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Characterisation of selective human oxytocin/ vasopressin ligands

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Leopold Dürrauer, BSc

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Abbreviations

ANT	arachnotocin
ANTR	*Varroa destructor* arachnotocin receptor
AST	asterotocin
AVP	arginine vasopressin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
[D-Glu8]-AST	[D-Glu8]-asterotocin
GFP	green fluorescent protein
$hV_{1a}R$	human vasopressin receptor 1a
$hV_{1b}R$	human vasopressin receptor 1b
hV_2R	human vasopressin receptor 2
HEK 239	human embryonic kidney cells 293
INT	inotocin
IP_1	inositol-1-phosphate
OT	oxytocin
OTR	oxytocin receptor
rcf	relative centrifugal force
Varroa	*Varroa destructor*

1 Introduction

1.1 Oxytocin/ vasopressin signalling system, an overview

Few molecules are as present in public discourse as oxytocin, where it is often referred to as the "cuddle or love hormone". Discovered at the beginning of the last century due to its uterine-contracting properties (hence the name oxytocin, derived from the Greek words 'oxys' and 'tokos', meaning "quick birth") (Dale 1906), it was also the first peptide hormone to be synthesized, a feat for which the American chemist Vincent du Vigneaud was awarded the Nobel prize for Chemistry in 1955 (Vincent du Vigneaud et al. 1954; The Nobel Foundation n.d.). The genes for the preprohormone (Oxt) and the corresponding receptor (Oxtr) have been cloned for more than 25 years (Ivell and Richter 1984; Kimura et al. 1992). Since then, this hormone has appeared in countless newspaper articles, live style journals and magazines due to its 'pleasurable' effects on social bonding and orgasms (Magon and Kalra 2011). A query on PubMed for "oxytocin" results in over 25.000 journal articles and almost 3000 reviews, which demonstrates the importance of this hormone in scientific research.

Oxytocin is part of a larger and exceptionally versatile neuroendocrine system called the oxytocin/vasopressin signaling system. These neuroendocrine systems facilitate interaction between the nervous and endocrine (hormonal) system via a process termed neuroendocrine integration. The oxytocin/vasopressin signaling system can be traced back at least 600 million years to a nonapeptide by the name of vasotocin (Gruber 2014).

The oxytocin/vasopressin system in humans consists of four G protein-coupled receptors (GPCRs): $V_{1a}R$, $V_{1b}R$, V_2R and OTR, that are activated by two neuropeptide ligands: oxytocin and vasopressin (Devost, Wrzal, and Zingg 2008).

Within this complex signaling system, oxytocin controls and influences numerous physiological functions such as labor (Dale 1906), orgasm (Magon and Kalra 2011), milk ejection (Schafer

et al. 1911), cardiac effects and feeding (Meyer-Lindenberg et al. 2011). Meanwhile, vasopressin performs two primary physiological functions: water retention and constriction of blood vessels.

The latter of which is achieved via an acute regulatory endocrine response to osmotic changes leading to enhanced water reabsorption in the kidneys. This so called anti-diuresis is triggered by increased plasma osmolality being sensed by osmosensors in the hypothalamus that releases AVP into circulation. AVP then binds to V2 receptors in the renal collection ducts of the kidneys, leading to an increased insertion of water channels, named aquaporin, in the plasma membrane of cells in that area. This results in increased water reabsorption in the kidneys and restoration of plasma osmolality (McCormick and Bradshaw 2006).

Vasopressin-induced vasoconstriction, the tightening of bloodvessles due to contraction of the muscular walls of the vessle, is currently attributed to two different concentration dependent pathways. On involves activation of phospholipase C and resulting Ca^{2+} release from the sarcoplasmic reticulum, the other relies on protein kinase C and L-type voltage-sensitive Ca^{2+} channels (Henderson and Byron 2007).

The psychological functions of the oxytocin/vasopressin signaling system are less well defined. In mammals it plays important roles in regulating social behaviours, such as social cognition (Kirsch et al. 2005), attachment (Insel and Young 2001), anxiety-related behaviour (Landgraf 2006; Liebsch et al. 1996), fear conditioning (Eckstein et al. 2015), fear extinction and social exploring (Meyer-Lindenberg et al. 2011), conditioned freezing behavior (Stoehr, Cramer, and North 1992), aggression (Bosch et al. 2005; Wersinger et al. 2002) and social recognition (Bielsky and Young 2004; Stoop 2016). Plasma oxytocin levels have also been lined to trust (Zak, Kurzban, and Matzner 2005), physical contact with partners (Grewen et al. 2005) and stress response (Taylor et al. 2006). Other implicated functions are memory and learning

(Alescio-Lautier and Soumireu-Mourat 1998) and attentional preference for faces in autism (Kanat et al. 2017).

As with all complex regulatory systems, failure of a small portion of the system can have vast and unforeseeable consequences. It is therefore no surprise, that the oxytocin/vasopressin signaling system is linked to a wide range of various disorders and diseases, including social anxiety disorder, autism, schizophrenia (Goldman et al. 2008; Keri, Kiss, and Kelemen 2008), borderline personality disorder, stress, depression (Scantamburlo et al. 2007; McQuaid et al. 2014), preterm-labor, cardiovascular diseases, and cancer (Meyer-Lindenberg et al. 2011).

To make this system more accessible for applied research and therapeutic intervention, a toolbox of selective ligands is needed, that specifically modulate different functional states of these receptors. Recently a novel vasopressin V_{1a}-receptor antagonist has been developed by taking advantage of the evolutionary conservation of this system and modifying related insect neuropeptides (Di Giglio et al. 2017). This thesis investigates new human oxytocin/vasopressin peptide drug leads derrived from invertrebrate species.

