CHAPTER 1

Evolutionary Aspects of Physiological Function and Molecular Diversity of the Oxytocin/Vasopressin Signaling System

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1.1 Evolution of peptidergic signaling

One of the major eukaryotic signal transduction machineries is composed of G protein-coupled receptors (GPCRs) and their associated signaling molecules (see Chapter 10). GPCRs share a seven α-helical transmembrane architecture with an extracellular N-terminus and an intracellular C-terminus, and are able to sense a diverse set of ligand molecules, including proteins, peptides, amino acids, nucleosides, nucleotides, ions, and photons. GPCRs are involved in many processes such as cell growth, migration, density sensing or neurotransmission (Gruber *et al.*, 2010).

Proteins comprising a seven-transmembrane topology have been identified as far back in the evolutionary timeline as prokaryotes; these are, for instance, light-sensitive proteo-, bacterio- and halorhodopsin proteins that are involved in non-photosynthetic energy harvesting in Archaea and bacteria. Structurally similar sensory rhodopsin proteins can also be found in eukaryotes, but to determine the phylogenetic relationship between prokaryotic and eukaryotic GPCRs is rather difficult, since (i) the prokaryotic and eukaryotic proteins have evolved independently for approximately 1.2 billion years, which resulted in low sequence conservation, and (ii) the occurrence of lateral gene transfer between prokaryotes and eukaryotes has been reported for certain microbial rhodopsins, which further complicates the analysis of phylogenic relations (Strotmann *et al.*, 2011).

Recent studies on the evolution of GPCR signaling systems in eukaryotes – covering not only the receptors and their cognate G proteins, but also upstream and downstream regulators of the system – concluded that the last eukaryotic common ancestor must have already expressed a complex repertoire of GPCRs. Furthermore, it has been suggested that different parts of the GPCR signaling system evolved independently, and that some of them have been lost or became simplified without disrupting overall signaling functionality. For instance, most organisms contain most of the known GPCR signaling components, but certain species have retained only a subset of those, whereas others are completely reduced. These findings suggest that the GPCR signaling system is modular and that during evolution, drastic rearrangements can occur without complete loss of functionality. Analyses of protein domain architectures additionally suggest that domain shuffling is a major mechanism of signaling system evolution (de Mendoza *et al.*, 2014).

Molecular Neuroendocrinology: From Genome to Physiology, First Edition. Edited by David Murphy and Harold Gainer.

Companion website: www.wiley.com/go/murphy/neuroendocrinology

Gene families and protein domain architectures of cytoplasmic transduction elements (for example, G proteins, arrestins, regulators of G protein signaling, guanine nucleotide exchange factors) are largely conserved between unicellular holozoans and metazoans. In contrast, receptors underwent a dramatic expansion in metazoans compared to their closest unicellular relatives. For instance, the human and mouse genomes code for more than 800 and 1300 GPCRs, respectively, which equals more than 1% of the total predicted genes, while yeast has as little as 10 GPCR genes, less than 0.2% of the total predicted genes (de Mendoza *et al.*, 2014; Fredriksson and Schioth, 2005). This could be due to adaptation of GPCR signaling systems for new functions, such as cell–cell communication, developmental control, and complex environmental sensing, from light to odor and taste (de Mendoza *et al.*, 2014). However, most GPCRs do not play a primary vital role in these organisms. Only 8% of GPCR genes in mice responded to gene disruption by embryonic or perinatal lethality; about 41% exhibited an obvious phenotype and more than 50% of knockout mice of individual GPCRs display no obvious phenotypical change (Schoneberg *et al.*, 2004). However, in humans, mutations in genes encoding GPCRs and G proteins result in pathological conditions, for instance severe vision impairment and blindness, and many other retinal, endocrine, metabolic or developmental disorders (Schoneberg *et al.*, 2004).

1.1.1 Evolution and diversity of peptide G protein-coupled receptors and their endogenous ligands

Of particular interest for this chapter are peptidergic systems, which are generally defined as a functional complex consisting of a cell that synthesizes and releases a peptide mediator, a cell that responds to that peptide by a certain physiological change, and the process of transferring the peptide from the site of synthesis to the site of action. In particular, we use the term **peptidergic signaling** for pathways that are mediated by peptides, their endogenous receptors and associated signaling molecules, which commonly belong to the family of G protein-coupled receptors. Many signaling peptides are released by the central nervous system and these neuropeptides are closely associated with the emergence of the first nervous system. Neuropeptides and the nervous system probably evolved in the common ancestor of cnidarians since sponges (the evolutionary older animal group) do not exhibit any physiological or anatomical signs of a nervous system. Neuropeptides are expressed in brains and are involved in the complex regulation of homeostatic processes and neuronal activity in metazoans. They may act as neurotransmitters, if released within synapses, or as neurohormones to activate receptors distal from the site of release. **Neuropeptides** are short (<50 amino acids) secreted polypeptides derived from larger precursor proteins which share defining features at the level of their primary sequence, which is useful for evolutionary studies, because short peptides sometimes lack sequence similarities (Mirabeau and Joly, 2013).

Recently, the evolutionary history of **bilaterian** neuropeptides and receptors was reconstructed to clarify the relationships between **protostomian** and **deuterostomian** peptidergic systems (Mirabeau and Joly, 2013). The results clearly indicated that the majority of peptidergic systems were present in the last common ancestor of bilaterians (the **urbilaterian**) (Table 1.1). This further supports the theory that the urbilaterian was an animal with a sophisticated physiology and a complex nervous system, capable of integrating sensory information. Another conclusion of this study was the existence of co-evolution between the majority of receptors and their ligands, although previously it has been suggested that, during evolution, novel ligands may outcompete existing ones for a given receptor (Mirabeau and Joly, 2013).

Table 1.1 Inferred evolutionary relationships between the different ancestral bilaterian peptidergic systems.

	Deuterostome					Protostome				
Family	Peptide name	V	T	B	A	L	D	I	N	Peptide name
1.	Vasopressin									Vasopressin
2.	Tachykinin									Tachykinin
3.	GnRH									AKH/corazonin
4.	Cholecystokinin									Sulfakinin
5.	Neuromedin U									Capability/pyrokinin
6.	Neuropeptide Y									Neuropeptide F
7.	Corticoliberin									Diuretic hormone 44
8.	Calcitonin									Diuretic hormone 31
9.	Orexin									Allatotropin
10.	Neuropeptide S									CCAP
11.	NPFF									SIFamide
12.	Endothelin/GRP									CCHamide
13.	Galanin									Allatostatin A
14.	Thyroliberin									Uncharacterized
15.	Kiss1									Uncharacterized
16.	QRFP									Uncharacterized
17.	PTH/blucagon/ PACAP									Uncharacterized
18.	Uncharacterized									Leucokinin
19.	Uncharacterized									Ecdysis-triggering hormone
20.	Uncharacterized									RYamide/luqin
21.	GPR139/142									Allatostatin B/proctolin
22.	Uncharacterized									PDF/cerebellin
23.	GPR19									Uncharacterized
24.	GPR83									Uncharacterized
25.	GPR150									Uncharacterized
26.	Uncharacterized									Uncharacterized
27.	Uncharacterized									Uncharacterized
28.	Uncharacterized									Uncharacterized
29.	Uncharacterized									Uncharacterized

