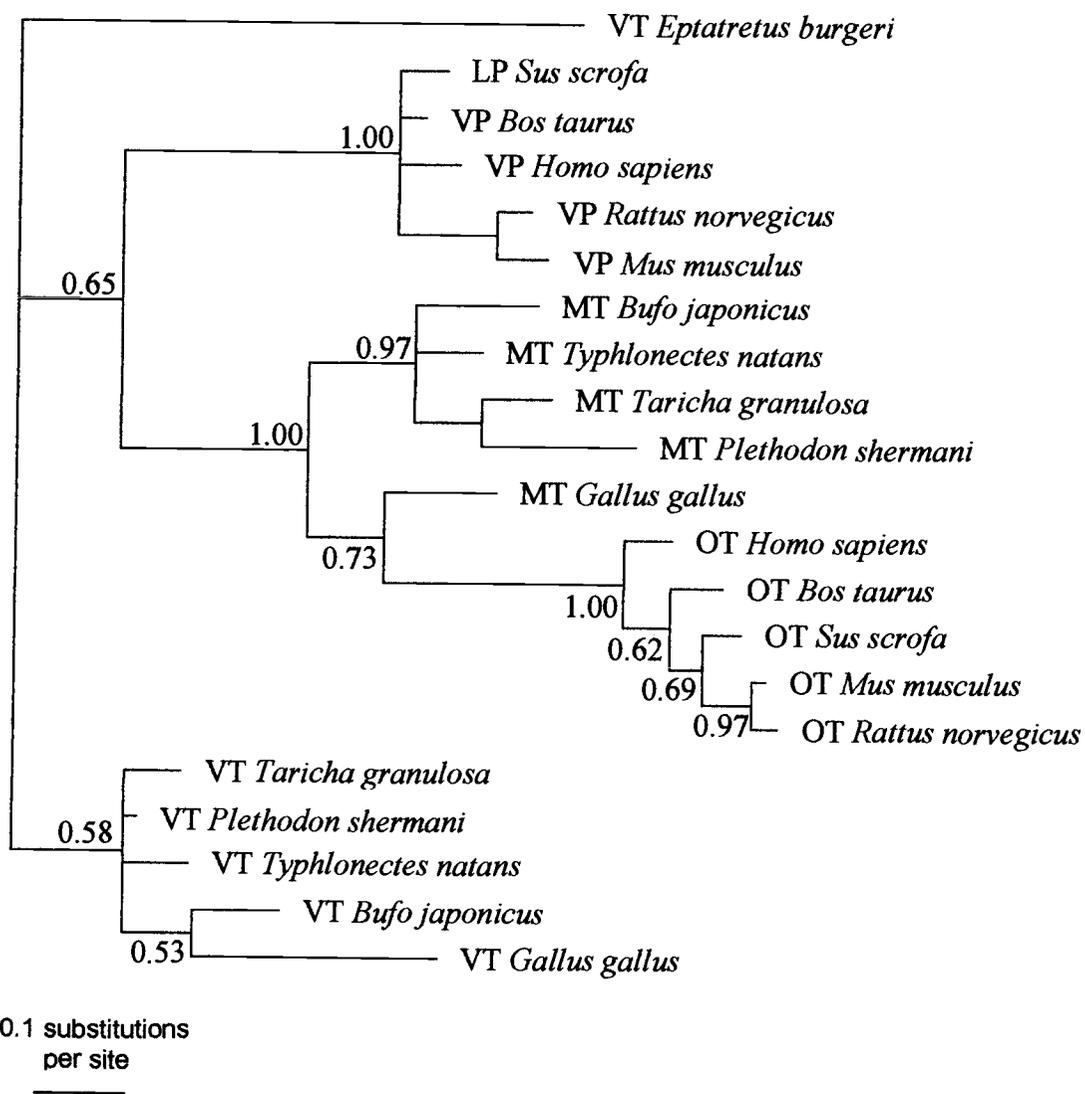
preproMT with the teleost preproIT sequences, and the preproVT and preproVP sequences (all vertebrates; 40-50% identity). Lowest identity was with the hagfish preproVT sequence (31.9%).

A phylogenetic tree based on the alignment of the full-length tetrapod preproNHP sequences was generated by Bayesian analysis. This tree clustered *Plethodon* and *Taricha* preproVT and preproMT sequences with the identified amphibian and avian preproVT and preproMT sequences, respectively (Fig 2.4A). In this analysis, the mammalian preproVP and preproOT sequences grouped together in a cluster distinct from the other tetrapod preproNHP sequences. These results may indicate that either the mammalian preproVT and preproOT genes arose from a duplication event that occurred after the divergence of mammals from other tetrapods, or, alternatively, the genes in this family do not evolve as a single unit. To examine these two possibilities, the section of the precursor proteins hypothesized to undergo gene conversion (see discussion and Fig 2.3) was masked from the phylogenetic analysis (Fig 2.4B). In this analysis, the mammalian preproOT clustered with the preproMT sequences, and the mammalian VP precursor protein clustered with preproVT sequences.

2.4A Full length deduced amino acid sequences



2.4B Putative gene conversion region masked.



2.4C Putative Gene Conversion Region Only

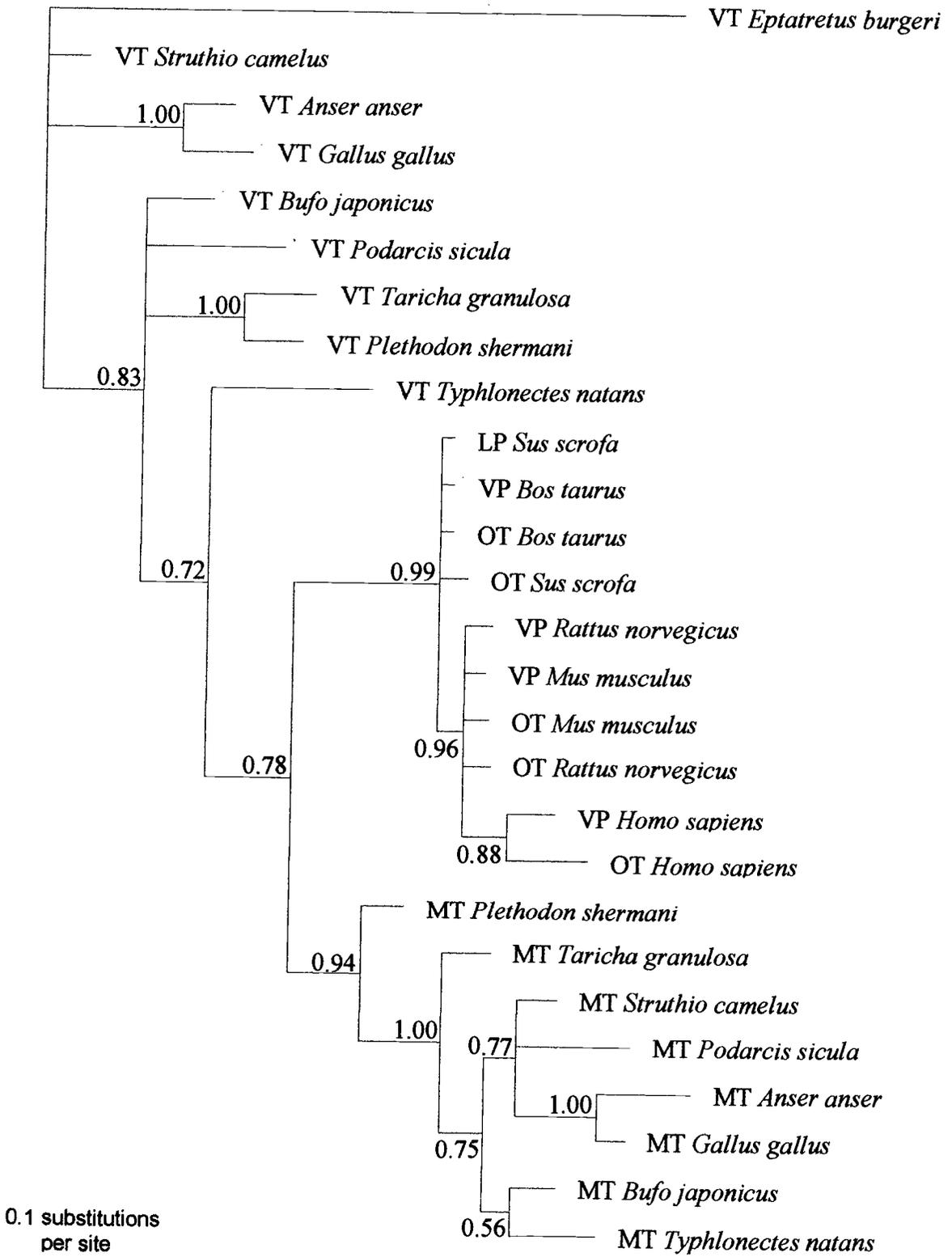


Fig 2.4 The proneurohypophysial hormone receptor phylogenetic trees. The 50% majority rule consensus tree obtained from the Bayesian analyses of the deduced amino acid sequences of representatives from each of the major classes of proneurohypophysial hormones (preprovasotocin (VT), prepromesotocin (MT), preprovasopressin (VP), preprooxytocin (OT), prepolysopressin (LP)). Posterior probability values are indicated at each node. Nodes with posterior probability values <50% were considered polyphyletic. **A.** A phylogenetic tree based on the complete neurohypophysial hormone sequence. **B.** A phylogenetic tree in which the putative gene conversion region of each sequence was masked from the analysis. **C.** A phylogenetic tree in which only the putative gene conversion region was included in the analysis. . . Non-*Taricha* or *Plethodon* sequences were obtained from Genbank; Accession Numbers: VT *Eptatretus burgeri*, BAA06668; VT *Struthio camelus*, P21916; VT *Anser anser* P19630; VT *Gallus gallus*, CAA38923; VT *Bufo japonicus*, P08163; VT *Podarcis sicula*, AAL15166; VT *Typhlonectes natans*, AAF76847; LP *Sus scrofa*, P01183; VP *Bos Taurus*, P01180; OT *Bos Taurus*, P01175; OT *Sus scrofa*, P01177; VP *Rattus norvegicus*, P01186; VP *Mus musculus*, NP_033862; OT *Mus musculus*, NP_035155; OT *Rattus norvegicus*, P01179 ; VP *Homo sapiens*, P01185; OT *Homo sapiens*, P01178; MT *Struthio camelus*, P15444; MT *Podarcis sicula*, AAL15165; MT *Anser anser*, P35519; MT *Gallus gallus*, (Barth et al. 1997); MT *Bufo japonicus*, P08162; MT *Typhlonectes natans*, AAF76848.

To examine the evolution of the putative gene conversion site in the preproNHP sequences, a phylogenetic analysis of this region was conducted (Fig 2.4C). This analysis found that the non-mammalian tetrapod sequences clustered by precursor type (preproMT or preproVT); whereas, the mammalian sequences (preproOT and preproVP) clustered together with no clear organizational pattern. Within the cluster of mammalian sequences the human preproOT and preproVP sequences formed their own distinct group. When the sequence identities between the putative gene conversion regions of the two precursor proteins (preproMT-like and preproVT-like) of each individual species are calculated (Table 2.1), the percent identity between these sequences is much higher in mammals (91-100%) than in other tetrapods (66-78%). This high identity between neurohypophysial hormone sequences within individual species is not maintained when the identity is calculated for either the sequences as a whole, or for only the portion of the sequence outside the putative gene conversion region (Table 2.1).

Table 2.1 Amino acid sequence identity between the two preproneurohypophysial peptides of individual species. Sequence identities were calculated for the deduced amino acid sequence of the two paralagous preproneurohypophysial peptide sequences from individual species. For each species, a sequence identity was calculated for the full-length sequence, the full-length sequence without the gene conversion region, and the gene conversion region alone. Non-*Taricha* and *Plethodon* sequences were obtained from Genbank. For Accession Numbers see Fig 2.4.

	<u>Full Sequence</u>	<u>Gene Conversion Region and Copeptin Masked</u>	<u>Gene Conversion Region Only</u>
<u>Mammalia</u>			
<i>Bos taurus</i>	56	48	100
<i>Sus scrofa</i>	58	55	100
<i>Mus musculus</i>	54	44	98
<i>Rattus norvegicus</i>	54	47	97
<i>Homo sapiens</i>	53	48	91
<u>Amphibia</u>			
<i>Taricha granulosa</i>	49	51	78
<i>Plethodon shermani</i>	50	52	78
<i>Typhlonectes natans</i>	49	53	75
<i>Bufo japonicus</i>	45	42	73
<u>Reptilia</u>			
<i>Podarcis sicula</i>	N/A	N/A	73
<u>Aves</u>			
<i>Struthio camelus</i>	N/A	N/A	72
<i>Anser anser anser</i>	N/A	N/A	70
<i>Gallus gallus</i>	44	50	66

Discussion

This is the first report describing the full-length precursor cDNA sequences for any neurohypophysial peptide in a caudate amphibian. The preproMT and preproVT sequences of both *T. granulosa* and *P. shermani* were amplified from brain cDNAs of males in breeding condition. Initially, internal fragments were amplified by PCR using degenerate primers. The cloning and sequencing of each of these four fragments allowed the design of species-specific and sequence-specific primers which were then used in conjunction with RACE PCR to amplify the 5' and 3' ends of each cDNA molecule. Each of the amplified VT and MT precursor cDNA sequences had highest identity with previously characterized VT-like and MT-like sequences, respectively. In addition, each of the four salamander sequences contained single large open reading frames that, when translated, encoded deduced amino acid sequences (Fig 2.2A, 2.2B) with structural configurations that are in agreement with neurohypophysial deduced preproNHP sequences reported previously (Fig 2.3).

The *Plethodon* and *Taricha* preproVT sequences each consisted of four regions: a short hydrophobic signal peptide, the neurohypophysial peptide, neurophysin-II, and at the C-terminus of neurophysin-II, a region homologous to the mammalian glycoprotein, copeptin (Fig 2.2A, 2.2B). Each sequence encoded the neurohypophysial peptide VT. Both the *Taricha* and *Plethodon* preproVT sequences contain an Arg residue at the

C-terminal end of the neurophysin-II sequence, present in mammals, and hypothesized to be involved in neurophysin-II/copeptin cleavage (Hyodo et al. 1997). Despite possessing this cleavage sequence, because non-mammalian tetrapods are thought to lack the proper enzymes to process the precursor protein at this site (Chauvet et al. 1988; Michel et al. 1990), these two caudate amphibians might produce a "big" neurophysin. Both frogs (*Rana esculenta*) (Chauvet et al. 1988) and birds (*Anser anser* (Michel et al. 1990) and *Struthio camelus* (Lazure et al. 1989)) have been found to release "big" neurophysin consisting of a combined neurophysin-copeptin sequence. Similar to the salamander preproVT sequences, an Arg residue is expressed at the homologous site of the toad (*Bufo japonicus*) preproVT sequence, another amphibian also thought to express "big" neurophysins (Chauvet et al. 1988).

The structure of *Plethodon* and *Taricha* preproMT sequences (Fig 2.2A, 2.2B) consists of three regions including a signal peptide, the neurohypophysial peptide, and neurophysin-I. The *Taricha* preproMT sequence contains the neurohypophysial peptide MT; whereas, the *Plethodon* preproMT sequence includes [Val⁴]-MT. Little sequence variation occurs between neurophysin-I and neurophysin-II, and this nomenclature is based primarily on the type of neurohypophysial peptide co-expressed with each neurophysin. Each salamander preproMT sequence contains a copeptin domain, which is consistent with findings in other tetrapod preproMT-like sequences. The lungfish preproMT (Hyodo

et al. 1997) and elasmobranch preproITs (Buchholz et al. 1995; Hyodo et al. 2004) also lack a copeptin moiety. In contrast, all vertebrate preproVT-like sequences, as well as the teleost preproIT proteins (Heierhorst et al. 1989; Hyodo et al. 1991; Suzuki et al. 1992; Warne et al. 2000) include a copeptin-like moiety (see Fig 2.3).