1.2 G protein-coupled receptors

1.2.1 GPCRs

GPCRs, sometimes referred to as seven-transmembrane receptors, named for their common seven transmembrane spanning helix architecture, are among the largest families of membrane proteins (Pierce, Premont, and Lefkowitz 2002). They act as biomolecular sensors, transducing extracellular stimuli into intracellular signals (Kroeze, Sheffler, and Roth 2003). In addition to

mediating most cellular responses to hormones, neurotransmitters and light, they also play important roles in taste and smell (Rosenbaum, Rasmussen, and Kobilka 2009). Because of their ability to selectively alter cellular behavior, GPCRs represent one of the largest group of drug targets in therapeutic use (Overington, Al-Lazikani, and Hopkins 2006). The human genome encodes for more than 800 different GPCRs (Fredriksson et al. 2003), for many of which no ligands have been identified yet. Investigation of these so called orphan GPCRs as drug targets have been going on for more than a decade (Wilson et al. 1998).

GPCRs consist of seven hydrophobic, transmembrane α-helices that are linked by alternating extracellular and intracellular loops, an extracellular N-terminal domain responsible for a ligand interaction and an intracellular C-terminal domain that is associated with a G protein which is responsible for downstream signaling. The vast majority of G proteins are heterotrimeric proteins comprising of an α, β and γ subunit, each of which is a gene family of its own. In the classical example of GPCR signalling, an agonist binds the extracellular part of the receptor, thereby forming a transient high-affinity complex of the agonist, the receptor and the G-protein. The thereby induced conformational change of receptor and in the G protein leads to a release of GDP bound to the G protein, which is replaces by GTP. This leads to the dissociation of the G protein into a monomeric α subunit and a dimeric βγ subunit. These free subunits then activate further effectors downstream depending on their subtype (Pierce, Premont, and Lefkowitz 2002). Measurement of the activity of these downstream effectors (like adenylyl cyclase, which is activated by $G\alpha_s$ or myo-inositol 1 phosphate production, which is induced by G_q) is the common method to quantify GPCR activity (Gabriel et al. 2003; Trinquet et al. 2006).

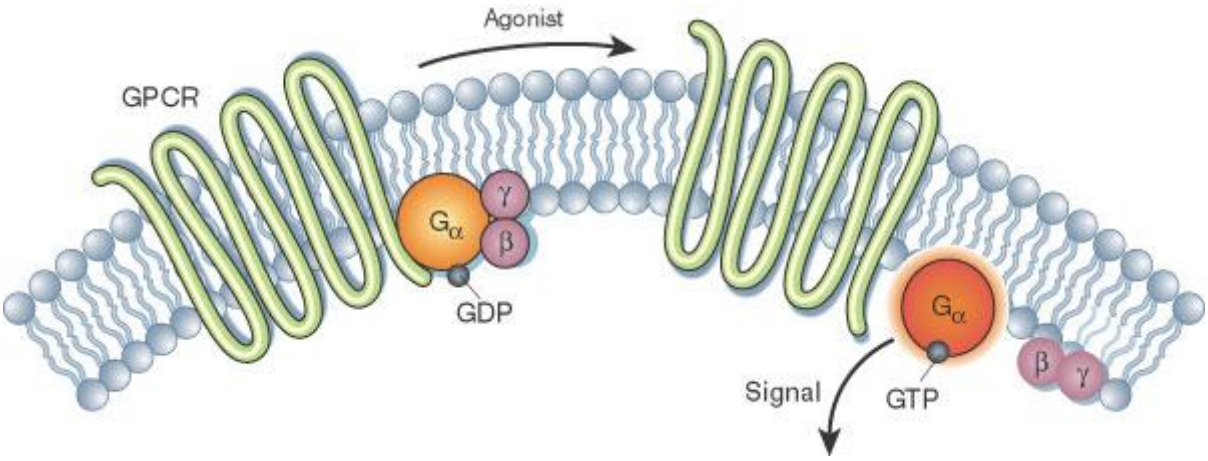

Figure 1: Activation of the G alpha subunit of a G protein-coupled receptor

In unstimulated cells, the state of G alpha (orange circles) is defined by its interaction with GDP, G beta-gamma (purple circles), and a G protein-coupled receptor (GPCR; light green loops). Upon receptor stimulation by a ligand called an agonist, the state of the receptor changes. G alpha dissociates from the receptor and G beta-gamma, and GTP is exchanged for the bound GDP, which leads to G alpha activation. G alpha then goes on to activate other molecules in the cell. [1]

1.2.2 OTR, $V_{1a}R$, $V_{1b}R$ and V_2R

The receptors of the human oxytocin/vasopressin-signaling system (OTR, $V_{1a}R$, $V_{1b}R$ and V_2R) belong to the GPCR class A: the rhodopsin-β adrenergic receptor family (Palczewski 2006). This family is by far the largest group of GPCRs and includes prototypic receptors such as rhodopsin and the adrenergic receptors (Pierce, Premont, and Lefkowitz 2002). The receptors

[1] Activation of the G alpha subunit of a G-protein-coupled receptor. Figure and description adopted (and modified) form Nature Publishing Group. Li, J. et al. The Molecule Pages database. Nature 420, 716-717, 2002.

of the human oxytocin/vasopressin-signaling system display a high degree of sequence similarity. About one hundred of the 370 to 420 amino acids of these receptors are conserved. All receptors except for V_2R, which's activity is mediated by the adenylate cyclase stimulating $G\alpha_s$, couple to $G\alpha_q$, which activates phospholipase C (Gimpl and Fahrenholz 2001). Additionally, OTR is also engages and activates $G\alpha_i$ (Busnelli et al. 2012).

1.2.3 Selectivity

The high similarity of the oxytocin/vasopressin-receptors, especially in the extracellular parts of the receptors, which display a homology of ~80%, is the main bottleneck in development of receptor specific ligands for this system (Koehbach et al. 2013). *In vivo*, selectivity is enhanced through various other factors such as receptor up- and down-regulation, ligand degrading enzymes, local ligand production and receptor expression patterns (Gruber, Koehbach, and Muttenthaler 2012; Manning et al. 2012).

1.3 Oxytocin, vasopressin and oxytocin/vasopressin-like peptides

Oxytocin (CYIQNCPLG-NH_2), vasopressin (CYFQNCPRG-NH_2) and oxytocin/vasopressin-like peptides in other species are nonapeptides (=peptides consisting of 9 amino acids) with a disulfide bond between the cysteine residues in position 1 and 6 and an amidated three residue C-terminal tail. They are generally derived from polypeptide precursors via cleavage (Gruber, Koehbach, and Muttenthaler 2012). In humans, oxytocin and vasopressin are manly synthesized in the posterior pituitary gland of the hypothalamus, hence can be referred to as neurohypophysial hormones, although they can also be synthesized locally. Both peptides have a half-life in serum of less than half an hour (Leng and Ludwig 2008).