Mirabeau and Joly suggested that 29 peptidergic systems (here shown as different families from 1 to 29) were present in the last common ancestor of bilaterians (the urbilaterian). A dark gray square denotes the presence of both peptides and receptors from a given peptidergic system, a light gray square denotes the presence of receptor or peptide and the white square shows that both peptides and receptors are absent for a given peptidergic system in the phylogenetic group. Subphylum Vertebrata (**V**) is composed of *Homo sapiens* and *Takifugu rubripes*, phylum Tunicata (**T**) of *Ciona intestinalis* and *Ciona savignyi*, superphylum Ambulacraria (**A**) of *Strongylocentrotus purpuratus* and *Saccoglossus kowalevskii*, Lophotrochozoa (**L**) of *Capitella teleta* and *Lottia gigantea*, class Insecta (**I**) of *Drosophila melanogaster*, *Tribolium castaneum*, and *Acyrthosiphon pisum*, phylum Nematoda (**N**) of *Caenorhabditis elegans* and *Pristionchus pacificus*, Branchiostoma (**B**) of *Branchiostoma floridae*, and Daphnia (**D**) of *Daphnia pulex* (Mirabeau and Joly, 2013).

1.1.2 Origin of the oxytocin (OXT)/arginine vasopressin (AVP) signaling system

One of the best known peptidergic systems is OXT/AVP signaling. Homologs of OXT/AVP receptors and ligands have been identified in diverse organisms such as hydra, worms, insects, and vertebrates. Across evolutionary lineages, the OXT/AVP neuropeptide signaling system shows conserved functions in water homeostasis, reproductive behavior, learning, and memory (see section 1.4 later in this chapter) (Gruber, 2014).

Phylogenetic grouping indicated that invertebrate crustacean cardioactive peptide (CCAP) receptor, neuropeptide S (NPS) receptor, and AVP-like receptors form a monophyletic family which is phylogenetically the closest to the gonadotropin-releasing hormone (GnRH) receptor superfamily (Figure 1.1). Although CCAP, NPS, and AVP-like peptides are not similar in sequences, analyses of precursors encoding those peptides showed the presence of neurophysin domains in some of the genes, which consolidates the common origin of the peptides. Furthermore, AVP and neuropeptide S are found in neighboring tandem position in the amphioxus (*Branchiostoma floridae*) genome, indicating that they are the product of ancient duplication. Accordingly, in an ancestor of bilaterians, the duplication of a single gene (AVP/NPS/CCAP-like) must have occurred, which gave rise to AVP-like and NPS/CCAP-like genes (Mirabeau and Joly, 2013; Pitti and Manoj, 2012).

The origin of the OXT-like system can be dated back to a common ancestor of bilateral animals – more than 600 million years ago. Invertebrates, with few exceptions, have only one OXT/AVP peptide homolog, whereas vertebrates have two. Although sharing high sequence similarities, historically the peptides were given different names in different species (for instance, lysipressin, phenypressin, vasotocin, mesotocin, isotocin, annepressin, conopressin, and inotocin – all of them belong to the family of OXT/AVP peptides) (Table 1.2). The ancestral vasotocin gene was duplicated before vertebrate divergence approximately 450 million years ago, forming OXT-like peptides. Within these lineages, the genes of the peptides are found in close proximity on the same chromosome. In all taxa, the short nine-amino acid peptide first is translated as longer precursor proteins, which contain a signal peptide, followed by the neuropeptide domain, a dibasic amino acid cleavage site, and a neurophysin domain; additionally, in some genes a copeptin sequence completes the precursor. Precursor genes are also characterized by similar intron sites and lengths (Acher *et al.*, 1995; Mirabeau and Joly, 2013).

Besides the peptide precursor proteins, there are four receptors in the vertebrate OXT/AVP receptor family: one OXT receptor (OTR) and three AVP receptors ($V_{1a}R$, $V_{1b}R$, and V_2R), whilst invertebrates commonly have only one receptor: VPR (invertebrate AVP-like receptor). OXT and AVP V_1 receptors are more similar to each other compared to the AVP V_2 receptor, which indicates that V_2R arose before the V_1R/OTR split. At the base of vertebrate lineage, two rounds of whole genome duplication (*2R*) have been described. It has been proposed that local duplication of an ancient vertebrate AVP receptor before *2R* gave rise to the V_2R and V_1R/OTR genes. After *2R,* these genes were quadrupled, although there have been several losses in different vertebrate classes. Interestingly, the spotted gar (*Lepisosteus oculatus*) has three V_2 receptors (V_2A, V_2B, and V_2C), indicating that three subtypes of the V_2 receptor arose early in vertebrate evolution, but since then they have been lost in some vertebrates. Eleost-specific tetraploidizations (*3R*) gave teleost fish additional gene family members of OXT/AVP receptors (Lagman *et al.*, 2013).

In mammals, OXT and AVP differ from each other at only two amino acid positions. Contrary to many other signaling systems, selectivity of the different receptors is not achieved via the ligands (because they are very similar and can cross-react), but mainly via interplay of factors including receptor up- or downregulation, release of specific ligand-degrading enzymes, local ligand production, and receptor clustering (Goodson, 2008; Gruber and Muttenthaler, 2012). However, ligand-receptor selectivity also has important implications for drug design and development of selective compounds for pharmacological applications.