The *Plethodon* cDNA sequence (Fig 2.2B) encodes for the ligand [Val⁴]-MT rather than the dominant amphibian form of this hormone, MT. Finding an alternative form in one of a class' dominant neurohypophysial peptides is unusual but not unique. [Ser⁵]-MT rather than MT was found in a toad (*Bufo regularis*) (Chauvet et al. 1995), and [Lys⁸]-VP rather than [Arg⁸]-VP occurs in pigs (Popenoe et al. 1952). In addition, the preproMT-like neurohypophysial peptides found in cartilaginous fishes vary considerably, with no one form conserved in the majority of species (for review see (Acher et al. 1999)). Amino acid 4 in the neurohypophysial peptide differentiates MT (Gln⁴) from the teleost IT (Ser⁴) (see Fig 2.1A). Although MT is the dominant OT-like peptide in non-mammalian tetrapods, because amino acid 4 distinguishes MT from IT, it may be a region of the neurohypophysial peptide that has less selective pressures. The substitution of Gln⁴ (present in MT, Fig 2.1A) to Val⁴ ([Val⁴]-MT) in *Plethodon* requires a double mutation, so at some point there may have been an intermediate form. Because Gln is a polar amino acid and Val is non-polar, the substitution of Val for Gln may have an impact on the affinity this peptide has for the *Plethodon* MT receptor. It would be

interesting to examine whether there have been parallel changes in MT receptor specificity in *Plethodon*, investigating whether the MT receptor has evolved a higher affinity for [Val⁴]-MT than MT. If the *Plethodon* MT receptor has higher affinity for MT than [Val⁴]-MT it may indicate that this change in ligand sequence is not exerting strong pressures on the evolution of the receptor.

Phylogenetic analyses of the neurohypophysial preproNHP sequences indicated that the salamander preproMT and preproVT sequences cluster with other amphibian and avian preproMT and preproVT sequences, respectively (Fig 2.4A, 2.4B), supporting the conclusion that these four sequences are indeed the preproVT or preproMT cDNAs. This conclusion also holds true when complete preproNHP sequences were analyzed (Fig 2.4A), and when only portions of preproNHP sequences were included in the phylogenetic analyses (Fig 2.4B, 2.4C).

The evolution of the preproNHP genes, as predicted by phylogenetic analysis of the full-length preproNHP sequences (Fig 2.4A), did not conform with the evolutionary model developed using the neurohypophysial peptide sequences alone (Fig 2.1B, see introduction) (Acher 1980; Acher, Chauvet et al. 1995), but did resemble the phylogeny that was constructed based on cDNA substitution rates (see introduction: (Hyodo et al. 1991; Suzuki et al. 1992)). One interpretation of the full-length preproNHP analysis is that, soon after the divergence of mammals

from other tetrapods, a gene duplication event gave rise to the OT and VP precursor genes from an ancestral VT precursor; in addition, the preproMT gene was lost. This model is not parsimonious and unlikely.

A second interpretation of the full-length preproNHP phylogenetic analysis is that preproMT/OT and preproVT/VP share a common ancestor but are not strictly orthologous genes. This could occur if the two neurohypophysial hormone genes within each species are not evolving as independent units, violating an assumption of phylogenetic analysis. One mechanism through which this might occur is gene conversion. Gene conversion is the non-homologous crossing over of a DNA sequence from one gene to a second closely related gene. Gene conversion has been proposed as an explanation of the nearly 100% nucleic acid identity between the neurophysin encoding portion of the preproOT/IT and the preproVP/VT genes within individual mammal and fish species (Ruppert et al. 1984; Ivell and Richter 1984; Sausville et al. 1985; Levy et al. 1987; Hyodo et al. 1991; Suzuki et al. 1992). There is potential for gene conversion to occur between preproNHP genes because of their sequence similarity and chromosomal location (reviewed (Ivell 1987)). In mammals, the OT and VP precursor genes are proximally located on opposite strands of the same chromosome, 3 – 12 kb apart (Ivell and Richter 1984; Sausville et al. 1985; Hara et al. 1990).

Gene conversion has been proposed as a mechanism though which Brattleboro rats suffering from diabetes insipidus are rescued from

this disease (van Leeuwen et al. 1989; Mohr et al. 1994). Diabetes insipidus is a genetic disease caused by the inability of individuals to release an active form of VP; in Brattleboro rats, diabetes insipidus is thought to be due to a mutation in the neurophysin encoding portion of the preproVP gene (van Leeuwen et al. 1989; Mohr et al. 1994). Gene conversion has been proposed as a mechanism to rescue individual adult rats from this form of diabetes. In somatic cells of adult rats, a functional neurophysin sequence from the OT precursor gene is thought to replace the equivalent sequence in the VP precursor gene by gene conversion, allowing the active form of VP to be released (van Leeuwen et al. 1989; Mohr et al. 1994).

If gene conversion has occurred within a family of genes that includes paralogs, and the portion of the sequence undergoing conversion is included in the phylogenetic analysis, the phylogenetic analysis will be inaccurate. Gene conversion will cause the sequences of paralogous genes to become more similar, and mask the original time of divergence between these two genes. This will cause the original point of divergence to appear to be more recent than it actually is. The full-length preproNHP phylogenetic analysis (Fig 2.4A) appears to match this prediction in regard to the mammalian sequences. Mammalian preproOT and preproVT cluster more by class and species than the other tetrapod preproNHP sequences.

In order to test whether the putative gene conversion region of the preproNHP sequences was adversely affecting the phylogenetic analysis, the preproNHP sequences were analyzed with the putative region of gene conversion masked from the analysis (fig 2.4B). Results from this analysis clustered the preproOT with preproMT sequences and separated them from preproVP and preproVT sequences. This result differs from the full length sequence analysis (fig 2.4A), conforming more closely to a model based on the neuropeptide sequences alone (fig 2.1B). In addition, this result further supports the hypothesis that gene conversion is occurring within this family of genes.

To further examine gene conversion within the preproNHP sequences, a phylogenetic analysis was conducted that was restricted to the putative gene conversion regions of the preproNHP sequences (Fig 2.4C). This analysis clustered the mammalian preproNHP sequences together. The preproNHP sequences of each mammalian species arise from the same node, with the human preproNHP sequences forming their own independent cluster. In contrast to the mammalian sequences, the other tetrapod gene conversion sequences clustered by preproNHP type, rather than species. This analysis supports the hypothesis that gene conversion is occurring in mammals at a higher rate of frequency in mammals than in birds, reptiles and amphibians. The most recent gene conversion in the non-mammalian tetrapods would have had to occur prior to the divergence of each of these vertebrate classes.

The gene conversion regions of each sequence were also examined by calculating the percentage sequence identity between the preproVT-like and the preproMT-like sequences of this region within each species. The mammalian species sequence pairs have a much higher level of sequence identity than other tetrapods (Table 2.1). This may indicate that the rate of gene conversion is higher among mammals than it is in other vertebrate classes. One possible explanation for this difference in rate may be that the chromosomal loci for the OT and VP genes in mammals are different from the chromosomal loci for MT and VT in other tetrapods. In the non-mammalian tetrapods, the two preproNHP genes could either be too close or too far apart on the chromosome to facilitate gene conversion. Although the sequence identity of the gene conversion region is higher than other regions in the non-mammalian tetrapods, this does not necessarily indicate that gene conversion has occurred in these classes; an alternative explanation is that the neurophysin regions of each gene are more strongly conserved than the other regions.

In summary, we cloned the full length cDNA sequences that encode two salamander preproVT and preproMT proteins. Whereas the *Taricha* sequences include the neurohypophysial peptides VT and MT, the *Plethodon* sequences include VT and the novel hormone [Val⁴]-MT. The phylogenetic analysis of these sequences indicates that while the mammalian preproOT appears to be derived from a preproMT precursor, and preproVP from preproVT, these genes may not be strict orthologues

due to the possibility of gene conversion causing the preproVP and preproOT sequences to evolve in parallel. More sequences need to be obtained from the more ancient classes of vertebrates to expand this analysis to the preproNHP genes of all vertebrates.

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Chapter 3

Identification and Characterization of the Vasotocin and Mesotocin Receptors from the Brain of the Rough-skinned Newt, *Taricha granulosa*

Abstract

The cDNA sequences encoding the mesotocin receptor (MTR) and vasotocin receptor (VTR) were amplified from the brain of a urodele amphibian, the rough-skinned newt, *Taricha granulosa*. Deduced amino acid sequence identities and phylogenetic analysis indicated that the newt MTR (nMTR) is orthologous to mammalian oxytocin receptors (OTRs); whereas, the nVTR is orthologous to mammalian V_{1a} vasopressin receptors (V_{1a}Rs). PCR using *Taricha* specific MTR primers amplified cDNA sequences from brain, heart, large intestine, lung, pituitary gland, skeletal muscle, and testes. PCR using *Taricha* specific VTR primers amplified cDNAs from brain, liver, sperm duct, lung, pituitary gland and testes. The primary difference for each *Taricha* receptor distribution with other species is that neither the nMTR nor the nVTR sequences were successfully amplified from kidney cDNA. [³H]-OT saturation binding with COS-7 cell membranes transiently expressing nMTR was best fit by a two site model predicting a high affinity-low abundance site and a lower affinity-high abundance site. Competition binding studies found the following rank-order affinities for the nMTR: mesotocin (MT) > oxytocin (OT) ≈ vasotocin (VT) > vasopressin (VP) > isotocin (IT). Inositol phosphate (IP) accumulation studies demonstrated functional activity of both the nMTR and nVTR. The rank order potencies of the endogenous vertebrate neurohypophysial peptides for the nMTR were MT>OT>VT≈VP>IT. Rank order potencies for the nVTR were:

VT>VP>MT≈OT>IT. Stimulation of IP accumulation was blocked by $d(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$ (Manning compound) and $d(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2, \text{Thr}^4, \text{Tyr-NH}_2]\text{OVT}$ (OTA); OTA was a stronger antagonist for the transiently expressed nMTR; whereas, Manning compound was a stronger antagonist of IP accumulation in nVTR expressing cells. The nMTR and nVTR are the first neurohypophysial hormone receptor sequences reported from a urodele amphibian. Analyses in this report indicate the high likelihood that all mammalian neurohypophysial receptor subtypes will also be found in amphibians. Pharmacological profiles of the nMTR and nVTR indicate that Manning compound is not a good selective antagonist for the V_{1a} -like VTR in amphibians, a finding with special relevance to interpretation of comparative behavioral studies investigating the actions of neurohypophysial peptides.

Introduction

Rough-skinned newts, *Taricha granulosa*, express mesotocin (MT) and vasotocin (VT) (see Chapter 2), orthologs of mammalian oxytocin (OT) and vasopressin (VP), respectively (Acher 1980; Urano et al. 1992; Acher et al. 1995). Most of these peptides consist of nine amino acids, and contain disulfide bridges between Cys residues at position one and six, creating a six amino acid ring-like structure with a tail three amino acids in length (Fig 2.1). These peptides have been found to act both as

hormones on peripheral targets, and as neuromodulators/neurotransmitters within the central nervous system.

The peripherally acting neurohypophysial peptides are primarily expressed in hypothalamic magnocellular and parvocellular cells, and are secreted at the posterior pituitary and median eminence, respectively. The hormones released at the posterior pituitary enter general circulation, whereas the peptides released at the median eminence are transported through a portal vessel to cells in the anterior pituitary (adenohypophysis). In addition to their hypothalamic magnocellular and parvocellular expression, the neurohypophysial peptides are also expressed at additional sites in the brain (for review see (Barberis and Tribollet 1996; Moore and Lowry 1998; Smeets and Gonzalez 2001)), where they have been shown to directly affect behavior (for review (Young 1999; Insel and Young 2000; Goodson and Bass 2001; Rose and Moore 2002)), memory and learning (Kinsley et al. 1999; Alescio-Lautier et al. 2000; Tomizawa et al. 2003; Winslow and Insel 2004), and thermoregulation (Wilkinson and Kasting 1987; Wilkinson and Kasting 1987; Kluger 1991; Pittman and Wilkinson 1992).

The neurohypophysial hormones were originally identified because of their effects on physiology. Some of the actions of VP in mammals include the regulation of water balance (Silveira et al. 2004), vascular tone (Holmes et al. 2004), adrenocorticotropin (ACTH) release (Aguilera and Rabadan-Diehl 2000), and depletion of glycogen in hepatocytes (George

1977). Similar to the physiological role of VP in mammals, VT in amphibians is known to act as an antidiuretic (Schmidt-Nielsen and Forster 1954; Eggena et al. 1968; Shoemaker and Nagy 1977; Warburg 1995) and a modulator of ACTH release (Larcher et al. 1989; Larcher et al. 1992). In mammals the peripheral actions of OT include the stimulation of the milk let-down reflex from mammary glands, and enhancement of uterine contractility during parturition. In amphibians the only known peripheral action of MT is as a diuretic, directly antagonizing the actions of VT on water balance (Pang and Sawyer 1978; Warburg 1995).

Some of the behavioral effects attributed to VP in mammals include the modulation of locomotor activity (Tendis et al. 1987; Di Michele et al. 1998), flank-marking (Albers et al. 1988), aggression (Ferris and Delville 1994; Ferris et al. 1997), parental behavior (Wang et al. 1994), pair bonding (Winslow et al. 1993), social recognition (review (Winslow and Insel 2004)), female receptivity (Sodersten et al. 1983; Albers and Rawls 1989), and learning and memory (Metzger et al. 1993; Alescio-Lautier et al. 2000; Winslow and Insel 2004). Similarly, the role of VT in amphibians has been found to include the stimulation of locomotor activity (Boyd 1991), frog calling behavior (Penna et al. 1992; Boyd 1994; Marler et al. 1995; Propper and Dixon 1997; Chu et al. 1998; Semsar et al. 1998; Tito et al. 1999), egg-laying behavior (Moore et al. 1992), and both male (Moore and Zoeller 1979; Moore and Miller 1983) and female sexual behaviors (Diakow 1978; Boyd 1992).

The mammalian behavioral actions of OT include the modulation of male (Arletti et al. 1985; Stoneham et al. 1985; Carmichael et al. 1987; Mahalati et al. 1991) and female sexual activity (Arletti and Bertolini 1985; Caldwell et al. 1986; Witt and Insel 1991), induction of maternal behaviors (Pedersen and Prange 1979), initiating anti-stress (anxiolytic) responses (McCarthy et al. 1996; Windle et al. 1997), reduction of feeding behavior (Arletti et al. 1989), and modulation of memory and learning (Tomizawa et al. 2003; Winslow and Insel 2004). No behavioral action has been attributed to MT in amphibians to date.

In non-mammalian behavioral studies examining the effects of neurohypophysial hormones, the pharmacologies of the neurohypophysial hormone receptors have frequently been assumed to match the pharmacologies of their mammalian counterparts. Although many behavioral studies have been conducted in non-mammals, the specific binding properties of the neurohypophysial receptors in most of these species are not known. It is therefore possible that some responses attributed to VT and the VTR are actually modulated by MT and the MTR, or the IT and ITR in fish.

The neurohypophysial hormones act through receptors that are members of the G-protein coupled receptor family. In mammals, four types of neurohypophysial hormone receptor have been identified; a single class of OT receptor (OTR) (Kimura et al. 1992) and three VP receptors; the V_{1a} -VP receptor ($V_{1a}R$) (Morel et al. 1992), the V_{1b} -VP

receptor ($V_{1b}R$)(Sugimoto et al. 1994) (also called the V_3R (Thibonnier et al. 1998)) and the V_2 -VP receptor (V_2R)(Birnbaumer et al. 1992; Lolait et al. 1992).

Only a limited number of homologs to the mammalian neurohypophysial hormone receptors have been identified in non-mammalian vertebrates. Receptors thought to be homologous to the mammalian OTR have been characterized from anuran amphibians (MT receptor (MTR))(Akhundova et al. 1996; Kohno et al. 2003; Acharjee et al. 2004) and teleosts (isotocin receptor (ITR) (Hausmann et al. 1995). Similar to the findings in mammals, multiple VP receptor types have been identified in non-mammalian vertebrates: a V_{1a} -like VTR has been identified in anuran amphibians (Acharjee et al. 2004) and teleosts (Mahlmann et al. 1994; Warne 2001), a V_{1b} -like VTR has been found in birds (Cornett et al. 2003), and a V_2 -like VTR has been identified in both anuran amphibians (Kohno et al. 2003), and birds (Tan et al. 2000). Birds are the only non-mammalian vertebrate in which two VTR subtypes have been reported for the same species (*Gallus gallus*) (Tan et al. 2000; Cornett et al. 2003).