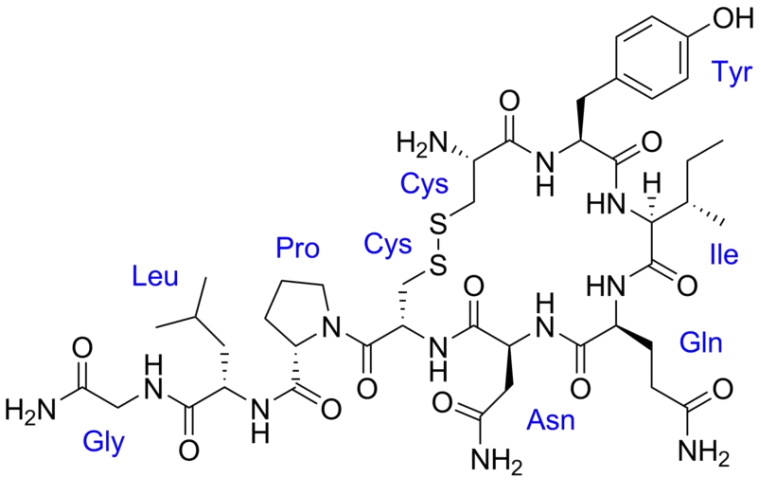

Figure 2: Oxytocin, structural formula

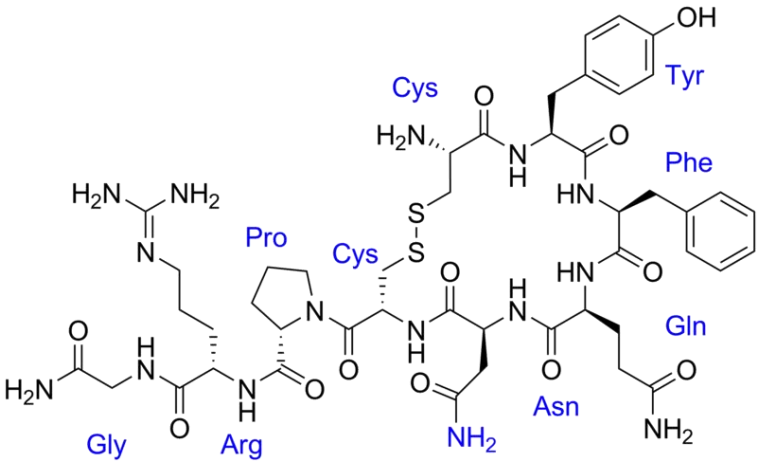

Figure 3: Vasopressin, structural formula

1.3.1 Crosstalk

Despite their differences, there is a significant degree of crosstalk between oxytocin and vasopressin receptors due to structural similarities of the two peptides and the previously mentioned high sequence homology of the corresponding receptors. Consequently, oxytocin not only binds its own receptor, but also activates vasopressin receptors to some extent. The same has been demonstrated for vasopressin (Arrowsmith and Wray 2014). This problem is further enhanced

by the formation of functional oxytocin and vasopressin receptor homo- and heterodimers (Terrillon et al. 2003). Accordingly, vasopressin has been repeatedly shown to induce contraction of the uterus in humans as well as in other mammals (Maggi et al. 1990; Mackler et al. 1999).

1.3.2 Evolutionary aspects

Oxytocin/vasopressin-like peptides and corresponding receptors have been identified in all vertebrates, including mammals, as well as fish and amphibians. They are evolutionary highly conserved and their molecular diversity in these species correlates well with the evolutionary diversity of their respective species (Hoyle 1999). However, oxytocin/vasopressin-like peptides are not limited to vertebrates and have been identified in a growing number of invertebrates such as mollusks, annelids, nematodes (Gruber 2014), insects, starfish and hydra, where they generally modulate social and reproductive behavior (Donaldson and Young 2008).

1.3.3 Nomenclature

Nomenclature of these oxytocin/vasopressin-like peptides is relatively straight forward as most peptides discovered were given unique names based on their phylogeny. However, it should be noted, that some classes display multiple different peptides.

Table 1 oxytocin/vasopressin-like peptide nomenclature

name	sequence	group	reference
Oxytocin	**C**YIQN**C**PLG	mammals	(V. du Vigneaud, Ressler, and Trippett 1953)
Arg-Vasopressin	**C**YFQN**C**PRG	mammals	(Acher and Chauvet 1953)
Lys-Vasopressin	**C**YFQN**C**PKG	pig	(Acher and Chauvet 1953)
Phenypressin	**C**FFQN**C**PRG	some marsupials	(Chauvet et al. 1980)
Inotocin	**C**LITN**C**PRG	insects	(Stafflinger et al. 2008)
Inotocin-like	**C**FITN**C**PPG	Arthropods	(Stafflinger et al. 2008)
Nematocin	**C**FLNS**C**PYRRY	Nematoda	(Beets et al. 2012)
Vasotocin	**C**YIQN**C**PRG	Non-mammalian vertebrates	(Acher et al. 1960)
Isotocin	**C**YISN**C**PIG	Osteichthyes	(Acher et al. 1962)
Annetocin	**C**FVRN**C**PTG	Oligochaeta	(Oumi et al. 1994)
Cephalotocin	**C**YFRN**C**PIG	*Octopus vulagris*	(Takuwa-Kuroda et al. 2003)
Octopressin	**C**FWTS**C**PIG	*Octopus vulgaris*	(Takuwa-Kuroda et al. 2003)
Arachnotocin (proposed name)	**C**FITN**C**PIG	*Varroa destructor*	(Gruber 2014)
Asterotocin	**C**LVQD**C**PEG	*Asterias rubens*	(Mayorova et al. 2016)

1.4 Agonists and antagonists

1.4.1 Atosiban

Atosiban, available under the names Tractocile, Antocin and Atosiban SUN, is a competitive oxytocin/vasopressin receptor antagonist first described in 1985. It is a nonapeptide, desamino-oxytocin analogue most commonly used intravenously to halt preterm labor (Åkerlund et al. 1985).