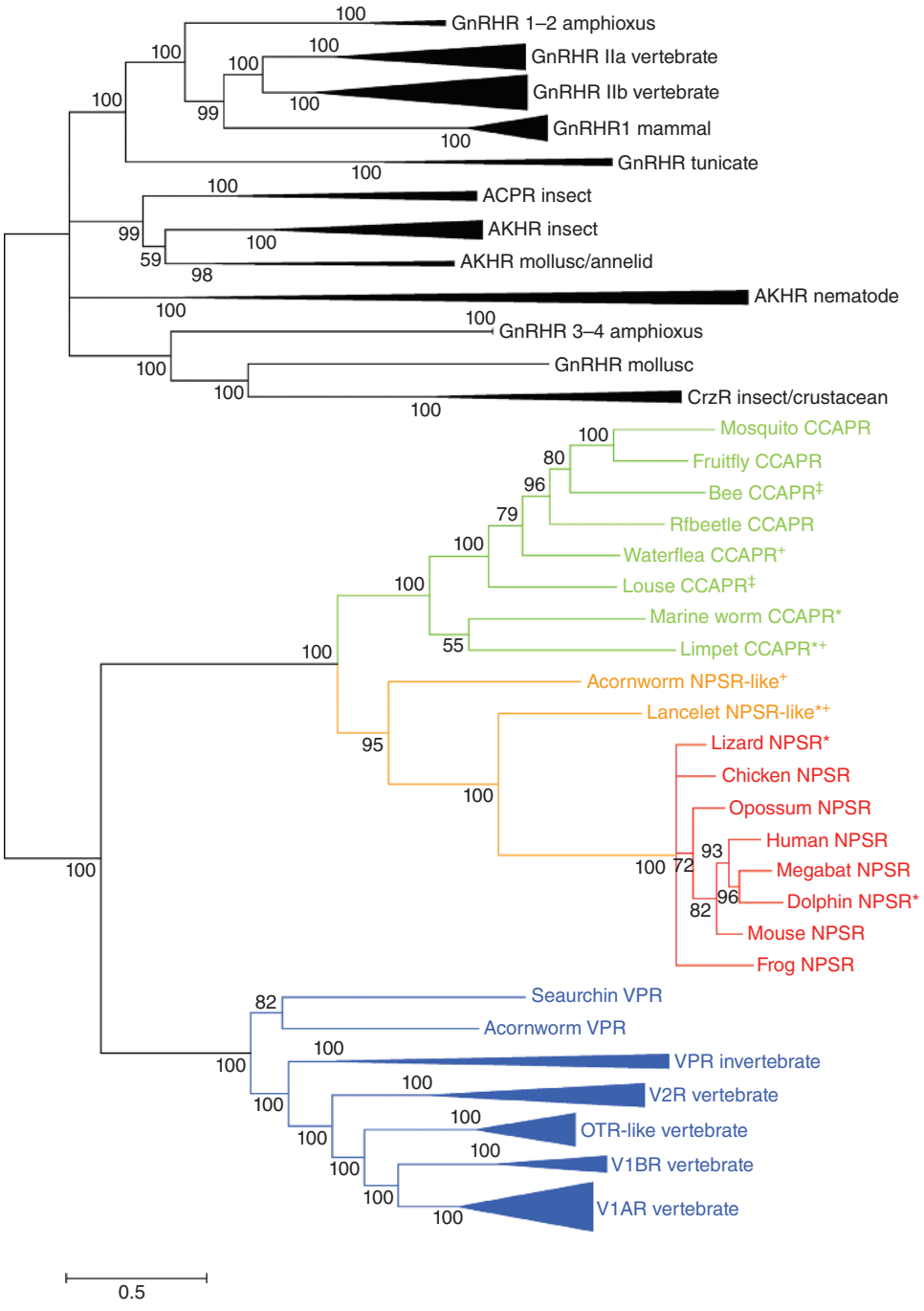

Figure 1.1 Phylogenetic relationship of the neuropeptide S, cardioacceleratory peptide, gonadotropin-release hormone, and OXT-/AVP-like receptors from vertebrates and invertebrates. Phylogenetic relationship is plotted as a Bayesian tree of neuropeptide S (NPSR; *red*), cardioacceleratory peptide (CCAPR; *green*), gonadotropin-release hormone (GnRHR; *black*), and OXT-/AVP-like (VPR, V_2R, $V_{1A}R$, $V_{1B}R$, OTR; *blue*) receptors from vertebrates and invertebrates. **Bayesian posterior probabilities** are marked near branches as a percentage, and are used as confidence values of tree branches. Nodes were compressed to represent the animal lineages. The scale bar represents the number of estimated changes per site for a unit of branch length. The sequence names marked with asterisk (*) and plus (+) symbols represent manually corrected sequences at the N-terminus and C-terminus, respectively. Sequence names marked with the double-cross (‡) symbol in this figure represent fragmented sequences. Adapted and modified from Pitti and Manoj (2012).

Table 1.2 OXT/AVP-like peptide sequences across the animal kingdom.

Animal groups		Peptide	Sequence
Vertebrates	Humans, mammals	Oxytocin	**C**YIQN**C**PLG*
		[Arg8]-Vasopressin	**C**YFQN**C**PRG*
	New World monkeys	[Pro8]-Oxytocin	**C**YIQN**C**PPG*
	Pigs, marsupials	[Lys8]-Vasopressin (Lysipressin)	**C**YFQN**C**PKG*
	Marsupials	[Phe2]-Vasopressin (Phenypressin)	**C**FFQN**C**PRG*
	Birds, reptiles, amphibians, fish	Vasotocin	**C**YIQN**C**PRG*
	Birds, reptiles, amphibians	Mesotocin	**C**YIQN**C**PIG*
	Fish	Isotocin	**C**YISN**C**PLG*
Annelids	Earthworms	Annetocin/annepressin	**C**FVRN**C**PTG*
	Leeches	Lys-conopressin G	**C**FIRN**C**PKG*
Molluscs	Cephalopods	Cephalotocin	**C**YFRN**C**PIG*
		Octopressin	**C**FWTS**C**PIG*
	Gastropods	Lys-conopressin G	**C**FIRN**C**PKG*
Nematodes	*Caenorhabditis elegans*	Nematocin	**C**FLNS**C**PYRRY*
Arthropods	Insects	Inotocin	**C**LITN**C**PRG*
		Inotocin	**C**LIVN**C**PRG*
	Arachnids	Arachnotocin†	**C**FITN**C**PPG*
		Arachnotocin†	**C**FITN**C**PIG*
	Myriapods	Myriatocin†	**C**YITN**C**PPG*
	Crustacea/brachiopods	Vasotocin-like	**C**FITN**C**PPG*

*indicates C-terminal amidation.

†Sequences were discovered by genome mining according to Gruber and Muttenthaler (2012). Table modified and adapted from Gruber (2014).

It is therefore intriguing to analyze the diversity of the OXT/AVP system by discovery of novel peptide sequences and to study evolutionary aspects of the molecular interaction of different ligand-receptor pairs.

1.2 The discovery of neuropeptide signaling components in the era of genomics

The increasing number of genome projects is generating an unprecedented amount of sequence information, which is, and will be, available in public databases for the identification of genes, such as the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). 'Genome mining' is a term that has been used to describe the exploitation of genomic information for the discovery of new processes, targets, and products. This technique is particularly valuable in the genomic era, since the number of available genomes is steadily increasing as whole-genome sequencing is becoming affordable and achievable. Furthermore, there are an even greater number of transcriptome projects, which offer the possibility to identify and annotate not only genes but also messenger RNA (mRNA) transcripts. According to the central dogma of molecular biology, when synthesizing a protein, the genetic information of DNA is transcribed into mRNA which is then translated into a protein. Therefore, 'transcriptome mining' provides information about the transcribed

and translated genetic pool of a cell, tissue, organ or, indeed, the whole organism at any one time. In addition, transcriptome sequencing is quicker and cheaper and the genetic information is easier to annotate.

1.2.1 Brief overview of finished and ongoing genome projects

The vast amount of publicly available genomic or RNA sequence information offers the opportunity to search for novel peptidergic signaling components, due to the steadily increasing number of ongoing genome sequencing projects as well as advances in bioinformatics tools. For example, one can mine these sequences for identification of neuropeptide precursor and receptor genes or transcripts. The number of total genome sequencing projects currently (August 2015) stands at 69,949, of which 7210 are listed as completed (www.genomesonline.org). Most of these sequences will be deposited in public databases mainly as non-annotated data. Hence it is the responsibility of the scientific community to make sense of this information. The first step is to identify and annotate genes or transcripts, and use them for comparative *in silico* studies or for functional genomics with the aim of elucidating their biological and physiological purpose.