G-protein coupled receptors transduce their signal through the activation of G-proteins, which then affect downstream actions in target cells. Mammalian OTR (Phaneuf et al. 1996), $V_{1a}R$ (Liu and Wess 1996), $V_{1b}R$ (Liu and Wess 1996), and the amphibian MTR (Kohno et al. 2003; Acharjee et al. 2004) and V_{1a} -like VTR (Acharjee et al. 2004), all appear to

associate primarily with the $G_{q/11}$ family of G-proteins. Once activated, these proteins initiate a signal that is transduced by the phospholipase C (PLC)/protein kinase C (PKC) pathway. Both the mammalian V_2R (Liu and Wess 1996) and the amphibian V_2 -like VTR (Kohno et al. 2003) associate primarily with the G_s protein (Liu and Wess 1996), which activates adenylyl cyclase, increasing both the production of cAMP and increasing protein kinase A (PKA) activity. In amphibians the exact identity of the G-protein associated with each receptor has not been identified, and has only been inferred by the specific pathway each receptor activates.

The present study with *T. granulosa* was designed to identify and characterize the MTR and VTR cDNA sequences from this species. Although the neurohypophysial hormone system of *T. granulosa* has been extensively studied (Moore and Zoeller 1979; Moore and Miller 1983; Zoeller et al. 1983; Tripp and Moore 1988; Boyd and Moore 1990; Boyd and Moore 1991; Moore et al. 1992; Lowry et al. 1997; Moore and Lowry 1998; Thompson and Moore 2000; Coddington and Moore 2003), the MT and VT receptors of this species have not been characterized. cDNA sequences encoding each of these receptors were amplified by PCR from the brains of breeding condition males. A phylogenetic analysis of the vertebrate neurohypophysial hormone receptors was then conducted, including the deduced amino acid sequences of the *Taricha* MTR and VTR, in an effort to further understand the evolution of this receptor family.

Because few physiological responses to VT and MT are known in amphibians, the tissue distribution of the receptors was examined by reverse transcriptase PCR (rtPCR) and compared with the distribution of each of these receptors in other vertebrates. Finally, the pharmacology of each of these receptors was determined in expression studies, looking at both agonist and antagonists affinities and/or activity. The pharmacological profiles of these receptors are compared with those from other species, and possible implications regarding behavioral studies are discussed. This is the first report characterizing the MTR and VTR cDNA sequences from a Urodele amphibian.

Materials and Methods

Study Animals

Adult rough-skinned newts (*Taricha granulosa*) were captured in Benton and Lincoln Co., Oregon, maintained in an environmentally controlled room (14 °C, 12 L/12D) for about 24 h, and held in large tanks containing dechlorinated water. The newts were fed earthworms upon arrival in their storage tank. The newts were cryoanesthetized and then sacrificed by rapid decapitation. Brains were quickly removed and snap-frozen in liquid nitrogen and then stored at -80 °C until proceeding with RNA preparation.

Preparation of RNA and cDNA

Total RNA was extracted from *T. granulosa* brains using a guanidinium thiocyanate based kit, ToTALLY RNA kit (Ambion Inc., Austin TX), according to the protocol recommended by the manufacturer. For each batch of RNA purified, six to ten *Taricha* brains were pooled. cDNA was obtained by RT-PCR using a cDNA first strand synthesis kit and primed with oligo-dT primers (Fermentas, Hanover, MD).

Degenerate PCR

Full-length cDNA sequences available from the National Center for Biotechnology Information (NCBI) databases for either the MT-like receptors or VT-like receptors from various species (amphibian, fish, and mammal sequences) were aligned using ClustalX, and degenerate oligonucleotide primers were designed to match highly conserved of each alignment. Degenerate primers were purchased from Gibco-BRL. The forward and reverse degenerate nMTR primers were: 5'-ATG TTY GCY TCH RCY TAY-3' and 5'-YRA GYY TGA CRC TRC TGA C-3', respectively. The forward and reverse degenerate nVTR primers were: 5'-GTS GTN CGN TTY TTY CAR GT-3' and 5'-GGY TCD ATR AAR TTN GCC CAR CAR TC. For degenerate PCR amplifications of both the nMTR and nVTR sequences, primers, dNTPs, *Taq* polymerase (Fermentas, Hanover, MD), and *Taricha* brain cDNA template were used in PCR reactions with the following cycling conditions: nMTR-PCR: 94 °C

x 5 min; 38 cycles of 94 °C x 45 sec, 47 °C x 45 sec, 72 °C x 1 min; terminal extension at 72 °C x 8 min. nVTR-PCR: 94 °C x 5 min; 38 cycles of 94 °C x 45 sec, 50 °C x 45 sec, 72 °C x 1 min; terminal extension at 72 °C x 8 min products were separated on a 1.3% agarose gel, stained with ethidium bromide, and visualized with UV light. PCR products of the appropriate size were ligated into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into TOP10 *Escherichia coli* competent cells. Following overnight incubation, plasmid DNA was isolated from mini-preps and purified by alkaline lysis using a Qiaprep kit (Qiagen, Valencia, CA). Purified DNA was sequenced by the Central Services Laboratory at Oregon State University. Sequences were analyzed with a BLAST-N 2.0 search tool provided by NCBI.

RACE PCR

Clones amplified by degenerate PCR that possessed the highest sequence identity (as determined by BLAST-N, NCBI) to either the known MTR-like or the known V1aR-like receptors were used to design gene-specific primers for RACE- (rapid amplification of cDNA ends) PCR. Sequence specific primers were purchased from One Trick Pony (Ransom Hill Bioscience, Ramona, CA). The 3' cDNA ends of both the nMTR and nVTR were amplified using the 3'-RACE protocol described in (Weis and Reynolds 1999). The nMTR specific sense primer 5'-CTG ACC TCA ACT TCC ACA CG-3' was paired with a 3' RACE primer (1-rev (Weis and

Reynolds 1999): 5'-CTC TAG AAC TAG TCT TTT TTT TTT TTT TTT TT-3') designed to anneal to the poly-A tail. Oligo-(dT) primed cDNA was used as a template for this reaction. The reaction conditions were as follows; 94 °C x 3 min; 2 cycles of 94 °C x 30 sec, 37 °C x 2 min, 72 °C x 2 min; 29 cycles of 94 °C x 30 sec, 62 °C x 2 min, 72 °C x 2 min; terminal extension at 72 °C x 5 min. To amplify the 3' cDNA of the nVTR, the following sequence specific primer was used: 5'-GGA GGT GAC CTA CCG TTT CC-3'. As with the nMTR 3' RACE, this primer was paired with 1-rev (Weis and Reynolds 1999). PCR conditions were identical to the nMTR 3'RACE with the exception that the 62 °C annealing steps occurred instead at 65 °C. 3' RACE PCR was also conducted for the nVTR using the FirstChoice RLM-RACE kit (Ambion, Austin TX). PCR products were assessed, cloned, and sequenced as above.

All 5' RACE PCRs were conducted using the FirstChoice RLM-RACE kit. 5' RACE-ready cDNA was prepared from newt brain RNA according to the FirstChoice RLM-RACE kit protocol. 5'RACE-ready cDNA, in conjunction with nMTR (5'-AGG ATC CAG GTG ACG ATG ACA TAC ACC-3') and nVTR (5'-TCG AGA AGA TGG CAT ACT GG-3') gene-specific reverse primers and the Ambion 5' "outer" RACE primer were used in a primary 5'-RACE PCR reaction under the following cycling conditions: 94 °C for 3 min; 4 cycles of 94 °C x 45 sec, 62 °C x 45 sec, 72 °C x 1 min; 34 cycles of 94 °C x 30 sec, 62 °C x 30 sec, 72 °C x 1 min; and 72 °C x 7 min. A second, "nested" PCR reaction was then set up using *T.*

granulosa specific nMTR (5'-TGA AGA GAC CGA AGA GGT TGG CAG ATG-3') and nVTR (5'-TAT GTA CCG GTC GGC AGT C-3') reverse primers designed to be internal to the initial PCR, paired with the Ambion 5' "inner"-RACE primer. Identical cycling conditions to the first reaction were used. PCR products were assessed, cloned, and sequenced as above.

Regions of overlapping, identical, sequences among the original degenerate PCR products, and the 5' and 3' RACE products were joined using SeqMerge software (Accelrys, San Diego, CA) to produce a contiguous sequence representing the full-length nVTR and nMTR cDNAs. Each region of both sequences was independently amplified and sequenced a minimum of three times.

Phylogenetic Analysis

Deduced amino acid sequences were determined for the open reading frames of the nMTR and nVTR cDNA sequences. These deduced *Taricha* amino acid sequences were then aligned with other neurohypophysial peptide receptor sequences obtained from the NCBI databases, using ClustalX v.1.83 (Thompson et al. 1997). The alignments were then manually adjusted prior to phylogenetic analysis to achieve optimal alignment. Phylogenetic webs were constructed by Bayesian inference with a Markov-chain Monte Carlo (MCMC) sampling method using MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003). The amino acid-

substitution model used in the analysis was Blossum (Henikoff and Henikoff 1992) with a gamma distribution of among-site rate variation and all amino acid substitution rates permitted to be different. One out of every 100 trees was sampled for 500,000 generations. The first 1000 trees were discarded as burn-in. The 50% consensus tree was computed by MrBayes using the last 4000 sampled trees. In each analysis we ran four simultaneous MCMC chains.

Tissue Distribution of the nMTR and nVTR

Non-quantitative reverse transcriptase PCR (rtPCR) was used to determine tissue distribution. Tissues were gathered from breeding condition males and snap frozen prior to RNA isolation and cDNA production, as described above. Sequence specific primers for each receptor were designed to amplify regions of low homology between the two receptors' cDNA. The nMTR primer pair was: forward 5'-CAT CAC CTT CCG GTT CTA TG-3' and reverse 5'-CTC AGG ATC CAG GAG-3', yielding an ~200 bp product. The nVTR primer pair was forward 5'-GGA GGT GAC CTA CCG TTT CC-3' and reverse 5'-TCG AGA AGA TGG CAT ACT GG-3', yielding an ~250 bp product. In addition, an actin specific primer was designed to be used as control to verify the quality of each cDNA preparation. The actin primers (generously donated by Dr. Joanne Chu) used were: forward 5'-AAG ACA GCT ACG TTG GTG ATG AAG-3, and reverse 5'-CAC GGA GCT CGT TGT AGA AGG T-3', yielding an

~140 bp product. The following cycling conditions were used for these PCRs: 94 °C x 3 min; 38 cycles of 94 °C x 10 sec, 60 °C x 30 sec, 72 °C x 1 min; terminal extension at 72 °C x 5 min. The sequence of each amplicon was verified from brain cDNA only.

Transient Expression of nMTR and nVTR in COS-7 Cells

New gene-specific primers were designed at the extreme 5' and 3' ends of the nMTR and nVTR open reading frames, and the complete coding regions of each cDNA was subsequently PCR-amplified using a high fidelity *Pfx* proof-reading polymerase (Invitrogen). The full-length nMTR and nVTR cDNAs were then sub-cloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen) and individual clones were analyzed for correct orientation with respect to the CMV promoter. COS-7 (African green monkey kidney) cells (American Type Culture Collection, Manassas, VA) were cultured in 10 cm tissue culture plates at 37 °C under 5% CO₂ in Dulbecco's Modified Eagle's Medium (Mediatech Cellgro, Herndon, VA) supplemented with 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (Hyclone, Logan, UT). Transient expression was achieved by transfecting plates (90-95% confluency) using Lipofectamine 2000 reagent (Invitrogen) diluted with Opti-MEM medium (Invitrogen). For each 10 cm culture plate, 24 µg of DNA in 1.5 ml Opti-MEM and 60 µl Lipofectamine 2000 in 1.5 ml Opti-MEM were added to a plate containing 12 ml growth medium. Six hours after transfection,

the lipofectamine mixture was aspirated and replaced with 14 ml of fresh growth medium.

Preparation of Transfected COS-7 Cell Membranes

48 hr post transfection, cells were washed twice with room temperature 1 x PBS and harvested in homogenization buffer (2 ml/plate; 25 mM HEPES (pH 7.45), 100 μ M PMSF, 100 μ g/ml trypsin inhibitor, 0.7 μ g/ml leupeptin, 100 μ g/ml bacitracin)) using a cell scraper. Suspended cells were homogenized in chilled buffer using an ice-cold glass-on-glass dounce. Homogenates were centrifuged for 10 min at 45000 x g at 4 °C. Supernatants were discarded and pellets were resuspended in homogenization buffer (1 ml/plate) and centrifuged a second time. Supernatants were discarded and cell membrane pellets were resuspended in binding buffer (250 μ l/plate: 25 mM HEPES (pH 7.45), 10 mM MgCl₂, 100 μ M PMSF, trypsin inhibitor (100 μ g/ml), leupeptin (0.7 μ g/ml), and bacitracin (100 μ g/ml)), snap-frozen in liquid nitrogen, and stored at -80 °C until use. The protein concentration of each membrane preparations was determined using the BCA protein assay kit (Pierce, Rockford, IL) prior to freezing of the samples.

[³H]-OT Binding Assays

Saturation and competition binding assays were performed in buffer consisting of 25 mM HEPES (pH 7.45), 10 mM MgCl₂, 100 μ M PMSF,

trypsin inhibitor (100 µg/ml), leupeptin (0.7 µg/ml), and bacitracin (100 µg/ml). The frozen transfected membrane pellets were thawed on ice and diluted with binding buffer to achieve a final protein concentration of 12.5 µg per binding reaction. For saturation experiments, various concentrations of [³H]-OT (33.0 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA) were used in the presence of 10 µM MT (Bachem Biosciences Inc., King of Prussia, PA). Competition binding experiments were performed with the transfected membranes using 1-2 nM [³H]-OT and various doses (10⁻¹² to 10⁻⁴ M) of non radioactive competitors. Mesotocin, oxytocin, vasopressin, vasotocin, and isotocin were purchased from Bachem. Non-specific binding was defined as radioactivity remaining bound in the presence of 10 µM non-radioactive MT. Triplicate samples were run at each dose for both total and non-specific binding. Assays were conducted at 30 °C for 30 min with gentle mixing (80 rpm on an orbital shaker). At the end of the incubation period, bound and free [³H]-OT were separated by rapid filtration over GF/C filters under vacuum using a BRANDEL (Gaithersburg, MD) cell harvester. Filters were washed twice with 4.5 ml of chilled (4 °C) 25 mM HEPES (pH 7.45), 10 mM MgCl₂ buffer. Radioactivity in the filters was determined by liquid-scintillation counting on a Beckman LS 6500 scintillation counter. Binding data were analyzed using Prism 3.0 (GraphPad Software, San Diego, CA).

Inositol Phosphate Accumulation Assays

Inositol phosphate accumulation was determined as previously described (Filtz et al. 1994) with the following modifications. COS-7 cells in 10 cm plates were transfected with the nMTR and nVTR plasmids as described above. 6 hours post-transfection, the cells from each 10 cm dish were split into 24-well plates using 1 x trypsin-EDTA (Invitrogen). These cells were then incubated in 0.5 ml fresh growth medium until 24 hours post-transfection, when the medium was changed, and transfected cells were labeled with inositol-free DMEM with NaHCO₃ (pH 7.2) containing 2 µCi/ml [³H]-inositol (0.5 ml labeling media per well). 48 hr post-transfection the labeling medium was aspirated from the cells and replaced with DMEM augmented with 10 mM LiCl and 10 mM HEPES (pH 7.4). The cells were incubated in this medium for 10 min prior to the stimulation with the various concentrations of each agonist (see binding assay above, similar doses used). Cells were then incubated for 20 min at 37 °C. The reactions were terminated by medium aspiration and addition of 0.5 ml ice-cold 5% trichloroacetic acid (TCA). Phospholipids were collected after solubilization of the TCA-precipitated cells with 0.5 ml 1 N NaOH. Antagonist studies using d(CH₂)₅[Tyr(Me)²]AVP (Manning compound) (Kruszynski et al. 1980) and d(CH₂)₅[Tyr(Me)², Thr⁴, Tyr-NH₂]OVT (OTA) (Elands et al. 1988; Terrillon et al. 2002) were conducted as above with one additional step. Prior to stimulation with agonists (10

μM MT for the nMTR and $10\mu\text{M}$ VT for the nVTR), cells were incubated for 10 min in varying concentrations of antagonist.