1.4.2 Carbetocin

Carbetocin, available under the names Duratocin, Pabal, Lonactene, Depotocin, Comoton and Decomoton, is an eight amino oxytocin receptor agonist used in the treatment of postpartum hemorrhage (Rosales-Ortiz et al. 2014; Su, Chong, and Samuel 2012).

1.4.3 Retosiban

Another oxytocin receptor antagonist used in the treatment of preterm labor is Retosiban, a cyclic dipeptide, which has a 1400-fold selectivity for the oxytocin receptor compared to vasopressin receptors. It is used to prevent preterm labor and premature birth (McCafferty et al. 2007).

1.4.4 Desmopressin

Desmopressin, sold under the names DDAVP and Minrin, sometimes referred to as 1-desamino-8-D-arginine vasopressin, is a synthetic human arginine-vasopressin derivative. In contrast to the original, the first cysteine residue has been deaminated via replacement with β-mercaptopropionic acid and the arginine residue in position eight has been switched from levo-rotation to dextrorotation. It is a selective V2R agonist developed for the treatment of diabetes insipidus (Vávra et al. 1968).

1.4.5 Future compounds

Although the development of synthetic oxytocin and vasopressin analogs has been going on for almost five decades (Sawyer and Manning 1973) and several agonists and antagonists against these receptors have already been brought to the market, companies like Merck, Pfizer and Sanofi have abandoned their development programs of non-peptide antagonists due to issues of receptor selectivity, species differences and *in vitro - in vivo* differences (Manning et al. 2012). Especially structural homologies between the endogenous peptides and receptors pose a major obstacle in the development of selective molecular probes. In this area however, peptide ligands pose significant advantages over small molecule ligands, due to their ability to interact with a larger surface area of the receptor as well as their greater stereochemical and structural complexity (Gruber, Koehbach, and Muttenthaler 2012).

Utilizing the evolutionary conservation of peptide-ligands as starting point for the development of selective ligands in humans and other species is a novel strategy that has recently been successfully employed in the development of a selective human $V_{1a}R$ ($hV_{1a}R$) antagonist. The group isolated the oxytocin/vasopressin orthologue inotocin (CLITNCPRG-NH_2) and

investigated its pharmacological properties on its native as well as on human receptors. They thereby discovered that inotocin activates $hV_{1b}R$ but inhibits $hV_{1a}R$. This functional dichotomy was then used built upon by replacing the arginine residue in position 8 with it's stereoisomer D-arginine, thereby creating a stable, competitive V1aR-antagonist with a 3,000-fold binding selectivity compared to the other threee receptor subtypes ($hV_{1b}R$, hV_2R, hOTR) (Di Giglio et al. 2017).

Figure 4: Lasius niger – the black garden ant[2]

[2] photographed by McIntosh Natura, distributed under Creative Commons Attribution-Share Alike, 3.0 Unported, 2.5 Generic, 2.0 Generic and 1.0 Generic license.

1.5 Arachnotocin

Like all oxytocin/vasopressin-like peptides, the *Varroa destructor* oxytocin/vasopressin-like peptide (CFITNCPIG) is a nonapeptide with a prominent disulfide bond between the conserved cysteine residues in position 1 and 6. This thesis will refer to this peptide by arachnotocin as an analogy to the existing phylum nomenclature.

Little is known about the physiological function of oxytocin/vasopressin-like peptides in arthropods. Due to their amino acid sequence similarity to vertebrate oxytocin/vasopressin-like peptides, arthropods might provide a worthwhile source for neuropeptide genome-mining in search for novel peptide-derived oxytocin/vasopressin-receptor ligands. As inotocin has already been shown to interact with the human oxytocin/vasopressin system, the same might be true for a neuropeptide from different class within the same phylum. This idea is encouraged by the high similarity of these two peptides

1.5.1 *Varroa destructor*

Varroa destructor is an external parasitic arthropod infesting honey bee (*Apis mellifera*) hives and one of the main culprits for colony collapse disorder and colony death in northern climates (Abbo et al. 2017; Guzmán-Novoa et al. 2010). Colony collapse disorder is characterized by the disappearance of adult bee population from a colony, leaving behind the queen, newly emerging adult bees, capped brood and food reserves, resulting in pronounced economic loss for beekeepers (Cox-Foster et al. 2007). About 9.5% of the global economic value of the world agricultural output for food ($153 billion) is dependent on pollination by insects, mainly honey bees (Gallai et al. 2008).

Originally from Asia, the mite switched host from *Apis cerana* to *Apis mellifera* at the beginning of the 20th century. It attacks adult workers as well as brood by feeding directly on their hemolymph which reduces weight, immunity and lifespan of the individual as well as the whole colony (Yang and Cox-Foster 2005). Additionally, *Varroa* destructor serves as vector for transmitting viruses. This is further amplified by suppression of the host immune response (Khongphinitbunjong et al. 2015).

Figure 5: Varroa destructor – honey bee mite[3]

[3] Public domain

1.6 Asterotocin

A vasopressin/oxytocin-type neuropeptide derived from a 147-residue polypeptide precursor in the common star fish, *Asterias rubens*, has been identified (Semmens et al. 2016) and named asterotocin (CLVQDCPEG) (Mayorova et al. 2016).

Asterotocin displays several structural characteristics that differentiate it from oxytocin-/vasopressin-like peptides in other animals. The most unusual of these is the presence of an acidic glutamate residue in position 8, as VP/OT-like neuropeptides commonly contain basic or hydrophobic residues in this positions (Semmens et al. 2016). Furthermore, leucin and valine at position 2 and 3 are atypical for this peptide type but are consistent with other hydrophobic residues usually found at these positions.