1.2.2 Data mining for the non-expert computational biologist

Genetic sequence data mining, in particular large-scale genome analysis or systems biology approaches, can be difficult and laborious, which requires expertise, powerful computing hardware and specialist analysis software that are usually only accessible to the expert computational biologist. However, many tools to mine genetic sequence data sets are readily available via online databases and are accessible for the non-expert biologist. The following section provides a simple description of this workflow, which is summarized in Figure 1.2. We would like to place particular focus on the identification of novel invertebrate neuropeptide precursor and receptor sequences (see section 2.3).

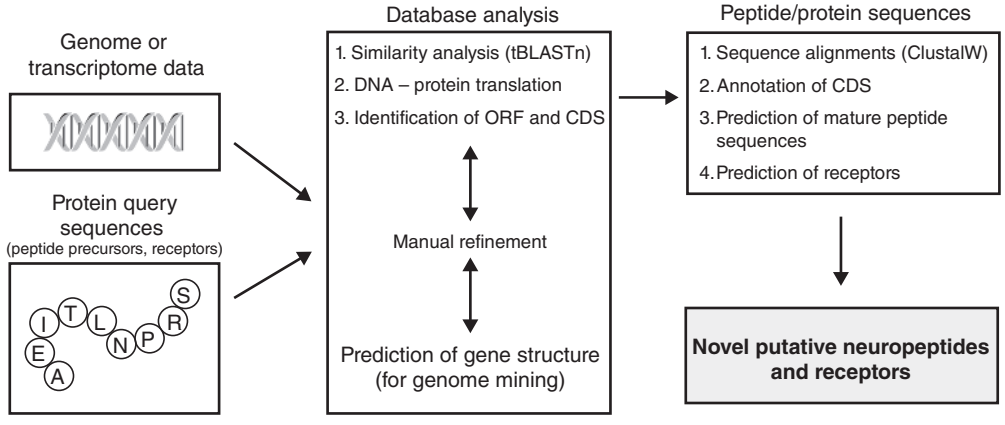

Figure 1.2 Flowchart of genome mining for the discovery of nonapeptides and their receptors. Whole genome shotgun or transcriptome data, and amino acid sequences of precursor proteins from nonapeptides or receptors of interest (for example, OXT-like neuropeptides and their receptors) were used for database analysis. This included similarity analysis of target DNA sequence and query protein amino acid sequence using tBLASTn, DNA to protein translation of discovered hit sequences and identification of open reading frames and coding sequence. The obtained automated results were refined and confirmed manually and used for gene structure prediction using the GeneWise algorithm. Database analysis yielded precursor protein and peptide sequences that were further annotated and analyzed by sequence alignments and similarity comparison to identify signal sequences, propeptides, and mature peptide chains. Using this genome-mining methodology, it is possible to predict the amino acid sequences of bioactive peptides in ants. Adapted and modified from Gruber and Muttenthaler (2012).

1.2.2.1 tBLASTn similarity search

Prior to the **BLAST** search, a number of suitable query sequences (peptide or protein sequences) need to be selected. It is advisable to choose query sequences that are related to the gene/transcript of interest of the target species, i.e. phylogenetic neighboring species, as well as the most homologous protein sequences. Sometimes including more distant and less related query sequences, i.e. sequences of another animal class, can provide additional information or can be used as a control. These query sequences will be used for initial tBLASTn analysis via GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&BLAST_PROGRAMS=tblastn&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) using the search set of interest, for example, whole genome shotgun contigs or transcriptome shotgun assemblies. The most similar hits (provided as genomic sequences) will be extracted and translated into their respective amino acid sequence (six-frame translation) using appropriate web tools (http://web.expasy.org/translate/). Open reading frames (ORFs) of the respective genes and coding sequences of transcripts can be identified via manual sequence assignments. For larger data sets, there are automated computing tools available, e.g. ORFPredictor, Dragon TIS, and MetWAMer. The annotated and identified amino acid sequences should be confirmed by another tBLASTn analysis against the search set and species of interest. These peptide/protein sequences will then be used for further identification and annotation.

1.2.2.2 Identification and annotation of novel genes

In case of genomic information, annotation of tBLASTn hits can be performed using the GeneWise2 algorithm (www.ebi.ac.uk/Tools/psa/genewise/) or gene structure prediction tools (for example, FGENESH available via Softberry Inc. http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind). In particular, for non-model organisms, these prediction tools may not be accurate enough and manual refinement will be necessary. Often gene structure analysis (intron/exon determination) fails completely, in which case a combined genome and transcriptome mining approach (if available for the same species) may be useful. If successful, the automated gene structure analysis will yield full or partial protein sequences, their open reading frames, and the corresponding DNA sequences. The predicted protein-coding sequences as well as the intron/exon structure from genomic data can be used for further annotation and similarity alignments, as well as functional genomics or physiological *in vitro* and *in vivo* studies (Gruber and Muttenthaler, 2012).

1.2.3 Case study: the discovery of novel inotocin peptides in ant genomes

Following in the footsteps of many successful genome-sequencing projects in animals, plants, and microbes, the genomes of seven ant species have recently been reported. These include the invasive Argentine ant *Linepithema humile*, the red harvester ant *Pogonomyrmex barbatus*, the fire ant *Solenopsis invicta*, the carpenter ant *Camponotus floridanus*, a basal ant *Harpegnathos saltator*, the leaf-cutter ant *Atta cephalotes*, and the farming ant *Acromyrmex echinatior*.

The aim of this study was to analyze three representative ant genomes for the discovery of peptide-encoding genes and their sequences using the above described workflow. We were able to identify numerous putative peptide sequences corresponding to partial or full-length precursors of ant neuropeptides. This included the first annotation of mature sequences of inotocin nonapeptides in social insects, which are OXT/AVP-related neuropeptides (Figure 1.3).