Following TCA solubilization, samples were purified by ion exchange chromatography as described in (Nakahata and Harden 1987). In brief, samples were added to hydrated columns (1 ml AG1-X8,200-400 mesh, formate form resin (Bio-Rad), and washed with 10 ml each dH_2O , and 50 mM ammonium formate (Fisher Scientific, Pittsburgh, PA). Samples were then extracted with 6 ml 1.2M ammonium formate/0.1 M formic acid (Fisher Scientific) into scintillation vials, mixed with Scintisafe gel (Fisher Scientific) and radioactive counts measured and analyzed as for the binding assay.

Results

Cloning of the Mesotocin and Vasotocin Receptors

Initial partial sequences encoding portions of the *Taricha* mesotocin receptor (nMTR) and vasotocin receptor (nVTR) were amplified from male *Taricha* brain cDNA by degenerate PCR. The degenerate VT primer pair amplified a 288 nucleic acid base pair (bp) product; whereas, the degenerate MT primer pair amplified a separate 435 bp product. The amplified nVTR partial sequence had highest identity with cDNA sequences encoding the mammalian V1a VP receptors. The amplified nMTR fragment had highest identity with other amphibian MTR and mammalian OTR cDNA sequences.

nVTR and nMTR sequence characteristics

The full-length nVTR cDNA consisted of 2266 bp sequence that included an open reading frame encoding a 418 deduced amino acid protein (Fig 3.1). The 5' untranslated region (UTR) was 72 bp in length and the 3' UTR was 930 bps in length. The 5' translation initiation sequence was a modified Kozak consensus sequence (Kozak 1981), with a 3' cytosine replacing the 3' consensus guanine in the nVTR cDNA sequence ((G/A)NNATGG consensus compared to the nVTR-ACCATGC). The nVTR cDNA sequence also contained a modified polyadenylation signal approximately 80 bp upstream of the 3' poly-a tail (consensus:AAU AAA compared to nVTR:AAC AAA).

The deduced amino acid (aa) sequence encoded within the nVTR's cDNA open reading frame contained the consensus signature sequence of a G-protein coupled receptor in the rhodopsin family (CDD:5814 (Marchler-Bauer et al. 2003)). In addition, a hydropathy analysis (results not shown) of the nVTR aa sequence predicts the presence of 7 putative transmembrane domains with an intracellular C-terminus, and an extracellular N-terminus. At the N-terminal of the protein there are consensus sequences of both N-linked glycosylation and N-linked myristoylation sites, while at the C-terminal end there are numerous phosphorylation sites.

atggaggggt tatgtctgaa ggagaacgat tgctggggtc ttttggccaa ccgctctctg 60
 M E G L C L K E N D C W G L L A N R S L 20
 gataactcaa ccatggagaa caggacgtat gctgtaaaca aaaccaggga ccccttgaaa 120
 D N S T M E N R T Y A V N K T R D P L K 40
 aggaatgagc acatggctcg cgtggaggtg agcgtgctgg ccctcatcct cttcctggct 180
 R N E H M A R V E V S V L A L I L F L A 60
 TM1
 ctggtgggta acgtgtgtgt gctgctggcc atccacatca accggaacaa gcactcagcg 240
 L V G N V C V L L A I H I N R N K H S R 80
 atgtacttct tcatgaagca cctcagcatc gctgacctgg tgggaggcat cttccaggctc 300
 M Y F F M K H L S I A D L V V A I F Q V 100
 TM2
 ctcccgcagc tcatctggga catcaccttc aggttctatg ggccggactt cctctgcccgg 360
 L P Q L I W D I T F R F Y G P D F L C R 120
 gcagtcaagt acttgacagt ggtggggatg ttccgctcca cctacatgct gctactgatg 420
 A V K Y L Q V V G M F A S T Y M L L L M 140
 TM3
 tccatggacc ggtgcctggc catctgccaa cctcttcggg ctcttcacag acgctcggat 480
 S M D R C L A I C Q P L R S L H R R S D 160
 cgggtgatg tcatcgtcac ctggatcttg agctttttgg taagcattcc tcagattcat 540
 R V Y V I V T W I L S F L V S I P Q I H 180
 TM4 □
 atcttctcct tgaaggagggt gggcaatggg gttcaggatt gttgggcaga cttcatccag 600
 I F S L K E V G N G V Q D C W A D F I Q 200
 ccttggggcg tgaagcgta cgtgacttgg atcaccgtga tgggtgtacat catccccgta 660
 P W G V K A Y V T W I T V M V Y I I P V 220
 TM5
 ctgacccca gcactctgcta tggcctcatc agcttcaaga tctggcagaa ctttcgctg 720
 L I L S I C Y G L I S F K I W Q N F R L 240
 aagacagtct gtgagaccaa tgtggcactg acctcaactt ccacacgcag gaccaccctg 780
 K T V C E T N V A L T S T S T R R T T L 260
 tcgagggtca gcagcgtgaa gctgatctcc aaggctaaaa tcaggactgt caagatgacc 840
 S R V S S V K L I S K A K I R T V K M T 280
 ttcacatcg tcttggccta catagtctgc tggacaccct tcttcttctg ccaaagtgg 900
 F I I V L A Y I V C W T P F F F V Q M W 300
 TM6
 tctgtctggg acatcaatgc acccaaagaa gcacccctgt tcatcattgc aatgctactg 960
 S V W D I N A P K E A S L F I I A M L L 320
 ggaagcctaa acagctgctg caaccctgg atctacatgc tcttcacggg acatctcttt 1020
 G S L N S C C N P W I Y M L F T G H L F 340
 catgacctct tgcagcgctt cctctgctgc tctgctcatt acttgaaatc caggcagcaa 1080
 H D L L Q R F L C C S A H Y L K S R Q Q 360
 ggggctgatc tcagcagtgc cagcaggaag agcaattcca ccgttgcctt gaggcgcaag 1140
 G A D L S S A S R K S N S T V V L S R K 380
 agtccagcc agaagagcat agcacagcca tccatggcat ga 1171
 S S S Q K S I A Q P S M A * 393

Fig 3.1 Coding cDNA and deduced amino acid sequence for the nMTR.

The putative transmembrane domains (TM1-TM7) are underlined. The stop codon is indicated by an asterisk. Target consensus sequences for post-translational modification are marked (symbol below the affected amino acid) with the following symbols: N-glycosylation (■), N-myristoylation (◆), PKC phosphorylation site (▼), cAMP and cGMP dependent protein kinase phosphorylation site (●), and casein kinase II phosphorylation site (○), tyrosine kinase phosphorylation site (□).

The full-length nMTR cDNA consisted of a 2495 bp sequence containing an open reading frame that encoded a deduced 393 aa protein (Fig 3.2). The 5' UTR was 550 bp in length and the 3' UTR was 765 bps in length. The nMTR cDNA sequence contained both a 5' Kozak initiation consensus sequence (GACATGG) (Kozak 1981), and, 18 bp upstream of the poly-a tail, a 3' consensus polyadenylation signal (AATAAA) (Zarudnaya et al. 2003). As with nVTR, the nMTR deduced aa sequence contained the consensus signature of a G-protein coupled receptor in the rhodopsin family. (CDD:5814 (Marchler-Bauer et al. 2003)). Hydropathy analysis (results not shown) of the nMTR aa sequence also predicted the existence of 7 transmembrane domains in the secondary structure of the deduced amino acid sequence. The predicted N-terminus of the nMTR aa sequence contained multiple N-glycosylation and N-myristoylation consensus sequences, while the C-terminus of the receptor includes multiple phosphorylation consensus sequences (Fig 3.2). In addition, there is a single tyrosine kinase phosphorylation consensus sequence in a region predicted to be a part of transmembrane domain 4 (Fig 3.2).

atgcgcttca ctggcagccc ggcaagcccc tggccatcct cgcaccccct gccaccccgc 60
 M R F T G S P A S P W P S S D P L P T R 20
 tggaactcca gcctgactgg ggggcccagc tgccccggcc tggccagctc aagcctgccc 120
 W N S S L T G G P S C P G L A S S S L P 40
 ■
 cccacctccc ccagcactaa ctctcgggac ctgttggggc gcgacgaggc gctggcgcg 180
 P T S P S T N S S D L L G R D E A L A R 60
 ■
 gtggagggtgg ccgttctggc gcttatcttc gtggcccggc tggttggcaa cggtgctg 240
 V E V A V L A L I F V A A V V G N G C V 80
 ctctggcat tgcagcgcag ccggcgcaag acgtcgcgca tgcacctctt catccgccac 300
 L L A L Q R S R R K T S R M H L F I R H 100
 ▼
 ctacgcctcg ccgacctggg ggtggcgctc ttccagggtgc tgccgcagct ctgctgggag 360
 L S L A D L V V A L F Q V L P Q L C W E 120
 ○
 gtgacctacc gtttccgagg cacggacgct ctctgcccgg tggtaagca cctgcaggctc 420
 V T Y R F R G T D A L C R V V K H L Q V 140
 ▼
 ttccggcatgt tcgcttcagc ctatatgctg gtggccatga ctgccgaccg gtacatagcc 480
 F G M F A S A Y M L V A M T A D R Y I A 160
 ◆
 gtttggcacc cgctaaagac gctgcagcag cctggcagac gctcgcagtc catgatcggc 520
 V C H P L K T L Q Q P G R R S H A M I G 180
 TM3
 ggcgccctggg cgctcagctc gctgctcagc acgccccagt atgccatctt ctcgatgcgc 540
 G A W A L S L L L S T P Q Y A I F S M R 200
 ◆
 gaggtgcgag aacctcaca ggtctacgac tgctggggcc acttcatcga gccctggggg 600
 E V R E H S Q V Y D C W A D F I E P W G 220
 gccagggttt acgtgacatg gatcgtggtc agcatctttg tggtgccagt cctcatcctg 720
 A R A Y V T W I V V S I F V V P V L I L 240
 TM5
 gctacctgct acggctttat ctgctacatc atctggacca acattcgtgg caagacgagg 780
 A T C Y G F I C Y I I W T N I R G K T R 260
 ccgtccaacg gcggggccca gctcctctcc acgtcggggc tcagcagcgt caagaccatc 840
 P S N G G A Q L L S T S A V S S V K T I 280
 ▼
 tccaggggcca agatccgcac cgtcaagatg accttcgctca ttgtgtccgc ctacgtgatc 900
 S R A K I R T V K M T F V I V S A Y V I 300
 TM6
 tgctgggctc cttcttcat cgtgcagacc tggtcggctc gggataagaa cttcacatgg 960
 C W A P F F I V Q T W S V W D K N F T W 320
 ○
 atcgactccg agaacacctc agtcaccgct acatccttgc tggccagtct aaacagctgc 1020
 I D S E N T S V T V T S L L A S L N S C 340
 ■
 tgcaaccctt gcatatacat gttcttcagc gggcaccttt ttcaagactt catccaaagt 1080
 C N P W I Y M F F S G H L F Q D F I Q S 360
 tttgtttgtt ggcggaagat gcaacacaac acccagaagg aggactcgga cagcagctgc 1140
 F V C W R K M Q H N T Q K E D S D S S C 380
 ○\▼
 cggcggcaga cttcttttac caggattaac aaccgtagtc cgaccaacag tacggacaca 1200
 R R Q T S F T R I N N R S P T N S T D T 400
 ●
 tggaaggagt cccccaagtc catccgctcc accaggttcc ttcccatcca gacctga 1257
 W K E S P K S I R S T R F L P I Q T * 418
 ▼
 ▼
 ▼

Fig 3.2 Coding cDNA and deduced amino acid sequence for the nVTR.

The putative transmembrane domains (TM1-TM7) are underlined. An asterisk indicates the stop codon. Consensus sequences for targets of post-translational modification are marked (symbol below the affected amino acid) with the following symbols: N-glycosylation (■), N-myristoylation (◆), PKC phosphorylation site (▼), cAMP and cGMP dependent protein kinase phosphorylation site (●), and casein kinase II phosphorylation site (○).

Sequence Identities of the nMTR and nVTR

The deduced aa sequences of the nVTR and nMTR were aligned with other identified neurohypophysial hormone receptor sequences. The nVTR aa sequence had highest sequence identity with the cloned frog (*H. japonica*) V_{1a}-likeVTR (64%), mammalian V_{1a}Rs (60 - 64%) and fish VTRs (47-56%), having lower identities with the mammalian V_{1b}Rs (42-43%), OTRs (40-41%) and V₂Rs (36-38%). The deduced amino acid sequence of the nMTR had highest identity with other identified amphibian MTRs (79-78%), fish ITR (66%) and mammalian OTRs (64-66%), and lower identity with the other classes of vertebrate neurohypophysial hormone receptor (V_{1a}R, V_{1b}R, V₂R: 34-44%). The predicted transmembrane domains of the nVTR and nMTR aligned well with each other (Fig 3.3.), as well as with the predicted domains of other cloned neurohypophysial hormone receptors (not shown).

Phylogenetic Analysis of the Neurohypophysial Peptide Receptors

A Bayesian analysis of the neurohypophysial peptide receptor deduced aa sequence alignment (representative alignment, Fig 3.3), rooted with invertebrate CPRs, produced a tree containing 5 groups (Fig 3.4): an invertebrate CPR group, a V_{1a}R-like group, a V_{1b}R-like group, a V₂R-like group, and an OTR-like group. The Bayesian analysis strongly grouped the nMTR aa with the other MTR, OTR, and ITR sequences. The nVTR aa groups with the V_{1a}-VP receptors as well as with all of the identified fish and the bullfrog (*Rana catesbeiana*) V_{1a}-like VTR.