1.6.1 *Asterias rubens*

The common star fish, *Asterias rubens*, is a member of the Echinodermata phylum, natively found in the northeastern Atlantic ocean and primarily described by Linnaeus, C. 1758 (Linné, Linné, and Salvius 1758). It is natively found in the northeastern Atlantic Ocean. Little is known about the endogenous function of this and other oxytocin/vasopressin-like neuropeptides. However, *in vitro* pharmacological tests indicate that similar molecules effect the contraction of the tube foot and the esophagus preparation in sea urchins, which are also members of the Echinodermata phylum (Elphick and Rowe 2009). While asterotocin is not expressed in the bipinnaria larvae stage of A. rubens, it is prominently expressed in the following mature brachiolaria stage. (Mayorova et al. 2016).

Figure 6: Asterias rubens – the common star fish.[4]

1.7 Aims of this thesis

The aim of this thesis was to study the pharmacological effects of two evolutionarily related ligands, arachnotocin and asterotocin, on the human the GPCRs of the human oxytocin and vasopressin signaling system. This was done using commercially available immunocompetitve secondary messenger quantification assays. The results of this investigation may serve as a vantage point for the design of peptide ligands for this neuroendocrine system.

Furthermore, the *Varroa destructor* arachnotocin precursor peptide as well as the *Varroa destructor arachnotocin* receptor were cloned and its pharmacological properties were investigated.

[4] photograph by Aldaron, a.k.a. Aldaron. - Own work, distributed under CC BY-SA 2.5

2 Materials and Methods

2.1 Cloning

2.1.1 Overview of the Cloning process

Two *Varroa destructor* arachnotocin receptor constructs as well as the *Varroa destructor* arachnotocin precursor sequence were cloned from *Varroa destructor* cDNA provided by Dr. Alan Bowman, University of Aberdeen, King's College, Aberdeen AB24 3FX. This was done by amplifying the desired sequences using PCR with sequence specific restriction site (EcoRI and KpnI respectively) containing oligonucleotide primers. The PCR products were then purified via gel purification, digested with EcoRI and KpnI and ligated into the vector pEGFP-N1. The resulting constructs were amplified in *E. coli* NEB 5-α and purified via midi preparation.

Table 2 materials for cloning

	Manufacturer
Enzyme/kit	ThermoFisher Thermo Scientific™ Phusion™ High-Fidelity DNA Polymerase
oligonucleotides	Sigma Aldrich
"Mastercycler" PCR cycler	Eppendorf
PCR purification kit	Thermo Scientific
Agarose	Sigma Aldrich

Gel electrophoresis marker and loading dye	Promega
Gel electrophoresis purification kit	Thermo Scientific
Sequencing	LCG / AGRF
Ligase	Promega
pEGFP plasmid	Addgene
DMEM media	Gibco, Thermo Fisher
+fetal bovine serum	Sigma Aldrich
+50U/mL penicillin	Sigma Aldrich
+50µg/mL streptomycin	

2.1.2 Polymerase chain reaction (PCR)

Table 3 PCR primers

	5′-3′ NUCLEOTIDE SEQUENCE
ANT precursor forward	AAAAGAATTCATGAAGCTTCACGTACTCGTACTAGCC
ANT precursor reverse	AAAAGGTACCTTAGATGATCATCAACGACTCGCG
6T-ANTR forward	AAAAGAATTCATGGCCGGCAACATCGT
6T-ANTR reverse	AAAAGGTACCCGCACCTCTATACGCTCTGGGC
ANTR forward	AAAAGAATTCAATGGATAGTAACAGCACGGGTTCGATTACTTCTAGCGCGTC
ANTR reverse	AAAAGGTACCCGCACCTCTATACGCTCTGGGC

Restriction sites are underlined.

PCR was performed using the Thermo Scientific™ Phusion™ High-Fidelity DNA Polymerase protocol and *Varroa destructor* cDNA provided by Dr. Alan Bowman, University of Aberdeen, King's College, Aberdeen AB24 3FX. Individual reactions contained 4 µL 5x Phusion™ HF Buffer, 0.4 µL 10 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 1 µL *Varroa* cDNA, 0.02 U/µL Phusion™ DNA Polymerase and ddH_2O to 20 µL. For cycling, the following 2-step protocol was used: Initial denaturation (98°C for 30 s), 25 cycles of denaturation (98°C for 10 s) and combined annealing (68°C for 30 s) and extension (72°C for 180 s) and a final elongation at 72°C for 5 min.

2.1.3 Gel purification

Table 4 materials for gel purification

	Manufacturer
Agarose	Sigma Aldrich
SYBR safe	Peqlab
TAE buffer	40 mM tris(hydroxymethyl)aminomethane 20 mM acetic acid 1 mM Ethylenediaminetetraacetic acid
GeneJET Gel Extraction Kit	Thermo Scientific

PCR products were purified via conventional agarose gel purification. (Lee, Costumbrado, Hsu, & Kim, 2012). Gel electrophoresis was performed on a 1%, SYBR safe stained agarose gel in TAE buffer at 100 V for 30-50 min depending on sample size. Afterwards the gel was analyzed under UV light and bands of correct size and sharpness were excised and purified via the Thermo Scientific GeneJET Gel Extraction Kit.

For this, gel fragments were dissolved in a 1:1 volume of Binding Buffer at 50°C for approximately 10min, transferred onto purification columns and centrifuged for 1 min at 13000 rcf. The columns were then washed with 100 µL Binding Buffer (centrifuging for 1 min at 13000 rcf), 400µL Wash Buffer (centrifuging for 1 min at 13000 rcf) and again with 400 µL Wash Buffer (centrifuging for 1 min at 13000 rcf). Finally, the purified DNA was eluted in 35 µL Elution Buffer (centrifuging for 2 min at 13000 rcf).