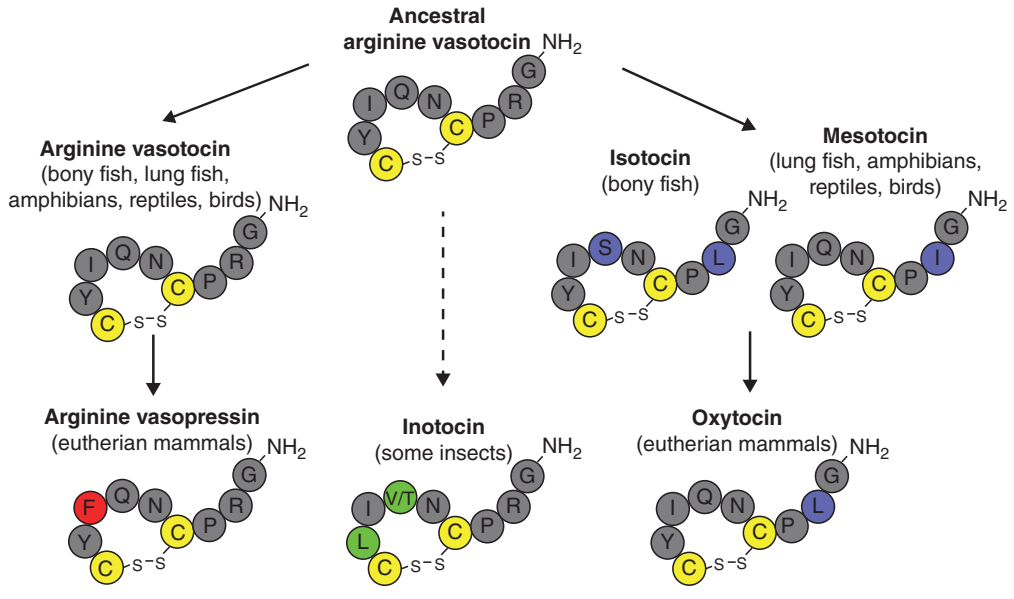

Figure 1.3 Discovery and origin of insect inotocin nonapeptides. (A) Arginine vasotocin is the presumed ancestral peptide of OXT and AVP. Mammalian OXT evolved via intermediate forms of isotocin (bony fish) and mesotocin (lung fish, amphibians, reptiles, and birds) (simplified illustration for clarity). It is yet to be determined whether invertebrate OXT/AVP-related peptides in insects or snails (e.g. conopressins, not shown) have also evolved from ancestral vasotocin (indicated as dashed line). The peptide sequences are shown in one-letter amino acid code. The highly conserved cysteine residues and disulfide bonds are colored in yellow. Residues in the ancestral arginine vasotocin and those that are identical to vasotocin are in dark gray. Residues that have changed during AVP evolution are colored in red, residues that have changed during OXT evolution are colored in purple and residues that are unique to insect inotocins are colored in green. Adapted and modified from Gruber and Muttenthaler (2012).

This study provided proof of concept for the use of a simple **genome mining** workflow to analyze endogenous neuropeptides from insects (Gruber and Muttenthaler, 2012). The newly identified ant inotocin peptide sequences displayed high similarity to vasotocin and OXT/AVP-like sequences from other species. In all analyzed ant genomes, the short mature nonapeptides are generated as a longer precursor protein which share molecular features with precursors of other insect inotocin proteins, and even human OXT and AVP precursors (see section 1.1). Besides the sequence of the mature nonapeptides, all precursors contain conserved protein domains for cellular secretion, enzymatic processing and physiological transport, and are characterized by similar position and lengths of introns (Gruber and Muttenthaler, 2012). Besides the mature nonapeptides, we also reported putative receptor sequences in ant genomes that share high similarity to other insects, like the beetle *Tribolium castaneum* (Gruber and Muttenthaler, 2012; Stafflinger *et al.*, 2008), indicating that possibly not only the genetic structure but also the function of these receptors and their nonapeptide ligands may be conserved across species. Recently, this work has been extended by characterizing OXT- and AVP-like precursors and receptor sequences from the genomes of several arthropod species, such as the red spider mite *Tetranychus urticae*, the predatory mite *Metaseiulus occidentalis*, and the centipede *Strigamia maritima* (Gruber, 2014).

These results offer the possibility to interpret the phylogenetic relationship and evolution of insect peptide hormone systems but, most importantly, the predicted mature peptide sequences could provide novel drug leads or tools to study similar and conserved receptor systems in humans.

1.3 Evolutionary aspects of OXT/AVP diversity

1.3.1 Diversity of OXT-like peptides

As introduced in section 1.1, the origin of the OXT and AVP signaling system is considered to date back at least 600 million years. OXT-like peptides are today present in many different species, including invertebrate animals, such as mollusks, annelids, nematodes and insects, non-mammalian vertebrates, such as amphibians, reptiles and birds, fish, mammals, and humans (Figure 1.4; see Table 1.2).

Invertebrates are considered to possess only one single OXT-/AVP-like nonapeptide form. However, the published information about OXT/AVP signaling in invertebrates as compared to vertebrates or mammals is vast and requires more detailed analysis. For example, it is not clear whether nematocin (= OXT homolog of *C. elegans*) is expressed as a single peptide or whether there exists a processing variant (Beets *et al.*, 2012). The earliest vertebrates probably possessed only a single nonapeptide, namely arginine vasotocin, although the vasotocin gene duplicated at about the same time that jaws evolved, so all jawed vertebrates now exhibit two nonapeptide forms in the brain – an OXT-like form and either vasotocin or AVP. The most common OXT-like forms are isotocin, which is found in bony fish, and mesotocin, which is found in birds, lungfish, reptiles, amphibians, and some marsupials. Interestingly, cartilaginous fish have evolved at least six OXT-like homologs (Donaldson and Young, 2008; Hoyle, 1999).

All members of the OXT/AVP/vasotocin peptide family share high sequence similarity, namely a N-terminal six-residue ring, formed by a disulfide bond between the two cysteines at positions 1 and 6, and a flexible C-terminal three-residue tail (exceptions are nematocin and tunicate vasotocin-like peptides). When comparing the intercysteine and tail sequences of those peptides from different organisms, it is obvious that certain positions are highly variable whereas others are highly conserved. For instance, positions 2 and 3 (hydrophobic or aromatic residues), positions 4 and 5 (polar or charged residues), as well as position 7 (proline) and position 9 (glycine) are conserved, whereas position 8 is highly variable (Figure 1.5; see Table 1.2). These amino acid variations are presumably responsible for species-selective recognition, binding, and activation of the different receptors. Subtle differences in the amino acid sequence of the peptide ligands may have significant effects on binding (K_d) and efficacy (EC_{50}) of the ligand to its receptor. Using an *in silico* approach, we recently attempted to correlate these interspecies differences of endogenous ligand with receptor sequence variations with the aim of providing a molecular understanding of recognition, binding, and activation of OXT and AVP receptors by their native ligands. This in turn could assist the design and development of novel selective ligands (Koehbach *et al.*, 2013c).

1.3.2 Molecular diversity of OXT-like receptor residues involved in ligand binding and activation

We previously compared 69 known OXT-like receptor sequences from a wide range of species. Figure 1.5 illustrates the comparison of the ligand sequence variation with the receptor sequence evolution. Consistent with the high receptor sequence similarity across

Figure 1.4 Evolution and diversity of the OXT receptor and its endogenous ligands. A phylogenetic tree consisting of 69 OXT, OXT-like, vasotocin, and human AVP receptors and their respective ligands is shown and has been prepared by sequence alignment using ClustalW. Receptor clusters for OXT, isotocin, mesotocin, and vasotocin/AVP receptors display high sequence similarity and are highlighted in dark/light gray. Receptors of species within the same class or order are highlighted by dashed lines. The UniProtKB entry numbers of the receptors are shown at the end of each branch. Originally published in Koehbach *et al.* (2013c).

closely related species (e.g. bony fish, amphibians or primates), it was found that also the respective ligands within these clusters were highly conserved. This is in agreement with the evolution of OXT- and AVP-related nonapeptides (Acher *et al.*, 1995; Goodson, 2008). Residues believed to be responsible for OXT/AVP ligand-receptor binding were analyzed and their degree of conservation was compared.