		<u>TM1</u>		
VTR <i>T. granulosa</i>	MRFTGSPASP	WPSSDPLPTR	WNSSLTGGPS	CPGLASSSLP
VTR <i>R. catesbeiana</i>	MGFSLKLGSSG	QELSLGNGST	LDNATSETPF	LFLSASPNE
V1aR <i>M. musculus</i>		MS FPRGSHDLPA	GNSSPWPLT	TEGANSSREA
V1bR <i>M. musculus</i>			MDSEP	SWTATPSGG
V2R <i>M. musculus</i>			MILVSTTS	AVPGALSSPS
VTR2 <i>H. japonica</i>			MSVTHSPSA	SISTNFTTED
OTR <i>M. musculus</i>		MEGTAAANWS	IELDLGSGVP	PGAEGNLTAG
MTR <i>B. marinus</i>		MEGLC	LNLDCSELN	SSWVNSSMEN
MTR <i>T. granulosa</i>		MEGLCLK	ENDCWGLLAN	RSLDNSTMEN
		<u>TM2</u>		
VTR <i>T. granulosa</i>	VAAVVGNGCV	LLALQR--SR	RKTSRMHLFI	RHLSLADLVV
VTR <i>R. catesbeiana</i>	VAAVLGNCSV	LLGLYK--SK	KMRSRMHLFI	KHLSLADLVV
V1aR <i>M. musculus</i>	VVAVLGNSSV	LLALHR--TP	RKTSRMHLFI	RHLSLADLVV
V1bR <i>M. musculus</i>	VLATGGNLAV	LLILGL--QG	HKRSRMHLFV	LHALTDLGV
V2R <i>M. musculus</i>	VAVALSNGLV	LGALIRRGRR	GRWAPMHVFI	SHLCLADLVV
VTR2 <i>H. japonica</i>	GFATFGNCLV	LFTLLR--RR	KHNALMHTFM	IHLCLADLVV
OTR <i>M. musculus</i>	FLALSNGACV	LLALRT--TR	HKHSRLFFFM	KHLSIADLVV
MTR <i>B. marinus</i>	FLALAGNICV	LLGIYI--NR	HKHSRMVFFM	KHLSIADLVV
MTR <i>T. granulosa</i>	FLALVGNVCV	LLAHI--NR	NKHSRMVFFM	KHLSIADLVV
		<u>TM3</u>		
VTR <i>T. granulosa</i>	QVFGMFASAY	MLVAMTADRY	IAVCHPLKTL	QQPG--RRSHA
VTR <i>R. catesbeiana</i>	QVFGMFASTY	MLVMTADRY	IAICHPLKTL	HQPT--KRSYL
V1aR <i>M. musculus</i>	QVFAMFASSY	MLVMTADRY	IAVCHPLKTL	QQPA--RRSRL
V1bR <i>M. musculus</i>	QVLSMFASY	MLLAMTLDRY	LAVCHPLRSL	QQPS--QSTYP
V2R <i>M. musculus</i>	QVMGYASSY	MILAMTLDRH	RAICRPMFLAY	RHGGRARWNR
VTR2 <i>H. japonica</i>	QVVGMFASSY	MIVAMTFDRH	QAICREMTTF	KKGS--ARWNI
OTR <i>M. musculus</i>	QVVGMFASY	LLLLMSLDRC	LAICQPLRSL	RR---RTDRL
MTR <i>B. marinus</i>	QVVGMFASY	LLLLMSLDRC	LAICQPLRSL	HR---RSDCV
MTR <i>T. granulosa</i>	QVVGMFASY	LLLLMSLDRC	LAICQPLRSL	HR---RSDRV
		<u>TM4</u>		
VTR <i>T. granulosa</i>	SQVYDCWADF	IEPWGARAYV	TWIVVSIFV	PVLILATCYG
VTR <i>R. catesbeiana</i>	DGVYDCWADF	ISPKGLKAYI	TWITISIFV	PVIILLTCYG
V1aR <i>M. musculus</i>	TKAQDCWATF	IPPWGTRAYV	TWMTSGVFEV	PVIILGTCYG
V1bR <i>M. musculus</i>	SGVLDCWADF	YFSWGPRAIY	TWTTMAIFLV	PVVVLTACYG
V2R <i>M. musculus</i>	SGVDFCWARF	AEPWGLRAYV	TWIALMVFA	PALGIAACQV
VTR2 <i>H. japonica</i>	PGVHDCWAHF	VKPWGPKEYV	TWITLAVLIL	PALFITTCQV
OTR <i>M. musculus</i>	DGVDFCWAVF	IQPWGPKEYV	TWITLAVYIV	PVIVLAACYG
MTR <i>B. marinus</i>	NGVYDCRADF	IQPWGPKEYI	TWITLAVYII	PVIVLAACYG
MTR <i>T. granulosa</i>	NGVQDCWADF	IQPWGVKAYV	TWITVMVYII	PVILLSICYG
		<u>TM5</u>		
VTR <i>T. granulosa</i>	----AQLLST	SAVSSVKTIS	RAKIRTVKMT	FVIVSAYVIC
VTR <i>R. catesbeiana</i>	---SNGLLST	S-VSSVRTIS	RAKIRTVKMT	FVIIVTAYIIC
V1aR <i>M. musculus</i>	GPFHKGLLVT	PCVSSVKISIS	RAKIRTVKMT	FVIVSAYIIC
V1bR <i>M. musculus</i>	AAAATRGLP	SRVSSISTIS	RAKIRTVKMT	FVIIVLAYIAC
V2R <i>M. musculus</i>	-----GHRT	GSPSEGAHVS	AAMAKTVRMT	LVIVIVYVLC
VTR2 <i>H. japonica</i>	-EKLVVGMNG	VPQVSDSGVT	KAMSKTVRMT	LAIVLIYVVC
OTR <i>M. musculus</i>	AAGGAGRAAL	ARVSSVKLIS	KAKIRTVKMT	FIIVLAFIVC
MTR <i>B. marinus</i>	---TSRRATL	SRVSSVRLIS	KAKIRTVKMT	FIIVLAFIVC
MTR <i>T. granulosa</i>	-STSTRRTTL	SRVSSVKLIS	KAKIRTVKMT	FIIVLAFIVC
		<u>TM6</u>		
VTR <i>T. granulosa</i>	-----DSE	NTSVTVTSL	ASLNSCCNPW	IYMFSGHLF
VTR <i>R. catesbeiana</i>	-----ENE	NTVTVSALL	ASLNSCCNPW	IYMFSGHLF
V1aR <i>M. musculus</i>	-----DSE	NPSTTITALL	ASLNSCCNPW	IYMFSGHLL
V1bR <i>M. musculus</i>	-----DST	NVAFTISMLL	GNLSSCCNPW	IYMGFNSHLL
V2R <i>M. musculus</i>	-----RPFVLLMLL	ASLNSCTNPW	IYAFSSSVS	SELSRLCCA
VTR2 <i>H. japonica</i>	-----HSAIQILMLL	ASLNSCTNPW	IYTFSSSVS	KDIQAILCCS
OTR <i>M. musculus</i>	KDEGTEVSEA	ASAFI IAML	ASLNSCCNPW	IYMLFTGHLF
MTR <i>B. marinus</i>	-----ASLFI IAML	GSLNSCCNPW	IYMLFTGHLF	HDLQRFLLC
MTR <i>T. granulosa</i>	-----ASLFI IAML	GSLNSCCNPW	IYMLFTGHLF	HDLQRFLLC
		<u>TM7</u>		
VTR <i>T. granulosa</i>	SFTRI--NNRS	PTNSTDTWKE	SPKSIRSTRF	LPIQT
VTR <i>R. catesbeiana</i>	SFTRIQT-RS	PTHSTDTWKD	SPKSSRSIKE	LPLQI
V1aR <i>M. musculus</i>	SYS---NNRS	PTNSTGTWKD	SPKSSKSIRF	IPVST
V1bR <i>M. musculus</i>	TLLLTHTCGP	STLRLSLNLS	LHAKPKPAGS	LKDLEQVDGE
V2R <i>M. musculus</i>	SSLMKDTPS			ATMETSIS
VTR2 <i>H. japonica</i>	TSFPKESLY			
OTR <i>M. musculus</i>	NSSTFVLSRR	SSSQRSQSOP	SSA	
MTR <i>B. marinus</i>	NSSTFVLSRK	SSSQKSITQP	STA	
MTR <i>T. granulosa</i>	SNSTVLSRK	SSSQKSIAQP	SMA	

Fig 3.3 Alignment of the deduced amino acid sequence for the nMTR and nVTR aligned with other neurohypophysial hormone receptor sequences. Sequences were initially aligned with ClustalX and then manually adjusted. Predicted transmembrane domains (as calculated by SOSUI) indicated by a solid overline for the nVTR and a dashed underline for the nMTR. Non-Taricha sequences were obtained from Entrez-Protein, NCBI. Accession numbers: VTR *Rana catesbeiana*, AAQ22364; V1aR *Mus musculus*, NP_036054; V1bR *Mus musculus*, NP_036054; V2R *Mus musculus*, CAC34589; VTR2 *Hyla japonica*, BAC23055; OTR *Mus musculus*, XP_144956; MTR *Bufo marinus*, Q90252.

Figure 3.4 Neurohypophysial hormone receptor consensus tree. The 50% majority rule consensus tree obtained from Bayesian analyses of the deduced amino acid sequence for representative sequences from each of the major classes of neurohypophysial hormone receptor (mesotocin receptor (MTR); isotocin receptor (ITR); oxytocin receptor (OTR); vasotocin receptor (VTR), chicken type 1 (VT1R), chicken type 2 (VT2R); vasopressin receptor type 1 (V1aR), type 2 (V2R), type 1b/3 (V1bR); conopressin receptor type 1 and 2 (CPR1, CPR2) indicates that the newt VTR(nVTR) clusters with the mammalian V1aRs and the newt MTR (nMTR) clusters with the MTR, ITR and OTRs. Posterior probability values are indicated at each node. Both snail CPRs were treated as the root for this tree. Accession numbers: CPR1 *Lymnaea stagnalis*, AAA91998; CPR2 *Lymnaea stagnalis*, AAC46987; VTR *Catostomus commersoni*, I50132; VTR *Astatotilapia burtoni*, AAM70493; VTR *Platichthys flesus*, AAF00506; VTR *Rana catesbeiana*, AAQ22364; V1aR *Homo sapiens*, NP_000697; V1aR *Mus musculus*, NP_036054; V1aR *Microtus ochrogaster*, AAD02821; VT2R *Gallus gallus*, AAG17937; V1bR *Mus musculus*, NP_036054; V1bR *Homo sapiens*, NP_000698; ITR *Catostomus commersoni*, Q90334; OTR *Homo sapiens*, NP_000907; OTR *Mus musculus*, XP_144956; MTR *Rana catesbeiana*, AAQ22365; MTR *Bufo marinus*, Q90252; MTR *Hyla japonica*, BAC23056; VTR1 *Gallus gallus*, AAF18344; VTR2 *Hyla japonica*, BAC23055; V2R *Homo sapiens*, P30518; V2R *Mus musculus*, CAC34589.

Tissue Distribution of nMTR and nVTR cDNA

Neither the nVTR specific primers, nor the nMTR specific primers, were found to amplify products encoding the paralogous receptors cDNA sequence. The nVTR specific primers were found to amplify an appropriately sized product from cDNA produced from male *Taricha* brain, liver, sperm duct, lung, pituitary gland and testes were each used as template. The nMTR specific primers were found to amplify an appropriately sized product when cDNAs produced from the male *Taricha* brain, heart, large intestine, lung, pituitary and testes were each used as template (Fig 3.5).

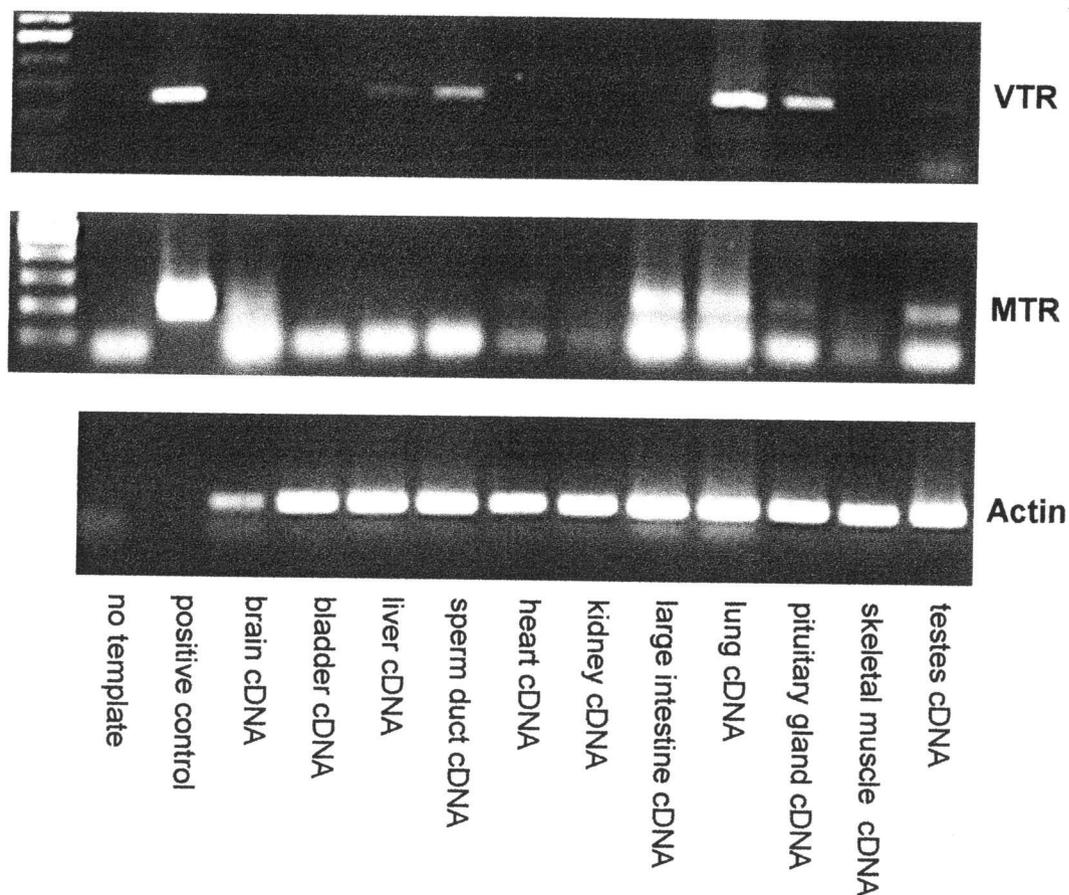


Fig 3.5 Distribution of nVTR and nMTR cDNA within *T. granulosa*. RNA was purified from various tissues and organs of male newts which was then converted to cDNA. Both the nVTR and nMTR primer pairs were designed to amplify a target sequence of 250 bp. In addition, the quality of the cDNA was screened with a primer pair designed to amplify a fragment of the α -actin sequence. There was a no template control, consisting of no added cDNA template, and a positive control consisting of a purified plasmid containing either the cloned nVTR or nMTR sequence.

Saturation and Competition Binding to the nMTR

In order to characterize the binding pharmacologies of the nVTR and nMTR, the coding sequences for the receptors were transiently transfected into COS-7 cells. No specific [³H]-OT binding was detectable in untransfected COS-7 cells. COS-7 cells transiently expressing nVTR specifically bound [³H]-OT, but a saturable binding site was not detected because a high enough concentration of [³H]-OT was unavailable (maximum attainable dose was 30 nM, data not shown). The transiently expressed nMTR had a specific binding site that saturated (Fig 3.6), and was best fit by a two site model (F-test; $p = 0.46$). Pilot studies indicated that maximum specific binding was reached after a 30 minute incubation at 30 °C (equivalent specific binding was found after a one hour incubation at 30 °C). In order to characterize the pharmacologies of the nMTR for a variety of ligands, competition binding studies were conducted in which cold ligands were used to compete with [³H]-oxytocin for the nMTR binding site. The lower affinity, higher abundance binding site (Table 3.1, $K_d = 4.27$ nM) was used in calculating the K_i of the cold competitors for the nMTR (Fig 3.7). The competition curve of each ligand was best fit by a single binding site model (F-test, $p > 0.15$). The relative affinity of the nMTR for these ligands was $MT > VT = OT > VP > IT$ (Table 3.1).

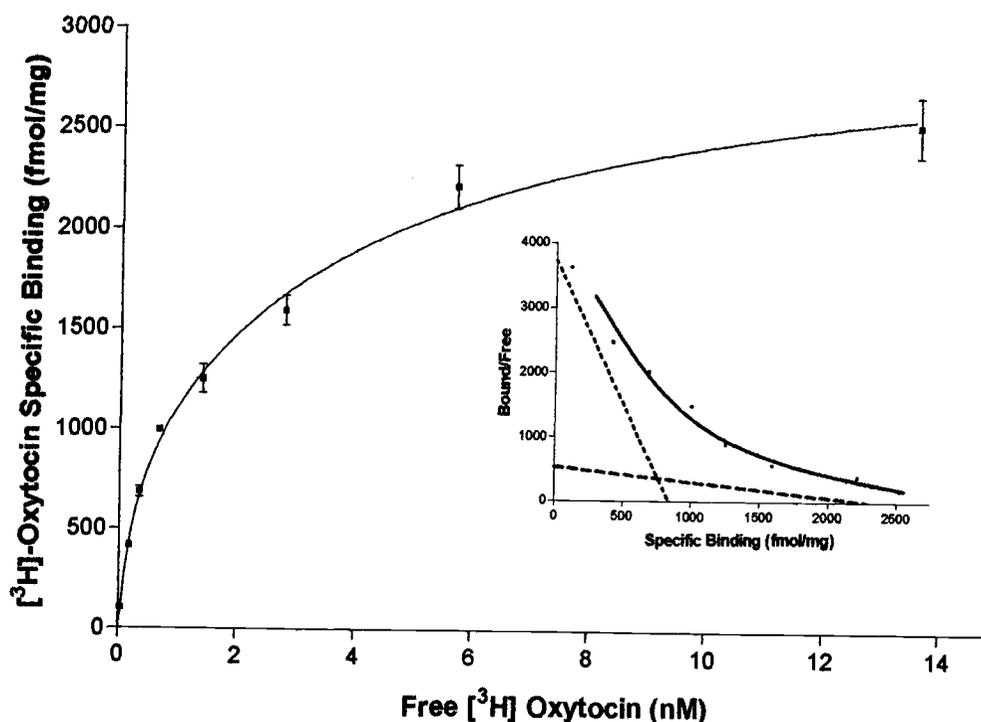


Fig 3.6 Saturation analysis of $[^3\text{H}]\text{-OT}$ binding sites in membranes isolated from COS-7 cells transiently expressing the nMTR. Saturation binding was conducted for 30 min. at 30 °C with 12.5 μg of prepared COS-7 cells transiently expressing the nMTR and increasing concentrations of $[^3\text{H}]\text{-OT}$. Non-specific binding for each dose was determined in the presence of 10 μM mesotocin. The resulting data was best explained by a two binding site model rather than an equation describing a single binding site (F-test, p value = 0.046). Each data point represents the mean \pm SEM. for triplicate measurements. The inset is a Scatchard analysis of the data.