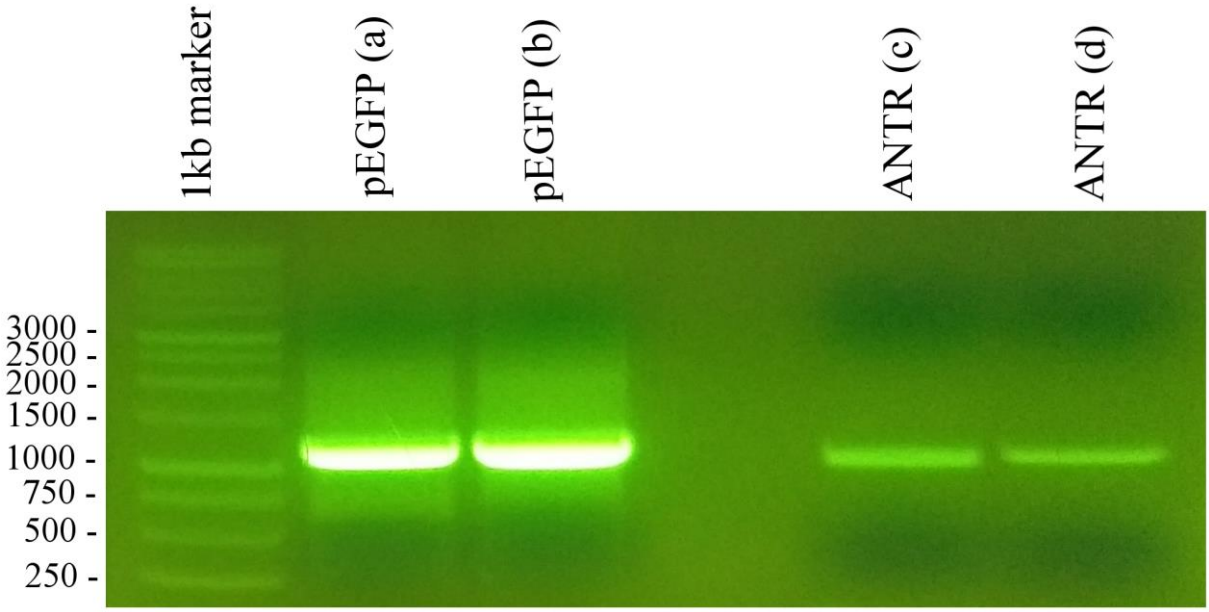

Figure 7: Agarose gel electrophoresis, example

Photograph of a preparative 1% agarose gel under UV light stained with SYBR safe after a 45 min run. 1 kb DNA marker by Promega (size depicted in base pairs); (a): supercoiled pEGFP; (b): supercoiled pEGFP; (c): ANTR PCR product; (d): ANTR PCR product with different primers. Note that supercoiled DNA runs significantly faster due to increased density and thus appears to be of smaller size then linear DNA of the similar length.

2.1.4 Restriction digests and ligation

Table 5 materials for restriction digests

	Manufacturer
EcoRI restriction enzyme	Promega
KpnI restriction enzyme	Promega
buffer C	Promega
acetylated Bovine Serum Albumin	Promega
T4 DNA Ligase	Promega
T4 ligase buffer	Promega

Restriction digests were performed with 1 µg DNA per reaction, as well as 2 µg acetylated Bovine Serum Albumin, 2 µL 10x Restriction Buffer, 5 u restriction enzyme and ddH_2O to 20 µL and were carried out at 37 °C for 3 hours. The digested products were then subjected to gel purification to remove (see above).

Ligations of purified ANTR digests were performed at a 3:1 molar ratio of vector:insert DNA with 200 ng of digested pEGFP vector and 1 µL 10x Ligase Buffer, 1 u (Weiss units) T4 DNA Ligase and ddH_2O to 10 µL. The reactions itself were carried out for 3 hours at room temperature.

2.1.5 Transformation

Table 6 materials for transformation

	Manufacturer
competent NEB-5α	New England Biolabs
SOC media	20 g/L tryptone 5 g/L yeas extract 20 mM glucose 10 mM NaCl 10 mM $MgCl_2$ 10 mM $MgSo_{4s}$ 2.5 mM KCl pH 7.0
LB media	10 g/L tryptone 10 g/L NaCl 5 g/L yeast extract pH 7.0
LB agar	LB media 15 g/L agar
Kanamycin	Sigma Aldrich

Per digest, 2 µL ligation products were added to 50 µL of competent NEB-5α cells and incubated for 30 min on ice. Heat-shock was carried out at 42°C for 30 sec and cells were incubated on ice for 2 min. Then 450 µL SOC media were added and the cells incubated at 37°C for 1 hour, before 100 µL aliquots were plated onto 50 µg/mL Kanamycin containing LB agar plates. Plates were incubated at 37°C overnight. Colonies of sufficient size and isolation were then picked for plasmid mini preparation.