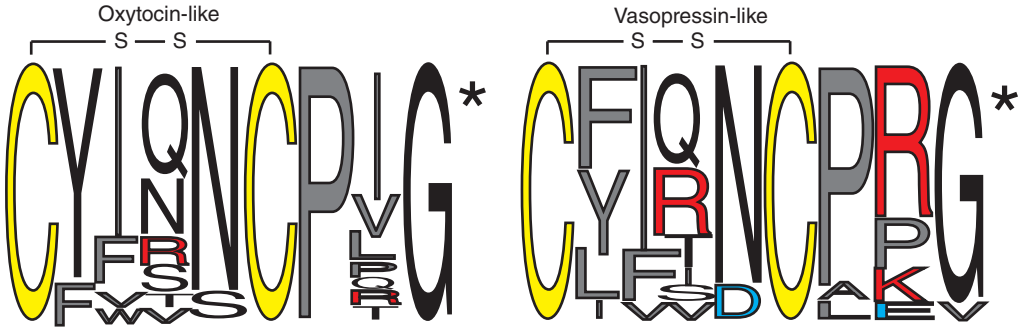

Figure 1.5 Molecular diversity of the OXT/AVP nonapeptide family. Sequence diversity of OXT/AVP-like nonapeptides is illustrated as sequence plots. Relative frequency sequence logo plots with conserved cysteine residues are highlighted in yellow, hydrophobic, aromatic and non-polar amino acids are labeled in gray, negatively charged residues are labeled in blue and positively charged residues are labeled in red. Modified and adapted from Gruber *et al.* (2012).

Previously, the residues arginine R34 (*numbering of all residues according to human OXT receptor*), phenylalanine F103, tyrosine Y209, and phenylalanine F284 were considered to be important for ligand binding and selectivity at the human OXT receptor. Additionally, aspartic acid D85 and lysine K270 are involved in receptor signaling (Zingg and Laporte, 2003). Interestingly, all OXT-like receptors share the latter two residues, which were reported to be important for receptor activation.

The N-terminal arginine residue (R34) is highly conserved in all OXT-like receptors, indicating its importance for ligand binding. Phenylalanine (F103) in the first extracellular loop has previously been demonstrated to be important for ligand selectivity in the OXT receptor and is considered to interact with the residue at position 8 of the peptide ligand (leucine in human OXT). However, a comparative study of 69 receptor-ligand pairs indicated that a direct interaction of this residue in the receptor and the residue at position 8 of the ligand may not generally be important for ligand selectivity (Koehbach *et al.*, 2013c). The two functionally important receptor residues located in the transmembrane region (Y209 and F284) have been considered as other important residues for ligand-receptor binding (Zingg and Laporte, 2003). Importantly, the residue in position 209 is always an aromatic residue and hence is highly conserved amongst all OXT-like receptors. Accordingly, all native peptide ligands contain an aromatic residue (Tyr or Phe) in position 2 and a hydrophobic or aromatic residue in position 3 (Ile, Phe, Val or Trp) of the peptide ligand (Gruber *et al.*, 2012), which confirms that this ligand-receptor interaction may indeed be conserved throughout evolution.

Overall, the analysis of ligand-receptor evolution with respect to the certain sequence variation of OXT ligand-receptor pairs is in agreement with previous biochemical studies (Zingg and Laporte, 2003), but it is likely that these molecular contacts may not be conserved in all native ligand-receptor pairs. Therefore, we developed an *in silico* approach to describe molecular interaction of OXT ligands and their receptors from an evolutionary perspective (Koehbach *et al.*, 2013c).

1.3.3 OXT ligand-receptor interaction: an *in silico* approach

Using publicly available GPCR crystal structures, sequence similarity alignments, ligand-receptor contact frequency plots, and receptor homology modeling, we were able to define a common binding site, deep within the **vestibule** of the OXT receptors. The receptor

residues with the highest frequency of direct contact with ligands included Q119 and M123 (in helix III), I204 and V208 (in helix V), W288, F291, F292 and Q295 (in helix VI), and residue M315 (in helix VII; *numbering according to the human OXT receptor*). Taken together with information from the literature, i.e. the four residues described to be important for human OXT ligand-receptor interaction (R34, F103, Y209, and F284), we hypothesize that it is likely that OXT binds to a common binding site that is located in between those four residues as proposed in the OXT receptor model.

Based on the three largest clusters of receptor-ligand evolution (see Figure 1.4) and with a focus on the residues forming the common binding site in GPCR-ligand structures, we also compared the sequences of OXT, isotocin, and mesotocin receptors and their respective ligands. Residues of the transmembrane helices that were oriented towards the ligand-binding site were found to be more conserved compared to the membrane-exposed residues. Interestingly, the degree of conservation was unevenly distributed: helices II, III, and VI were the most conserved, while residues in helix VII displayed the largest variability.

Biochemical studies with the human OXT receptor and its native ligand have identified four residues important for ligand binding and recognition (Zingg and Laporte, 2003). We compared 69 OXT, OXT-like, and vasotocin receptor sequences to gain further insight into peptide ligand binding. We utilized an *in silico* approach to map the common ligand interaction sites of recently published GPCR structures to a model of the human OXT receptor and compared the interacting residues within different receptor sequences. Our analysis indicates the existence of a binding site for OXT peptides within the transmembrane core region. Previous evolutionary studies of OXT receptors pointed out a number of conserved residues in helices II, III, IV, VI, and VII that may be important for ligand binding, but their analysis included only a few different receptor sequences. It is evident from our comparison that transmembrane helix VII displays the greatest sequence variability, which might be the source for interspecies selectivity. This helix also experiences the biggest structural movements when the receptor is activated and certainly is important for G protein signaling (Koehbach *et al.*, 2013c). While this information brings together empirical data, molecular sequence comparison, and homology modeling, there is still a lack of structure activity and mutagenesis studies to propose a working OXT receptor model that could explain selectivity differences observed in binding studies. In summary, only a ligand-bound crystal structure of OXT and its receptor will be able to shed light on the mechanism of interaction and provide the means for future design of novel and selective ligands.

To conclude, the quality and accuracy of such phylogenetic and evolutionary analysis are determined by the number and distribution of available genetic information about OXT and AVP signaling systems in different animal classes. To date, there are only a few examples of invertebrate peptide and receptor genes that have been characterized. Hence there is a need to study these genes and their function in the invertebrate animal class, which will be described in the following section.

1.4 Physiology of OXT and AVP signaling: from worm to man

Following the description of recent efforts aimed at the discovery of OXT- and AVP-like peptides in several kingdoms of life, as well as the molecular analysis of OXT/AVP diversity, we will summarize available information about the physiology and behavioral role of the OXT and AVP signaling systems, focusing on invertebrate animals, which are by far the biggest group of animals living on Earth.