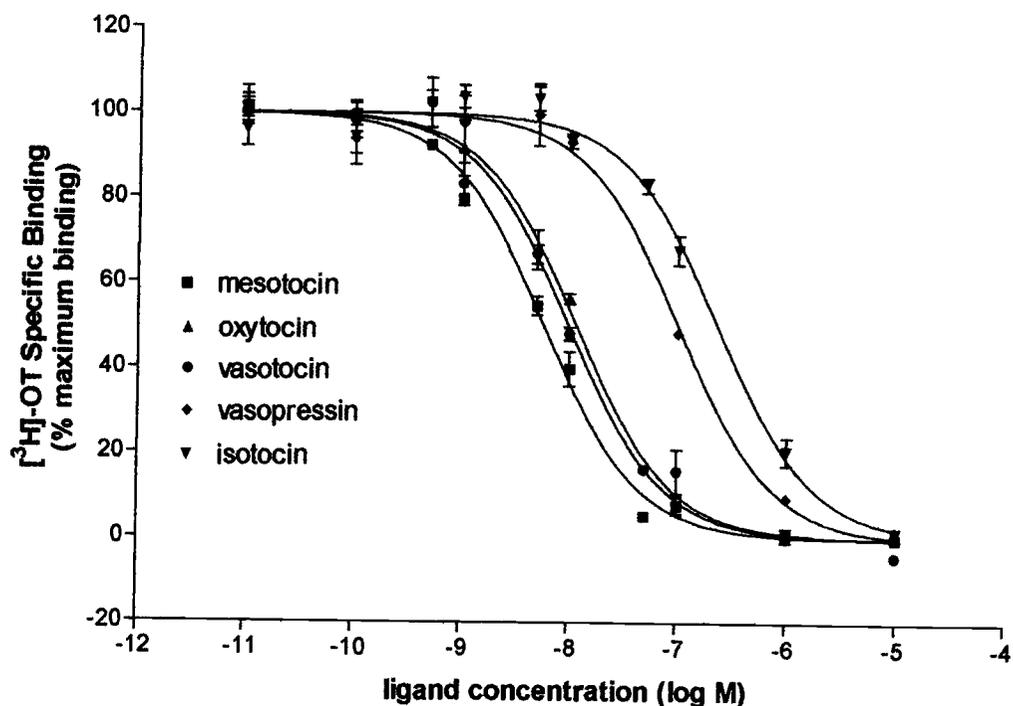


Fig 3.7 Inhibition of specific $[^3\text{H}]\text{-OT}$ binding in isolated COS-7 cell membranes transiently expressing the nMTR. $[^3\text{H}]\text{-OT}$ binding was measured in isolated cell membranes from COS-7 cells transiently transfected with the nMTR. Membranes were incubated with approximately 1 nM $[^3\text{H}]\text{-OT}$ (range 0.68 nM - 1.56 nM) plus increasing concentrations of each ligand for 30 min at 30 °C. Each data point represents the mean \pm SEM for each measurement in triplicate.

Table 3.1 Summary of saturation and inhibition binding data of [³H]-Oxytocin in COS-7 cells transiently expressing the nMTR. [³H]-oxytocin binding to COS-7 cells transiently expressing the nMTR was measured for both saturation binding analysis and for competition analysis. For both the saturation and competition assays, incubation of the isolated COS-7 cell membranes with the [³H]-oxytocin was for 30 min at 30 °C. In the saturation binding analysis various concentrations of [³H]-OT were used and non-specific binding was determined in the presence of 10 µM MT. For the inhibition binding data, various concentrations of cold competitors were tested to measure their efficacy at displacing [³H]-OT for the nMTR expressing membrane. Results are presented as the mean ± SEM for triplicate measurements.

[³ H]-Oxytocin	binding site 1	<u>K_d (nM)</u>	<u>B_{max} (fmol/mg)</u>
	binding site 2	4.27 ± 2.6	2276 ± 360.1
		0.23 ± 0.22	839.4 ± 510.8
<u>Ligand</u>		<u>IC₅₀ (nM)</u>	<u>K_i (nM)</u>
Mesotocin		5.83 ± 0.5	4.76 ± 0.5
Vasotocin		9.84 ± 1.3	8.19 ± 1.3
Oxytocin		11.6 ± 1.4	8.52 ± 1.4
Vasopressin		102 ± 18	74.5 ± 18
Isotocin		231 ± 35	200 ± 35

Induction of Inositol Phosphate Accumulation by the nMTR and nVTR

To test functionality, and further characterize the pharmacology of the nVTR and nMTR, [³H]-inositol phosphate (IP) accumulation assays were conducted with the endogenous vertebrate neurohypophysial hormone receptor ligands, as well as several receptor defining antagonists that have been frequently used in behavioral studies. No effect on [³H]-IP accumulation by 100 nM MT was detected in untransfected COS-7 cells. All ligand stimulations of [³H]-IP accumulation were between 2 and 5 fold over background levels. The rank order potency of the endogenous ligands on cells transiently expressing nMTR was MT>OT>VT=VP>IT (Fig 3.8, Table 3.2). The nVTR transiently expressing cells were successfully stimulated by the neurohypophysial peptides (Fig 8) with a rank order potency of VT>VP>MT=OT>IT (Fig 3.9, Table 3.2).

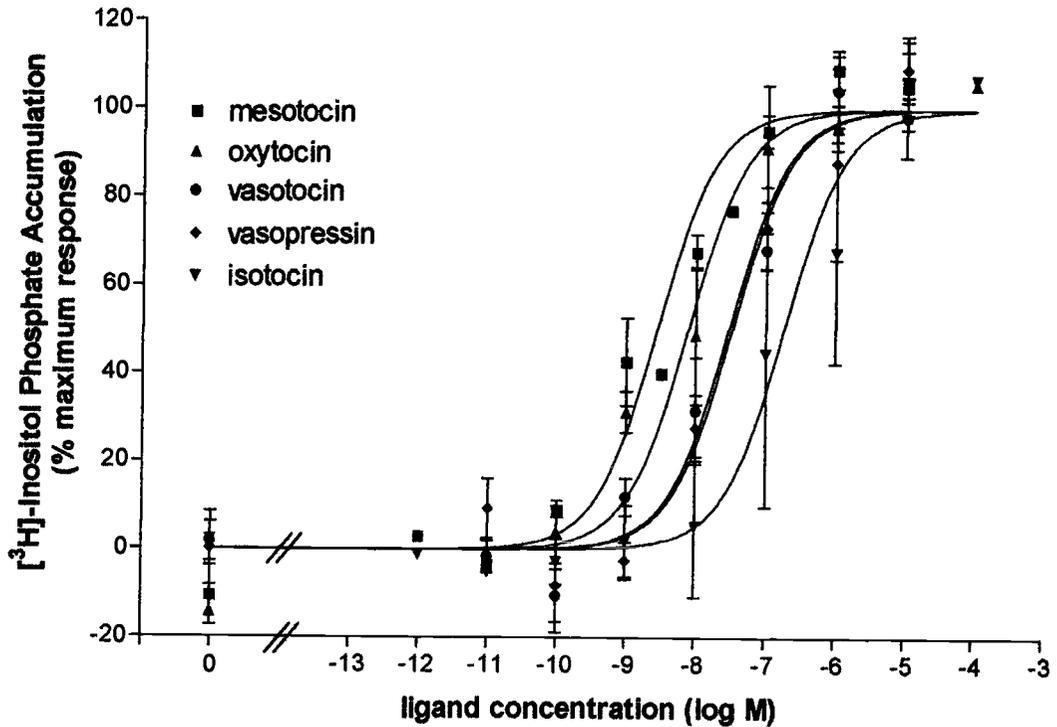


Fig 3.8 Functional effects of neurohypophysial hormone agonists on mesotocin receptor-transfected COS-7 cells. Inositol phosphate accumulation was measured in $[^3\text{H}]$ -inositol-prelabeled COS-7 cells transiently transfected with nMTR. Dose-response curves were generated by treating cells for 20 min with varying concentrations of agonists. Curves represent mean data \pm se obtained from two to three experiments assayed in triplicate.

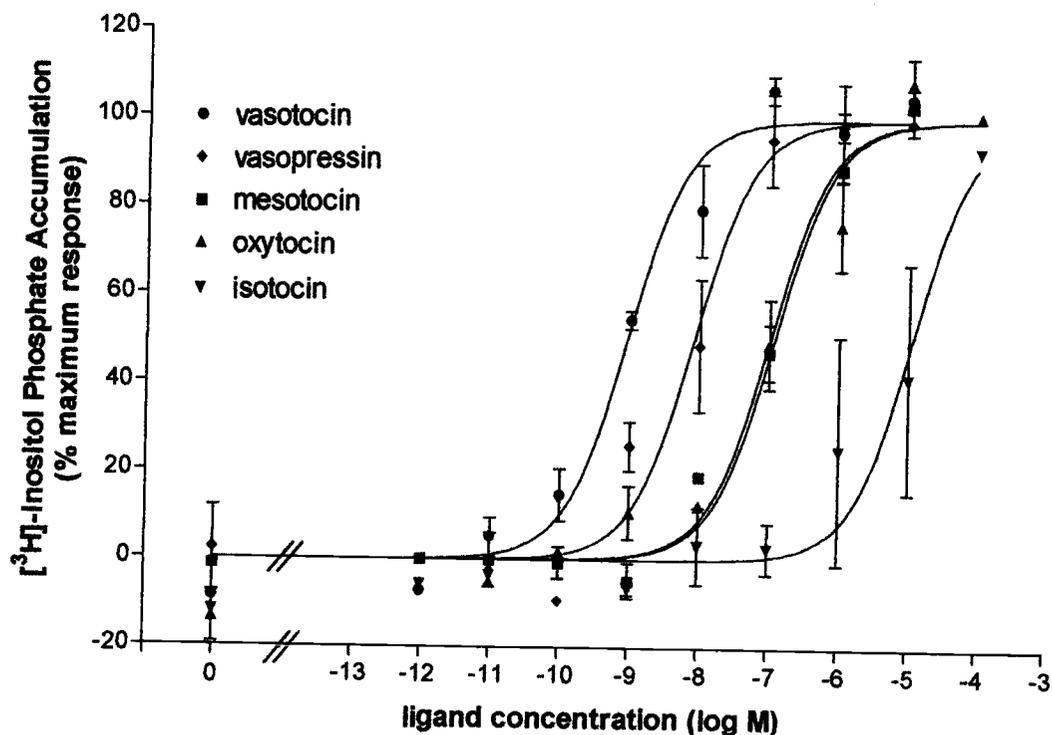


Fig 3.9 Functional effects of neurohypophysial hormone agonists on vasotocin receptor-transfected COS-7 cells. Inositol phosphate accumulation was measured in [³H]-inositol-prelabeled COS-7 cells transiently transfected with nVTR. Dose-response curves were generated by treating cells for 20 min with varying concentrations of agonists. Curves represent mean data +/- se obtained from two to three experiments assayed in triplicate.

The antagonist studies were conducted by stimulating the nVTR and nMTR with 10 nM VT or MT, respectively, and then using the antagonists to inhibit the stimulation. For each receptor, the dose of VT and MT used in the inhibition studies was the lowest approximate dose of each receptors ligand that produced the maximum level (100%) of [³H]-IP accumulation. In the nMTR studies, OTA had a lower IC₅₀ than Manning compound (Fig 3.10, Table 2) while in the nVTR studies the situation was reversed (IC₅₀ of OTA > Manning compound)(Fig 3.11,. Table 3.2). The calculated IC₅₀ for Manning compound was lower for nMTR expressing cells than nVTR expressing cells.

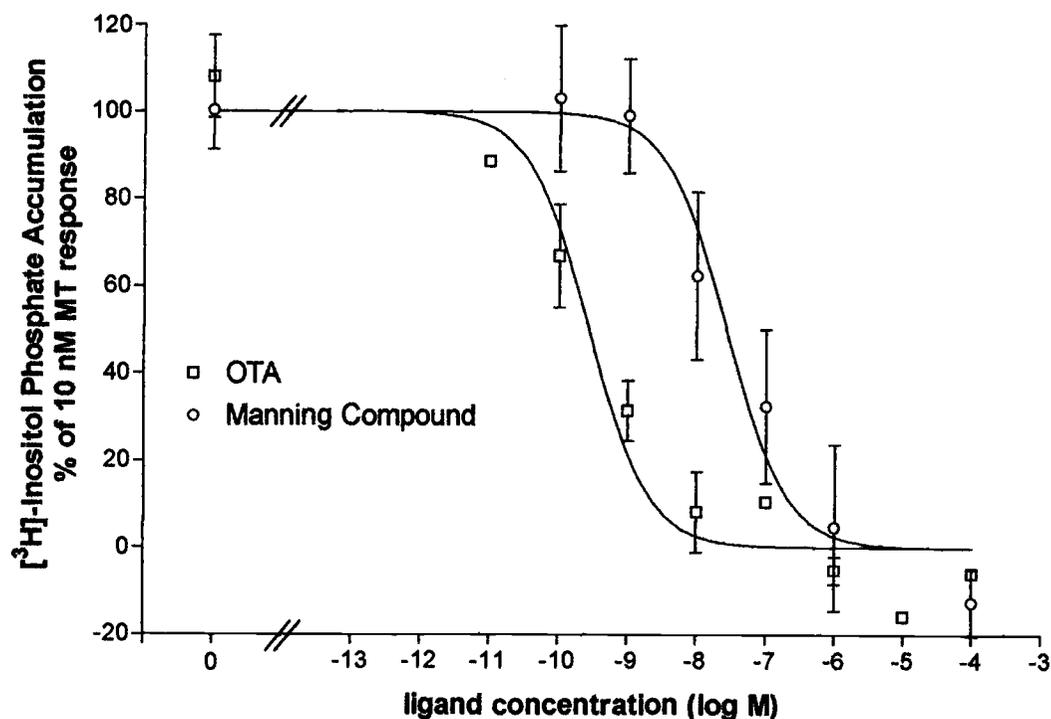


Fig 3.10 Functional effects of neurohypophysial hormone antagonists on mesotocin receptor-transfected COS7 cells stimulated with 10 nM mesotocin. Inositol phosphate accumulation was measured in [³H]-inositol-prelabeled COS-7 cells transiently transfected with the nMTR. Dose-response curves were generated by pretreating the cells for 10 min with varying concentrations of the antagonists, and then stimulating the cells for 20 min with 10 nM mesotocin. Levels of [³H]-inositol phosphate at 0% accumulation were no different than levels found in unstimulated nMTR transfected COS-7 cells (data not shown). Curves represent mean data +/- se obtained from three experiments assayed in triplicate