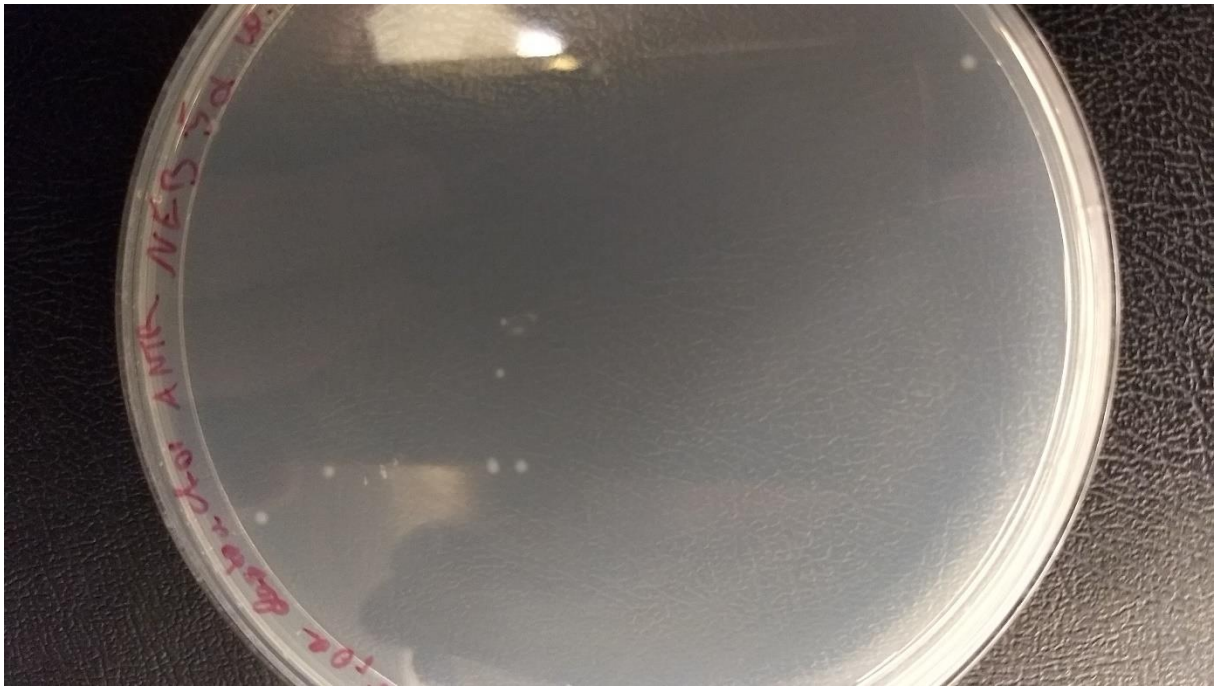

Figure 8: NEB-5α colonies on a LB-Kanamycin plate

Photography of NEB-5α colonies (white dots) on a LB-agar plate containing 35 µg/mL Kanamycin. This plate was incubated for 18 hours at 37°C after transformation.

2.1.6 Plasmid preparation and sequencing

Table 7 material for plasmid preparations and sequencing

	Manufacturer
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen
PureLink™ HiPure Plasmid Midiprep Kit	Invitrogen
PureLink™ HiPure Precipitator Module	Invitrogen
LB media	10 g/L tryptone 10 g/L NaCl 5 g/L yeast extract pH 7.0
Nanodrop	Thermo Fisher

Plasmid purifications were carried out on mini- and midi-preparation scale. Mini-preparations were used to verify the integrity of ANTR receptor constructs.

Suitable colonies were picked from LB-agar plates (containing 50 µg/mL) Kanamycin and incubated in 3 mL LB-media at 37 °C overnight. 800 µL overnight culture was used to make glycerol stocks. The remaining cells were harvested via centrifuging for 20 min at 3260 rcf and resuspended in 250 µL Resuspension buffer. 250 µL Lysis buffer were added and incubated for 5min at room temperature. 350 µL Precipitation buffer were added and the mixture centrifuged at 12000 rcf for 10 min. The supernatant was then transferred onto a spin column. The column

was centrifuged at 12.000 rcf and washed with 500 µL Wash buffer (centrifuged at 12.000 rcf for 1min) and 700 µL Wash buffer (centrifuged at 12.000 rcf for 1 min). All flow-throughs were discarded, and the column was centrifuged at 12.000 rcf for 1 min to remove remaining Wash buffer. The spin column was then transferred onto a recovery tube. 75 µL TE buffer were added to the column and incubated at room temperature for 1 min. Purified DNA was then eluted via centrifuging at >12.000 rcf for 2 min and the concentration determined via nanodrop.

600 ng DNA diluted in $_{dd}H_2O$ were sent to sequencing in a total volume of 12 mL containing 3.2 µM primer.

Glycerol stocks of correct clones were used to start 30 mL overnight cultures in LB media for midi preps. Cells were grown with agitation at 37°C overnight and harvested via centrifuging at 3260 rcf for 15 min. Cells were resuspended in 4 mL Resuspension buffer and lysed with 4 mL Lysis buffer for 5 min at room temperature. 4 mL of Precipitation buffer were added, and the lysate centrifuged at 3250 rcf for 20 min. The supernatant was then transferred onto an HiPure Midi column that had been equilibrated with 10 mL Equilibration buffer. The column was washed twice with 10 mL Wash buffer and the plasmid DNA eluted with 5 mL Elution buffer.

To decrease the final volume of Midi-preps, the PureLink™ HiPure Precipitator Module was used. 10.5 mL isopropanol were added to 5 mL previously obtained DNA solution and incubated for 2 min at room temperature. This mixture was then pushed through the precipitator with a syringe and washed twice with 3 mL 70% ethanol. The purified DNA was finally eluted in 1 mL ddH_2O. The final DNA concentration was determined via nanodrop.

2.1.7 Glycerol stocks

Table 8 material glycerol stocks

	Manufacturer
50% glycerol	Sigma Aldrich
LB media	10 g/L tryptone 10 g/L NaCl 5 g/L yeast extract pH 7.0
Cryogenic vial	Corning

Glycerol stocks of bacterial cells were made from monoclonal overnight cultures in LB-media by mixing 800 µL culture with 800 µL 50% glycerol in a 2 mL cryogenic vial. Stocks were stored at -80°C.

2.2 Cell culture

Table 9 materials cell culture

	Manufacturer
DMEM	Gibco, Thermo Fisher
TrypLE	Gibco, Thermo Fisher
Tissue culture flask	Sarstedt
Trypan Blue	Sigma Aldrich

All work eukaryotic cell culture work was done with Human embryonic kidney cells 293 (HEK-293). Unless stated otherwise, cells were incubated at 37°C and 5% CO_2 and all work requiring sterility was performed in a class II biosafety cabinet.

Cells were grown in 10 cm^2 flasks with 10 mL media and split every 2-3 days using the following protocol: All media was aspirated, and cells were detached via agitation in 2 mL of TrypLE for 2 min. Then 3 mL media were added and all liquid, but 1 mL was removed, and 10 mL of additional media was added to the flask. Cells were counted in a 1:1 dilution of cells in Trypan blue using a hemocytometer.

2.2.1 Transfection

Table 10 materials for transfection

	Manufacturer
lipofectamine	Thermo Fisher
Serum free media (DMEM)	Gibco, Thermo Fisher

About 0.48 million HEK-293 cells were seeded onto 6-well plates and incubated overnight. The next day transfections were performed as follows. 100 µL of serum free media was mixed with 7.5 µL lipofectamine and incubated 5min. Then 200 µL of serum free media were mixed with 2.5 µg DNA, added to the lipofectamine mix and dropwise added to the cells.

Further handling depended on the assay type performed on the cells.