The function of OXT- and AVP-like peptides has been studied across several animal groups. The effects of both peptides – OXT/OXT homologs and AVP/AVP homologs – appear

Table 1.3 Overview of OXT and AVP nonapeptide physiology.

Animal groups		Functional role of OXT/AVP-like peptide signaling
Mammalian vertebrates	Humans, mammals, marsupials	*Peripheral:* water homeostasis, blood pressure regulation, and reproduction (uterus and mammary gland contraction; ejaculation) *Central:* regulation of learning, memory, stress and anxiety, and complex social behavior (maternal care, bonding)
Vertebrates (non-mammalian)	Birds, reptiles, amphibians, fish	Reproductive physiology, osmoregulation, social communication, affiliation behaviors, aggression, and multiple aspects of stress responses
Invertebrates	Annelids	Reproductive behavior, osmoregulation, metabolism
	Mollusks	Reproductive behavior, memory and metabolism (osmoregulation)
	Nematodes	Reproductive behavior and associative learning
	Insects	Regulation of water homeostasis, other unknown functions(?)

to be distinctive in different species. For example, AVP-like peptides have a major role in regulating reproductive behavior in fish and amphibians, while in birds and mammals OXT-like peptides are more important for this. This situation becomes even more complicated in invertebrate animals, where often only one peptide precursor and only one or two OXT-like receptors exist as compared to humans and mammals which express two peptide ligands and up to four receptors. In the following, we will provide a brief overview of the physiology of OXT and AVP signaling in invertebrates, non-mammalian vertebrates, and mammals, including man (see Table 1.3 for a summary).

1.4.1 Function of OXT and AVP homologs in invertebrates: annelids, arthropods, nematodes, and mollusks

The function of OXT-like peptides has been studied across several invertebrate model organisms, such as annelids, arthropods, nematodes, and mollusks (see Table 1.3). OXT- and AVP-like signaling seems to be involved in neurotransmission, metabolism, and osmoregulation in mollusks and annelids, and in osmoregulation and possibly neurotransmission in insects. In nematodes, this neuropeptide system facilitates gustatory associative learning and coordinates reproductive behavior. Moreover, OXT and AVP show their function during reproduction of leeches, earthworms, and snails (Gruber, 2014). The most comprehensive and detailed studies regarding OXT/AVP physiology in invertebrates came from studies of the model worm *Caenorhabditis elegans* and the red flour beetle *Tribolium castaneum*. Whereas there is only little information on the function of those neuropeptides in insects (Aikins *et al.*, 2008; Stafflinger *et al.*, 2008), there is compelling evidence that this signaling system has a crucial function during reproduction and behavior in nematodes.

The recent discovery and functional characterization of two so-called nematocin receptors and their endogenous peptide ligand showed that this signaling system is involved in the worm's gustatory associative learning (chemotaxis towards NaCl and gustatory plasticity; Beets *et al.*, 2012) and reproductive behavior (regulation of reproductive efficiency, mating behavior, mate searching, brood size, and locomotion behavior; Garrison *et al.*, 2012). However, most studies in invertebrates used molecular biology, immunohistochemistry or *in vitro* pharmacology to describe the physiology of the OXT/AVP neuropeptide system. Functional genomics (for example, gene knock-down) and detailed biochemical analysis have generally not been carried out yet (except for *C. elegans* and *T. castaneum*), but are

required to obtain *in vivo* understanding of the physiological and biological role of this neuropeptide system in invertebrates.

In summary, the OXT and AVP neuropeptide signaling system shows conserved functions in invertebrate physiology (water homeostasis) and reproductive behavior, but more detailed studies are required for comparative analysis of OXT/AVP physiology across invertebrates. Especially from an evolutionary perspective, it is interesting to note that the OXT/AVP peptide hormone system is present in some **holometabolous** insects (beetles and ants), whilst these genes are absent in related insect species, such as the honeybee (*Apis mellifera*) and fruit fly (*Drosophila melanogaster*) (Gruber, 2014; Gruber and Muttenthaler, 2012).

1.4.2 Function in non-mammalian vertebrates: fish, reptiles, amphibians, and birds

In non-mammalian vertebrates, OXT, AVP, and their homologs modulate reproductive behavior. OXT-like peptides are involved in the induction of vocalization, courtship behavior, female sexual receptivity, alternative mating, and many more types of behaviors. Based on comparative studies of non-mammalian vertebrates, it is evident that this nonapeptide system has effects on reproductive physiology, osmoregulation, social communication, affiliation behaviors, aggression, and multiple aspects of stress responses (Donaldson and Young, 2008; Gimpl and Fahrenholz, 2001; Goodson *et al.*, 2012; Gruber *et al.*, 2010; Knobloch and Grinevich, 2014). Generally, the OXT/AVP signaling system exhibits extensive conservation of function, but there is at least some variation. For instance, whereas these OXT- and AVP-like peptides are anxiogenic in rodents, they are strongly anxiolytic in male zebra finches (Goodson *et al.*, 2012). In addition, the independent evolution of multiple behavioral characters is associated with evolutionary convergence in the anatomy of nonapeptide systems and their behavioral effects. This is observed in (i) the convergent roles of mesotocin (an OXT homolog of birds, amphibians, and reptiles) and OXT in the extended maternal care of mammals and most birds, (ii) the independently derived effects of mesotocin and OXT on pair bonding in female prairie voles and zebra finches, and (iii) the convergent patterns of nonapeptide receptor distributions in certain finch species that have independently evolved similar patterns of grouping behavior (Goodson *et al.*, 2012).

1.4.3 Function in mammals and man

Since the 1920s it has been known that an 'extract of the posterior lobe and pituitary gland' can be separated into two fractions, rich in oxytocic and pressor activity, respectively. Subsequent comparative studies between numerous species conducted in the first half of the 20th century revealed that in both mammalian and non-mammalian species, OXT stimulates the activity of smooth muscle in reproductive tracts, sperm movement and ejaculation, as well as uterus contraction during parturition and milk ejection from the mammary glands for breastfeeding. Besides these peripheral effects, it is now evident that OXT is a key player for orchestrating complex reproductive, prosocial, and in-group supporting behavior in the central nervous system, such as trust, maternal care and bonding, as well as stress and anxiety (Knobloch and Grinevich, 2014). However, AVP regulates peripheral fluid balance and blood pressure (see Chapter 15). For instance, the lack of AVP production or mutations of the AVP 2 receptor located in the kidney that prevent its correct folding and secretion to the membrane can cause diabetes insipidus. Centrally AVP is involved in memory and learning, stress-related disorders, and aggressive behavior. Both peptides act on GPCRs: OXT signals via one OTR and AVP via three AVP receptors (vasopressor $V_{1a}R$, pituitary $V_{1b}R$, renal V_2R) and these receptors are expressed in various tissues and organs (Frank and Landgraf, 2008; Gruber *et al.*, 2010; Meyer-Lindenberg *et al.*, 2011).