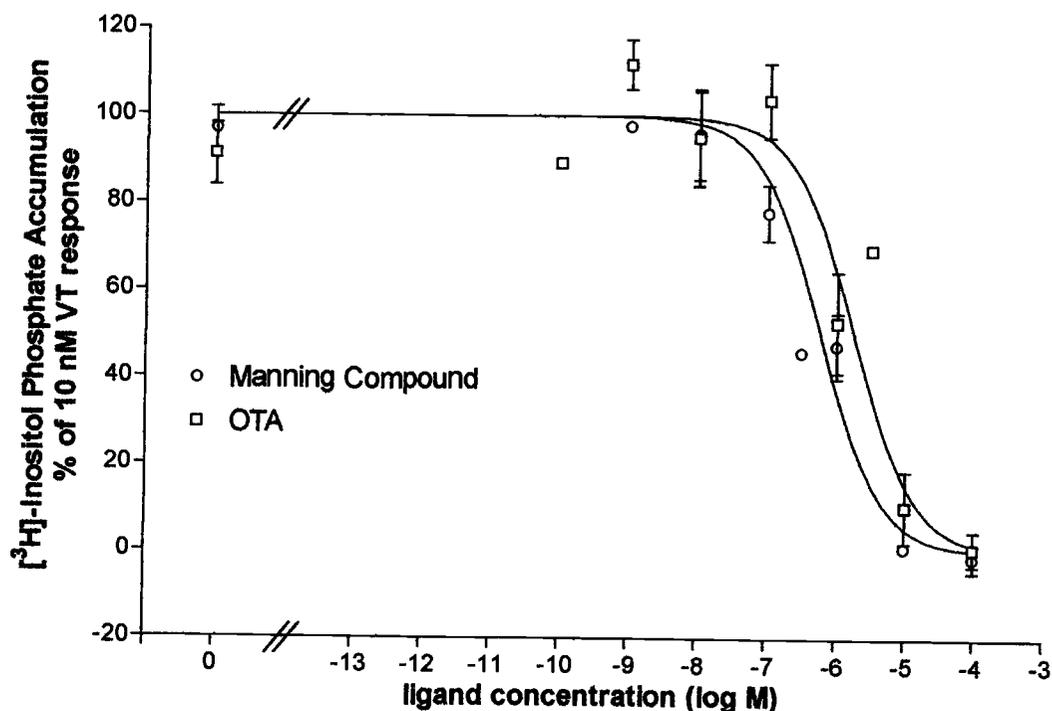


Fig 3.11 Functional effects of neurohypophysial hormone antagonists on vasotocin receptor-transfected COS7 cells stimulated with 10 nM vasotocin. Inositol phosphate accumulation was measured in [³H]-inositol-prelabeled COS7 cells transiently transfected with the nVTR. Dose-response curves were generated by pretreating the cells for 10 min with varying concentrations of the antagonists, and then stimulating the cells for 20 min with 10 nM vasotocin. Levels of [³H]-inositol phosphate at 0% accumulation were no different than levels found in unstimulated nMTR transfected COS-7 cells (data not shown). Curves represent mean data +/- se obtained from three experiments assayed in triplicate

Table 3.2 Relative potencies of the neurohypophysial hormone ligands for the newt mesotocin receptor and vasotocin receptor. [³H]-inositol phosphate accumulation was measured in nMTR and nVTR transfected COS-7 cells as described in Materials and Methods. Dose response curves were generated for the agonists by incubating [³H]-inositol prelabeled cells with varying concentration of agonist for 20 min (see Figs 3.10, 3.11). Antagonist dose response curves were generated by preincubating [³H]-inositol prelabeled cells for 10 min with antagonist followed by a 20 min incubation with 10 nM of either mesotocin or vasotocin for the nMTR and nVTR, respectively. EC₅₀ values were calculated as the concentration of ligand required to achieve half maximal effect as determined by non-linear regression analysis. Data shown are the mean +/- s.e. of EC₅₀ for two or three independent experiments.

	<u>EC₅₀: MTR</u>	<u>EC₅₀: VTR</u>
<u>agonists</u>		
Mesotocin	2.75 nM +/- 0.74	100 nM +/- 19.5
Oxytocin	7.68 nM +/- 2.6	115 nM +/- 35.8
Vasotocin	29.3 nM +/- 9.0	0.89 nM +/- 0.23
Vasopressin	32.7 nM +/- 13.1	8.15 nM +/- 2.5
Isotocin	181.6 nm +/- 89.3	11.2 μM +/- 5.9
<u>antagonists</u>		
Manning Compound	26.8 nm +/- 13.2	0.59 μM +/- 166
OTA	0.27 nm +/- 0.10	1.80 μM +/- 0.64

Discussion

The cDNAs encoding the OTR-like MT receptor (nVTR), and the V1a-like VT receptor (nVTR) were amplified by PCR from the brains of male *T. granulosa*. A phylogenetic analysis and description of the tissue distribution of these receptors was then included. These cDNA sequences were then separately transfected into COS-7 cells, allowing the pharmacological profile of each receptor to be defined. These are the first identified neurohypophysial peptide receptor sequences from urodele amphibians, and the pharmacological profile of each of these receptors provides the first such profile in this behaviorally relevant species.

Both the nMTR and nVTR sequences appear to be G-protein coupled receptors (GPCRs). The deduced amino acid sequences of the nMTR and nVTR each possesses the primary conserved elements required for its classification as a GPCR. The nMTR and nVTR sequences each has a consensus signature of a GPCR in the rhodopsin family (CDD:5814 (Marchler-Bauer et al. 2003)), the family of GPCRs that includes the mammalian neurohypophysial hormone receptors. Hydropathy analysis of the nMTR and nVTR sequences predicts that each of these proteins will form seven transmembrane domains, another feature common to all GPCRs. In addition, the hydropathy analysis of the *Taricha* receptor sequences also predicts that the N-terminus of each receptor will be extracellular; whereas, the C-terminus of each receptor will be intracellular, a configuration that also conforms to all other GPCRs.

Supporting the hydropathy analyses of the *Taricha* receptors, in an alignment of the identified neurohypophysial peptide receptor deduced amino acids sequences, the predicted transmembrane domains of the nMTR and nVTR line up with each other (Fig 3.3), as well as with the putative transmembrane domains of other neurohypophysial hormone receptors. Combined, these results support the hypothesis that we have amplified cDNA sequences encoding distinct GPCRs, and that these receptors are in the same family of GPCRs as the mammalian neurohypophysial peptide receptors.

The amplified nMTR and nVTR cDNA sequences both appear to represent actively expressed mRNAs from *Taricha* brain. Because each of these sequences was amplified from a brain cDNA template, and this cDNA was derived from total RNA, each sequence is predicted to represent an mRNA that is actively transcribed in the *Taricha* brain. The cDNA templates were all derived from DNase treated RNA, thus reducing the possibility of genomic DNA contamination. As further support that the nMTR and nVTR cDNAs are actively being transcribed, each sequence includes a polyadenylation signal, a conserved sequence required for proper addition of poly-A tails. A poly-A tail on the 3' end of each mRNA is thought to be needed for correct translation, stability, and transport of the mRNA (Beaudoing et al. 2000). While the nMTR cDNA sequence possesses an exact consensus polyadenylation signal (Beaudoing et al. 2000; Zarudnaya et al. 2003) the nVTR sequence contains a variation of

this sequence. Because the polyadenylation consensus sequence (AAT AAA (Zarudnaya et al. 2003)) differs only slightly from the nVTR sequence (ATT AAA), and the nVTR sequence was found to occur in 14% of 244 human poly(A) signals screened (Zarudnaya et al. 2003), the nVTR signal is most likely still functional, and would not greatly alter the expression efficiency of this receptor.

The nMTR and nVTR cDNAs each contain consensus sequences consistent with the hypothesis that each receptor sequences has the potential to be actively translated from mRNA into protein. The nMTR and nVTR cDNA sequences each includes a Kozak initiation signal (Kozak 1981; Kozak 1987), a feature thought to be required for optimal translation of proteins. As was the case with the polyadenylation signal, the nMTR cDNA exactly matched the consensus Kozak initiation sequence but the nVTR cDNA contains a variation of this sequence. Despite not matching the consensus sequence, because the nVTR initiation sequence occurs within the normal variation of the vertebrate translational initiation sequences (Kozak 1987), it is most likely translated effectively as well. The nVTR initiation sequence occurred in 16% of the 699 translational vertebrate initiation sequences screened (Kozak 1987).

In brief, both the nVTR and nMTR sequences possess key attributes supporting the conclusion that each of these receptors has the potential to be actively expressed and translated. Despite the nVTR sequence not containing either the Kozak initiation or the polyadenylation

consensus sequences, the nVTR sequences for each of these sites still falls into the normal range of variation and it is likely that the nVTR is still able to be actively translated in *T. granulosa*.

The correct classification of the nMTR cDNA as encoding the *Taricha* MTR ortholog is supported both by sequence identities and phylogenetic analysis. The identities of the nMTR sequence are highest with other described amphibian MTR sequences, indicating that the nMTR is properly classified as an MTR. In addition, the nMTR identities are also high with the other vertebrate MTR-homolog sequences (mammalian OTR and teleost ITR)(Acher 1980; Acher et al. 1995) and lower with the vertebrate VT-like receptor sequences. The phylogenetic analysis of the neurohypophysial hormone receptors (Fig 3.4) further supports this verdict, grouping the nMTR with the other identified amphibian MTRs, and includes a larger cluster that includes the mammalian OTRs and the teleost ITR.

The categorization of the nVTR cDNA sequence as encoding a V_{1a} -like VT receptor is also based on sequence identity and phylogenetic analysis. The deduced amino acid sequence encoded by the nVTR is highest identity with both the mammalian V_{1a} Rs and one of two previously identified amphibian VTR sequences. This previously identified amphibian VTR sequence, from the bull frog *Rana catesbeiana*, has also been categorized as a V_{1a} -like VTR (Acharjee et al. 2004). Sequence identity of the deduced nVTR sequence is lower with other vasopressin receptor

subtypes ($V_{1b}R$, V_2R), and with the MT-like receptors (see results). In support of the sequence identity based conclusion, phylogenetic analysis of the neurohypophysial hormone receptors also groups the nVTR with the V_{1a} -like receptors (Fig 3.4).

The phylogenetic analysis of the neurohypophysial hormone receptors (Fig 3.4) also indicates that there is a high probability that amphibians will be found to express all three subtypes of the mammalian VP receptors ($V_{1a}R$, $V_{1b}R$ and V_2R). Because the node at which the nVTR branches from the phylogenetic tree is to the right of the node separating the mammalian $V_{1a}R$ and $V_{1b}R$ s, this analysis predicts that the nVTR is not an ancestral form common to both VP receptor subtypes; therefore a V_{1b} -like VTR is also likely to be found in amphibians. There has already been an amphibian V_2 -like VTR (Kohno et al. 2003) and, supporting its correct categorization, this analysis groups it with the mammalian V_2R s. Although no more than one VTR subtype has been identified in any single species of amphibian, because none of the receptors identified appear to be a common ancestor to the mammalian VP receptor subtypes, it is likely that amphibians will also express multiple VTR subtypes in each species. To date, there have been two complete V_{1a} -like VTRs identified (*T. granulosa* and *R. catesbeiana* (Acharjee et al. 2004)) and one V_2 -like VTR (*H. japonica* (Kohno et al. 2003)), but no V_{1b} -like VTRs in amphibians. The chicken is the only non-mammal in which two forms of VTR have been identified. Chickens express both a V_{1b} -like VTR (VT2R, second chicken

VTR identified, original nomenclature based on order of identification in chickens (Cornett et al. 2003)) and a V_2 -like VTR (VT1R, first chicken VTR identified (Tan et al. 2000)). Only one form of oxytocin-like receptor has so far been identified in any single species, mammal, or otherwise and the current study provides no evidence that any additional OTRs will be found.

The distribution of nMTR and nVTR cDNA expression in the bodies of male *T. granulosa* was examined by rtPCR. The nMTR and nVTR sequence specific primers used in these experiments were designed to anneal to regions of low homology between these two receptor types. Supporting the specific amplification of each receptors sequence the nVTR primers were unable to produce a product using a full-length nVTR cDNA clone as template; the MTR primers were also unable to amplify a product using an nVTR cDNA clone as template. Although PCR products from every tissue were not cloned and sequenced, amplicons from the brain cDNA matched their respective receptor sequences. Although there is a possibility that the primers used in these experiments amplified other neurohypophysial receptor subtypes, such as a V_{1b} -like and a V_2 -like VTR, because the apparent distributions of the nMTR and nVTR cDNAs are reduced, as compared to other species, this possibility is unlikely.

The nMTR specific primers amplified cDNA products from the brain, heart, large intestine, lung, pituitary gland, and testes. The expression pattern of OTR in mammals is very similar to the distribution of the nMTR cDNAs; the OTR is found in all the same tissues with the addition of also

being expressed in the kidney (review (Gimpl and Fahrenholz 2001)). Most of the evidence demonstrating the presence of the OTR in the mammalian intestine is based on physiological responsiveness (Wu et al. 2003) rather than detection of cDNA or receptor proteins. Among other amphibians, areas that have been shown to express the MTR that differ from *Taricha* include the bladder (Akhundova et al. 1996; Kohno et al. 2003), kidney (Kohno et al. 2003; Acharjee et al. 2004), and skeletal muscle (Akhundova et al. 1996).

The rtPCR studies revealed that the nVTR cDNA is found in the brain, pituitary, liver, testes, sperm duct, and lung. The difference with the nMTR distribution is that the nVTR is found in the liver but not the large intestine. V_{1a} -like VTRs and V_{1a} Rs have been found in all the tissues in which the nVTR is found, although each individual species does not mimic the nVTR distribution exactly. The nVTR distribution pattern agrees with the expression pattern of the V_{1a} R in mammals in all but the kidney (review (Thibonnier et al. 1998)), tissues in which mammals express the V_{1a} R, but *Taricha* does not express the nVTR cDNA. In mammals all three subtypes of VP receptor have been found to be expressed in the kidney (review (Thibonnier et al. 1998)). The only other V_{1a} -like VTR tissue distribution that has been described in amphibians is in *Rana catesbeiana*, the bullfrog. In *R. catesbeiana* the primary difference between the V_{1a} -like VTR expression pattern and the nVTR cDNA distribution is that the bullfrog VTR is expressed in the kidney and

adrenals, but not in liver (Acharjee et al. 2004), reversing the distribution pattern of the nVTR in these tissues. A more similar expression pattern to nVTR was found in the teleost, *C. commersoni*, which had detectable expression in the liver, but not in the kidney (Mahlmann et al. 1994). Another teleost, *Platichthys flesus*, has an expression pattern of V_{1a}-like VTR that combines the nVTR and *R. catesbeiana* V_{1a}-like VTR distribution, with its V_{1a}-like VTR expression detected in both the liver and the kidney (Warne 2001). It is unusual that the nVTR is not found in the kidney, but there is still the possibility that *Taricha* expresses the nVTR at low levels in the kidney and our rtPCR did not detect it. An alternative explanation is that *Taricha* may also only express the unidentified new V₂-like VTR in the kidney, similar to the V₂-like VTR identified in *H. japonica* (Kohno et al. 2003).

To characterize the binding pharmacologies of the nMTR and nVTR, and to verify that each receptor could be functionally expressed, the coding regions of each of these cDNAs were ligated into mammalian expression vectors, and transiently expressed in a mammalian tissue culture cells. Although both the nMTR and nVTR appeared to specifically bind [³H]-OT, because the affinity of the nVTR for [³H]-OT was too low, we were unable to saturate the nVTR binding site with this ligand; therefore, binding studies were only conducted with the nMTR expressing cells. Inositol phosphate accumulation assays were conducted with cell transiently expressing each receptor type.

In binding studies with the nMTR, the ligand found to have highest affinity for the nMTR was MT, supporting our classification of this receptor as a MTR. The nMTR binding pharmacology was determined using [³H]-OT as a label. The [³H]-OT saturation binding curve for the nMTR (Fig 3.6, Table 3.1) was best fit with a model that predicted the presence of two binding states; a high affinity-low abundance state, and a lower affinity-high abundance state. Both the chicken MTR and the mammalian OTR have previously been found to have multiple binding states (Takahashi et al. 1996; Takahashi et al. 1997) (Okuda et al. 1992). One possible explanation for this phenomenon, in the transiently expressed nMTR, is that the multiple sites are the result of various states of nMTR association with G proteins. The affinity of GPCRs for their ligands is thought to vary depending on whether receptor-ligand binding is occurring in the presence or absence of a G-protein (De Lean et al. 1980). Because there is high potential for over-expression of receptors in transiently transfected cells, the endogenous G-proteins in the nMTR expressing COS-7 cells may have become limiting. The nMTR alone, unassociated with a G-protein, may present a lower affinity binding site for [³H]-OT than when G-proteins are available to associate with it. High affinity states of other transiently expressed neurohypophysial peptide receptors may have not been detected because the concentrations of ligand used in previous studies may have been too great to detect the higher affinity state. In the

nMTR studies only the two lowest doses indicate the possibility of a second higher affinity state.

Binding affinities of the nMTR for the dominant vertebrate neurohypophysial hormones were determined using competition bindings studies. Because of the higher abundance of the low-affinity [³H]-OT binding state, the K_d of the lower affinity state was used to calculate the K_i for each of the tested receptor agonists. The relative binding affinities of the neurohypophysial hormones for the nMTR (MT>OT≈VT>VP>IT; Fig 3.7, Table 3.1) matched the pharmacology of the other characterized MTR (*Bufo marinus*, (Akhundova et al. 1996)). While the rank-order of the ligand affinities were the same between the two MTRs, the absolute affinity of each ligand for the nMTR was approximately twofold lower than the same ligands binding affinity in the *B. marinus*. This difference may be partially accounted for if the *Bufo* MTR has two binding states that were averaged to create a single K_d .

Inositol phosphate accumulation was successfully induced in cells transiently expressing the nMTR, indicating the functional capacity of this identified receptor type. Based on these assays the rank order potencies of the ligands for the nMTR (MT>OT>VT=VP>IT; Fig 3.8, Table 3.2) agreed exactly with the rank-order potencies of these ligands for the bullfrog MTR (Acharjee et al. 2004) but did not match the rank-order potencies with the fish ITR (IT>VT~MT>OT>VP (Hausmann et al. 1995)).

Surprisingly, the rank-order IP accumulation potencies and the rank-order binding affinities of the neurohypophysial peptide ligands, for the nMTR, did not agree. This difference between ligand binding affinities and potencies with the same receptor may be explained if VT were to act as partial agonists of the nMTR. In mammals, VP has been found to be a partial agonist of the OTR (Chini et al. 1996); capable, at most, of stimulating OTR 60% of maximal OT stimulation (Chini et al. 1996). Similar to the current findings with the nMTR, the human OTR shows similar binding affinities with both OT ($K_i = 0.79$ nM) and VP ($K_i = 1.65$ nM), but the EC50 values of the IP accumulation assays differed dramatically ($EC_{50,OT} = 4.8$ nM; $EC_{50,VP} = 81$ nM; studies in COS-7 cells) (Chini et al. 1996). Because the VT-MTR results resemble the VP-OTR results, VT may also be acting as a partial agonist. The data acquired in these current experiments were unable to be used in testing this hypothesis because of experimental design.

IP accumulation was also successfully induced in nVTR expressing cells, indicating functionality of this receptor. Of the ligands tested, VT had the highest potency for activating the nVTR, supporting this receptor's identity as the *Taricha* VTR. The rank order potencies of the neurohypophysial hormones with the nVTR differed from the rank order potencies for the nMTR (Table 3.2), also confirming the nVTR's independent identity and pharmacology. Although the rank order potencies of the ligands for the nVTR ($VT > VP > MT \approx OT > IT$, Fig 9, Table 2)

did not agree with the previously characterized amphibian V1a-like VTR (VT>OT>VP≈MT (Acharjee et al. 2004)) or fish V1a-like VTR (VT>OT>MT>VP>IT (Mahlmann et al. 1994)), the order of ligand potencies was similar to the rank order potencies of the amphibian V2-like VTR (VT>VP>MT=OT>IT (Kohno et al. 2003)) and very similar to both the V1b-like VTR (VT>VP>MT>OT (Cornett et al. 2003)) and V2-like VTR (VT≈VP>OT≈MT>IT (Tan et al. 2000)) characterized in chickens. The differences in rank order potencies between the nVTR and the other V1a-like VTRs is most likely a species differences and can largely be accounted for by sequence variation (see analysis below). It is interesting to note that species differences appear to have a stronger influence on receptor-peptide ligand pharmacology than receptor subtype.

The neurohypophysial hormone receptors have been the subject of detailed mutational studies comparing amino acid sequence and ligand binding affinity or activation potential. Primarily these studies have been conducted on the mammalian receptors, although several studies have also examined how the amino acid sequences of the amphibian (Acharjee et al. 2004) and fish (Hausmann et al. 1996) VTRs relate to receptor binding and/or activation. Some of the conserved elements thought to be required in all neurohypophysial peptide receptors, and found in both the nMTR and nVTR include: an N-terminal Arg residue (nMTR⁴¹, nVTR⁵⁰), thought to be required for agonist association with the receptors (Hawtin et al. 2002); three amino acids distributed between transmembrane (TM)

domains two, three, and four (Gln-TM2 (nMTR⁹⁹, nVTR¹¹²), Lys-TM3 (nMTR¹²³, nVTR¹³⁶), Gln-TM4 (nMTR¹⁷⁸, nVTR¹⁹³)), hypothesized to be key for both agonist and antagonist binding (Cotte et al. 2000)); and a Lys residue located in TM3 (nMTR¹²³, nVTR¹³⁶), thought to be required for G_{q/11} binding in the human OTR (Yang et al. 2002). The nVTR matches 11/12 of the amino acids hypothesized to form the binding pocket for VT in the fish VTR (Hausmann et al. 1996), while the nMTR only conforms with 8/12 of these sites. The lower homology of the nMTR to the fish VTR at these sites may explain the differences in VTs potency with nMTR versus nVTR in the inositol phosphate accumulation assays; in these assays VT was a more potent ligand for nVTR than the nMTR (Table 3.2).

At the key amino acids in the neurohypophysial peptide receptor sequences, thought to be responsible for conferring low VP potency to the bullfrog V1a-like VTR (Acharjee et al. 2004), the nVTR and bullfrog VTR sequences differ. Because the inositol phosphate accumulation assays indicated that VP has higher potency for the nVTR than the bullfrog VTR, these sequence differences may account for the disparities in rank order potency of the various ligands between these two V1a-like receptors. At one of the two key *R. catesbeiana* VTR amino acids thought to reduce its affinity for VP, the nVTR sequence (Phe-TM6 (nVTR³⁰⁵)) is more similar to the mammalian V1aRs, which has a higher affinity for VP than VT. At the second *Bufo* VP affinity reducing site, the nVTR matches the bullfrog VTR

sequence (Tyr-TM7 (nVTR³³¹)), and not the mammalian V_{1a}R sequence (Acharjee et al. 2004).

Mutational studies have also defined two amino acids that are critical in causing VP to act as a partial agonist of the human OTR (Chini et al. 1996). While the nVTR amino acid sequence predicts full activity of VP, the nMTR matches the sequence of the human OTR at one of these two sites (Chini et al. 1996). This sequence similarity to the mammalian OTR lends support to the hypothesis that VT acts as a partial agonist of the nMTR.

Antagonist studies for the nVTR and nMTR were conducted using the V_{1a}R/OTR antagonist [β -Mercapto- β , β -cyclopentamethylenepropionyl¹,)-Me-Tyr², Arg⁸]-Vasopressin (Manning compound (Kruszynski et al. 1980)) and d(CH₂)₅[Tyr(Me)², Thr⁴, Tyr-NH₂]OVT (OTA) (Elands et al. 1988; Terrillon et al. 2002). The rank order potencies of these two compounds for each receptor matched previous findings, with Manning compound having higher potency against the nVTR than OTA, and the inverse being true for the nMTR. Unexpectedly, Manning compound had relatively high potency in inhibiting inositol phosphate production with the nMTR, whereas it had lower potency against the nVTR. This difference in potency may be caused by an absolute difference in affinity of Manning compound for each receptor, or it may be an artifact caused by the differing potencies of the ligands used to stimulate each of the antagonized receptors.

Mutational studies have also examined the effects of receptor sequence on antagonist binding and potency. The antagonist [125 I]-OTA has been found to require a conserved Leu-Val-Lys motif in mammalian OTR, thought to be the contact site for OTA on this receptor (Breton et al. 2001). Neither the nVTR nor the nMTR sequence exactly match this motif, but the nVTR and nMTR sequences at this site differ from each other; the nVTR amino acid sequence is Val¹³⁴-Val¹³⁵-Lys¹³⁶ while the nMTR is Ala¹²¹-Val¹²²-Lys¹²³. If this sequence is the primary contact point for OTA with the neurohypophysial hormone receptors, this sequence difference may account for difference in potencies of OTR for each receptor. In addition, the sequence differences and potencies differences between nMTR and nVTR for OTA would predict that OTA should also have higher affinity for the mouse V₂R (accession number CAC34589), and the mouse V_{1b}R (accession number NP_036054), based on their sequences at this site, which exactly match the nMTR sequence. Both the nMTR and nVTR have many, but not all of the amino acids thought to be required for optimal interaction with Manning compound (Cotte et al. 2000), each diverging from optimal interaction sequence in TM7.

Manning compound has frequently been used to specifically block the VTR system in behavioral studies of non-mammalian systems (Moore and Miller 1983; Semsar et al. 2001; Salek et al. 2002; Goodson et al. 2004). The findings in this paper require that these results be reinterpreted in light of the cross reactivity by Manning compound for the

VTR and MTR. In addition, the rank order potencies of the various neurohypophysial hormone ligands for each receptor do not always match the potencies on the homologous receptor in another species, even if those predictions are made based on the relative potencies of these same ligands within a member of the same class of animal. For each species, testing should be conducted to determine whether specific agonists and antagonists for one receptor will act in the manner they are predicted to perform, prior to basing interpretations on these assumptions.

The results of this study indicate that we have identified the cDNA sequences encoding the nVTR and nMTR, orthologs of the mammalian V_{1a}R and OTR, respectively. Phylogenetic analysis of the neurohypophysial hormone receptor amino acid sequences indicates that multiple VTR subtypes will likely be found in amphibians, and that the gene duplication that gave rise to the vasopressin receptor subtypes most likely occurred in a vertebrate class more ancestral than amphibia. The pharmacological studies reported in this study characterized the binding-affinities and activation-potencies of neurohypophysial hormones for the VTR and MTR in a species that is a comparative model for behavioral effects of neurohypophysial peptides, providing a useful reference for future behavioral studies. Our findings indicate that there is high potential for risk by inferring pharmacological profiles of these receptors from other species, even from within the same class. Most significant of these findings is the non-selectivity of Manning compound for the nVTR.

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Chapter 4

Summary and Future Directions

Results and Conclusions

The research reported in this thesis describes the identity and characterization of the neurohypophysial peptides and receptors of Urodele amphibians. In Chapter 2 the identity of the cDNA sequences encoding preproMT and preproVT from two species of salamander, *Taricha granulosa* and *Plethodon shermani* was reported. Sequence analyses supported our correct identification and classification of the cDNAs encoding the preproneurohypophysial peptides from each of these species. The *P. shermani* preproMT cDNA was found to encode the novel neurohypophysial peptide [Val⁴]-MT. Phylogenetic analysis of the preproNHP amino acid sequences, masking the putative gene conversion region from the analysis, grouped the preproOT and preproVP of mammals with other tetrapod preproMT and preproVT sequences, respectively. Gene conversion is the non-homologous crossing over of gene sequences. Analysis of the gene conversion region in each of the preproNHP sequences found that, whereas gene conversion appears to occur with relatively high frequency between the preproNHP genes in mammals and fish, it occurs much less frequently in non-mammalian tetrapods.

In Chapter 3, the cDNA sequences encoding VTR and MTR from *T. granulosa* were reported. Sequence analyses indicated correct identification and classification of each of these receptor's cDNA sequences. Tissue distribution of the nVTR and nMTR cDNAs in *Taricha*

matched previous findings in other species, with one exception; neither nVTR nor nMTR cDNAs were detected in the kidney. In Chapter 3, the binding affinities of a variety of ligands for the newt neurohypophysial peptide receptors were also reported. [³H]-OT binding to the nMTR was found to be specific, high-affinity, and best described with a two-site model. The peptide-ligand found to have highest affinity for the nMTR was MT. The functionality of the nMTR and nVTR were demonstrated using inositol phosphate accumulation assays; in addition, this assay allowed the calculation of the rank order potencies of several peptide agonists and antagonists for each receptor. VT was the ligand with highest potency for the nVTR. MT was the ligand with the highest potency for the nMTR. Because the affinities and potencies of the agonists and antagonists for the *Taricha* neurohypophysial peptide receptors did not match the findings with other orthologous amphibian neurohypophysial receptors, our results indicate that the pharmacologies of neurohypophysial peptide receptors cannot be inferred from comparative studies, even when the receptors from a species within the same class have been characterized. This warning is particularly relevant to comparative studies characterizing the behavioral effects of the neurohypophysial peptides.

Future Directions

The findings reported in Chapter 2 raise several intriguing questions that could be answered with future studies. First, whereas the

phylogenetic analysis of the deduced amino acid sequences for the preproNHP sequences was able to delineate the evolution of the neurohypophysial peptide genes in tetrapods, a full analysis of all vertebrate sequences was not reported. This was because phylogenetic analysis did not generate a statistically significant tree with the available vertebrate sequences.

Second, the reported identification of [Val⁴]-MT encoded in the *P. shermani* preproMT cDNA sequence allows questions to be raised regarding ligand/receptor co-evolution such as: do changes in the ligand sequence drive evolution of the receptor, or does the receptor sequence allow for changes to occur in the ligand sequence? By conducting a pharmacological characterization of the *Plethodon* MTR, specifically looking at this receptor's affinities for MT and [Val⁴]-MT, and comparing these findings with the affinities of the *Taricha* MTR for these same ligands, this question could be addressed. If the two receptors' pharmacologies are similar, then the *Plethodon* receptor sequence may have permitted the changes in the ligand sequence to occur; if the receptor pharmacologies do not match, then perhaps the receptor sequence is adapting to the novel ligand. The co-evolution of receptors and their ligands is a topic of frequent speculation (van Kesteren et al. 1996; van Kesteren and Geraerts 1998; Darlison and Richter 1999), and studying the *Plethodon* [Val⁴]-MT/MTR relationship may provide some insights into the mechanisms behind this type of evolution.

A third question generated from Chapter 2 involves the study of gene conversion. Why does gene conversion occur frequently in mammals and not non-mammalian tetrapods? To address this question, the genomic sequences of preproNHP need to be characterized in non-mammalian tetrapods. If the genomic architecture is different in non-mammalian tetrapods from mammals, this may provide the explanation. If the architecture of these genes is similar in all tetrapods, including mammals, other explanations would need to be explored.

The results, presented in Chapter 3, also suggest a number of intriguing topics for future study. First, the phylogeny of the neurohypophysial hormone receptors is not complete for vertebrates; this is due to a limited number of identified sequences. Because our analysis indicates that there is a high likelihood that at least three VTR subtypes will be found in each amphibian species, efforts should be exerted to identify these receptors. In addition, the identity of the neurohypophysial peptide receptors needs to be determined for less derived vertebrates, specifically the elasmobranchs and cyclostomes. By identifying receptor sequences in these less derived vertebrates, the evolution of these receptors within all vertebrates could be characterized.

Second, the multiple binding states found in the transiently expressed nMTR need to be further characterized. Because of the possibility that G-proteins are a limiting factor, this receptor should be co-transfected with $G_{q/11}$ proteins. If this co-transfection increases the

abundance of the high affinity state of the nMTR, this would support the G-protein limitation hypothesis. In addition, the higher affinity state would most likely reflect the biologically relevant condition of the nMTR *in vivo*, providing a more relevant profile for behavioral and physiological studies of MT and VT responses.

A third direction that would be interesting to investigate based on the results of Chapter 3 is the possibility of steroid binding to the nMTR. The OTR in rats and humans has been reported to bind progesterone (Grazzini et al. 1998), and although this finding has been disputed (Burger et al. 1999), it still remains a viable possibility. If progesterone binding could be reconstituted using the newt MTR, it would demonstrate the conserved evolution of the neurohypophysial peptide receptors as a non-genomic target for steroids. Because it appears unlikely that OTR directly binds progesterone by itself, other proteins may need to be involved in order to detect this interaction. A steroid binding globulin may need to be present for progesterone to be functionally presented to the OTR

Finally, because no behavioral actions have been attributed to MT, despite this peptide (Smeets and Gonzalez 2001) and its receptor (Tripp and Moore 1988) being detected in the brain, MTR specific agonists and antagonist need to be identified. Some of the behavioral actions currently attributed to the VTR may in fact be elicited by the activation of the MTR. The identification of MTR and VTR specific agonists and antagonist would allow the specific receptor type responsible for a given response to be

recognized. The pharmacological profiles of these two receptors, as defined in Chapter 3, are too similar to allow conclusive identification of the specific receptor responsible for eliciting a response based on their currently profiles.

If all these future studies were pursued, they would greatly expand our understanding of the evolution of the neurohypophysial peptides and receptors, increase our understanding of the co-evolution of receptors and ligands, characterize potentially novel membrane steroid binding sites, and allow the determination of specific behavioral responses elicited by both MT and VT.

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