Due to this physiological importance, ligands of OXT and AVP receptors have potential therapeutic applications for novel treatment approaches in mental disorders characterized by social dysfunction, such as autism, social anxiety disorder, borderline personality disorder, and schizophrenia (Meyer-Lindenberg *et al.*, 2011), childbirth-related conditions, such as premature labor and postpartum hemorrhage (Gruber and O'Brien, 2011), osmoregulatory dysfunction, such as diabetes insipidus, as well as cardiovascular disorders, such as congestive heart failure or vasodilatory shock states (Gruber *et al.*, 2010; Manning *et al.*, 2008).

OXT and AVP are closely related, highly conserved, multifunctional neurohypophysial peptides. The high extracellular receptor homology and ubiquitous receptor distribution constitute a major hurdle for the development of selective ligands and therapeutics (Gruber *et al.*, 2012). For example, OXT is still the ligand of choice in the clinic, although it is well established that OXT also signals via the AVP receptors, which can lead to unwanted side effects. A selective OXT/AVP ligand hence has enormous potential for therapeutic development and it will be intriguing to explore natural OXT-like peptides (and their modifications) for selectivity and potency on human receptors in the future (Gruber *et al.*, 2012).

1.4.4 Excursus: Peptides from nature to mimic OXT and AVP peptides

1.4.4.1 Cyclotides from plants

We recently identified the first OXT-like peptide from plants (Koehbach *et al.*, 2013b). The peptide kalata B7 belongs to the family of plant **cyclotides**. They were originally discovered in the Rubiaceae species *Oldenlandia affinis* based on its use in traditional African medicine to accelerate labor. Since then, cyclotides have been identified in numerous plant species of the coffee, violet, cucurbit, pea, potato, and grass families (Koehbach *et al.*, 2013a). Chemically, cyclotides are peptides with an average length of approximately 30 amino acids that contain three conserved disulfide bonds linked together in a knotted arrangement. In addition, they are characterized by a head-to-tail cyclized backbone. This unique structural topology is known as a cyclic cystine-knot motif, which makes them extremely stable against biochemical degradation.

Using bioactivity-guided fractionation of a herbal peptide extract, the cyclotide kalata B7 was identified as a strong uterotonic agent on human uterine smooth muscle cells. Radioligand displacement and second messenger-based reporter assays confirmed that kalata B7 is a partial agonist of human OXT and AVP V_{1a} receptors. In addition, cyclotide sequences have been used as templates for the design of selective peptide ligands by generating nonapeptides with nanomolar affinities to the human OXT receptor (Koehbach *et al.*, 2013b).

We designed several OXT-like nonapeptides and the peptide [G5,T7,S9]-OXT turned out to be a potent and selective agonist of the human OXT receptor. This is of great interest from a drug design point of view, since OXT, AVP, and many analogs synthesized to date have lacked receptor selectivity (Gruber *et al.*, 2012; Manning *et al.*, 2012). Our findings thus highlight the potential of exploiting cyclotides as templates for the design of peptide GPCR ligands. Given the diversity of plant cyclotides as well as the overall number of GPCRs (the human genome encodes for at least 800 different GPCRs; Gruber *et al.*, 2010), many of which are promising drug targets, our results provide the proof of principle for the development of cyclotide-based peptide ligands using a combination of two disciplines, ethnopharmacology and chemical biology (Koehbach and Gruber, 2013).

1.4.4.2 Conopressins from cone snail venom

Rich sources of venom peptides can be found in spiders, scorpions, snakes, and marine animals, in particular cone snails. Evolutionary pressures have afforded a preoptimized, structurally sophisticated collection of disulfide-rich peptide toxins that have been produced

in a combinatorial fashion and fine-tuned over millions of years. Hence it does not come as a surprise to find that within the venom of these animals, structural scaffolds are found that give rise to a very large number of agonists and antagonists, which act on functionally diverse targets such as ion channels, transporters, and GPCRs (Gruber *et al.*, 2010).

The two AVP-like conopressins from the venom peptides of the predatory marine cone snail are a good example. The original discovery of two of these AVP analogs was later characterized by the observation of grooming and scratching behavior upon intracerebral injection into mice. Although the sequences of conopressins are similar to human AVP, they have an additional positive charge in position 4, which is only found in two other endogenous AVP analogs, cephalotocin and annetocin. Conopressin-S was isolated from *Conus striatus*, whereas conopressin-G was first isolated from *Conus geographus* venom, but later also found to be present in the venom of *Conus imperialis* as well as in tissue extracts of the non-venomous snails *Lymnea stagnalis* and *Aplysia californica*, and the leech *Erpobdella octoculata*. It is not clear what evolutionary advantage is conferred by the presence of these peptides in the venom of the cone snail. Nevertheless, the discovery and characterization of conopressin-T in comparison with the human neuropeptides AVP and OXT led to the identification of an interesting agonist/antagonist switch, which is currently being investigated towards novel antagonist design for the human receptors (Dutertre *et al.*, 2008).

1.5 Perspectives

OXT- and AVP-like peptides mediate a range of physiological functions that are important for osmoregulation, reproduction, complex social behaviors, memory, and learning. The origin of the OXT/AVP signaling system is considered to date back more than 600 million years. Today these nonapeptides are present in vertebrates, including mammals, birds, reptiles, amphibians, and fish, as well as several invertebrate species, including mollusks, annelids, nematodes, and arthropods. Members of this peptide family share high sequence similarity, and it is possible that they are functionally related across the entire animal kingdom. In humans, OXT and AVP are structurally very similar; they differ only by two amino acids. Both nonapeptides contain an N-terminal cyclic six-residue ring structure, stabilized by an intramolecular disulfide bond, and a flexible C-terminal three-residue tail. They mediate their distinct functions by signaling through four GPCRs, which share ~80% sequence homology.

The structural similarity of OXT and AVP together with the high sequence conservation of their receptors results in significant cross-reactivity. This **selectivity dilemma** constitutes a major burden for the development of receptor-specific agonists and antagonists. In addition, it is known that OXT and AVP receptors signal via multiple G protein-coupling modes, and they can form functional homo- and heterooligomers, which further complicates the quest for selective and biased ligands. Although over the last decades, over 1000 OXT and AVP peptide ligands have been synthesized and characterized for therapeutic applications, there is still a great demand for selective and biased ligands that activate or block a specific receptor or a specific cellular pathway. As demonstrated with two examples, i.e. cyclotides and conopressins, peptide sequences identified from natural sources can provide evolutionary advantage over random chemical synthetic approaches and may yield novel lead compounds for therapeutic applications.

An even more promising and very timely approach will be the discovery of endogenous OXT and AVP-like nonapeptides in invertebrate species and in particular in insects, which represent more than half of all known living organisms. We are convinced that the discovery and functional characterization of OXT- and AVP-like neuropeptides from natural

sources, even plants, will not only have applications in drug development to target human OXT and AVP receptors, but may also provide novel information for comparative studies to identify common features of OXT and AVP physiology throughout the animal kingdom that may yield translational insights into evolutionary aspects of human behavior.

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