2.3 **Pharmacological assays**

Depending on the receptor, pharmacological assays were carried out using either the Cisbio Bioassays' IP-One Gq kit or the LANCE® *Ultra* cAMP measurement. Both kits rely on homogenous time-resolved fluorescence energy transfer and competitive binding of labeled components to quantify the corresponding metabolites.

Concentration response curves of ANT, AST and [D-Glu8]-AST at $hV_{1a}R$, $hV_{1b}R$ and OR were measured using the IP-One assay kit (CisBio), which detects intracellular myo-inositol-1-phosphate-accumulation in response to $G\alpha_q$ coupled GPCR activation. Because hV_2R does not couple to $G\alpha_q$, but to $G\alpha_s$, the same approach could not be used to quantify hV_2R activity. Concentration response curves of ANT, AST and [D-Glu8]-AST at hV_2R were therefore constructed using the LANCE Ultra cAMP assay kit (Perkin Elmer), which facilitates the measurement of modulation of adenylyl cyclase activity by G-protein coupled receptors by measuring cAMP. All exogenous peptides were tested at concentrations ranging from 30 pM to 30 µM and all graphs were normalized to the maximum activation by the endogenous peptide (AVP in case of $hV_{1a}R$, $hV_{1b}R$ and hV_2R; OT in case of OTR) and to minimum activity above baseline. All regression curves where fitted with a slope of 1 and sigmoidal shape.

Antagonist activity screens were performed using the same kits. All receptors ($hV_{1a}R$, $hV_{1b}R$, hV_2R, OTR) were stimulated with the endogenous peptides (AVP and OT respectively) at the corresponding EC_{50} in presence of 1 and 10 µM ANT, AST and [D-Glu8]-AST. All graphs were normalized to the maximum activation by the endogenous peptide (=0%) and a vehicle control (=100%).

All peptides were manufactured synthetically and kindly provided by Dr. Markus Muttenthaler, Institute for Molecular Bioscience, University of Queensland, 306 Carmody Road, St. Lucia QLD 4072, Australia.

2.3.1 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is an energy transfer between two light-sensitive molecules. An excited donor chromophore transfers energy to an acceptor chromophore via nonradiative dipole-dipole coupling.

Because the efficiency of this process is inversely dependent on the distance between donor and acceptor, FRET can be used to verify interactions between chromophore labeled antigens and antibodies. In an unbound state the excited donor emits fluorescence at a given wavelength. The interaction between antigen and antibody allows FRET to occur which leads to fluorescence of the acceptor chromophore at a different wavelength. This can be relatively quantified by the ratio of acceptor/donor fluorescence (Clegg 1995).

The quality of signals obtained via FRET is reduced by noise derived from background fluorescence of other sample components such as cell lysate and buffers. This impediment can be counteracted by introducing a 50-150 µsec time delay between excitation and measurement, which allows for the expiration of short-lived, non-specific emissions. The detected signal thus displays a significantly better signal to noise ratio and is referred to as Time-Resolved FRET (TR-FRET) or Homogeneous Time-Resolved Fluorescence HTRF (Degorce et al. 2009; Cisbio 2017).

2.3.2 IP-One assay

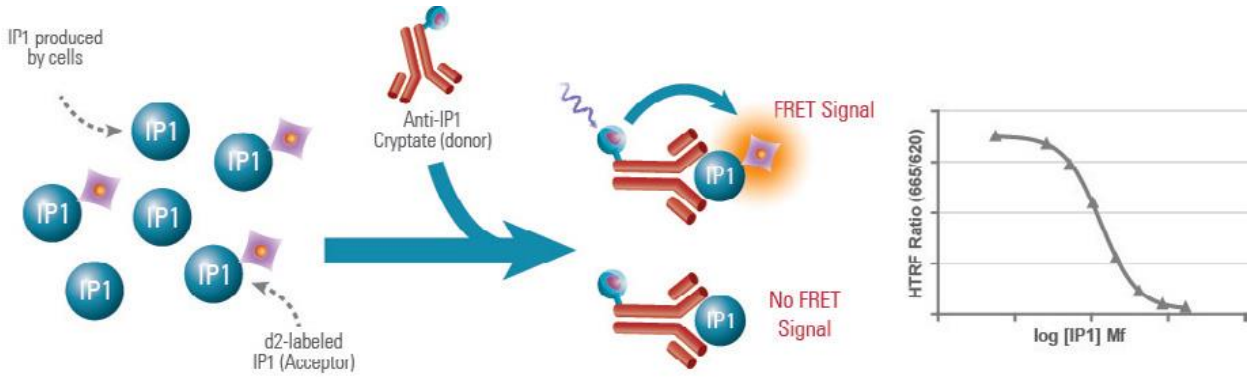

Figure 9: HTRF IP_1 competitive binding assay principle[5]

The Cisbio Bioassays' IP-One Gq kit facilitated measurement of the activation of G_q-coupled receptors by measuring myo-Inositol 1 phosphate (IP_1) accumulation in cells via homogenous time-resolved fluorescence resonance energy transfer (HTRF). Cellular produced IP_1 and d2-labled IP_1 (FRET acceptor) compete for binding of anti-IP1-Cryptate (FRET donor). The change of the HTRF ratio (665 nm/620 nm) is therefore inversely proportional to the concentration of IP_1 in the sample.

[5]©Cisbio

Table 11 materials for IP_1 assay

	Manufacturer
IP-One - Gq Kit	Cisbio
384 well plate	Greiner
Flexstation 3	Molecular Devices

4 hours after transfection, cells were dissolved in 5mL media, transferred onto a 384 well-plate at 40 µL per well and incubated for 2 days. Afterwards all media was removed from the cells, 6 µL stimulation buffer was added per well and the plate incubated for 15 minutes. Then 6 µL 2x peptide dilutions in stimulation buffer were added and the assay incubated for 1 hour. 4 µL of IP_1-d2 and 4 µL AB-cryptate dilutions (3/4 of 1:20 IP_1-d2/AB in lysis buffer plus ¼ stimulation buffer) were added and the plate incubated for 1 hour or o/n at room temperature before reading on a Flexstation 3 at a wavelength of 620 nm and 665 nm.

2.3.3 cAMP assay

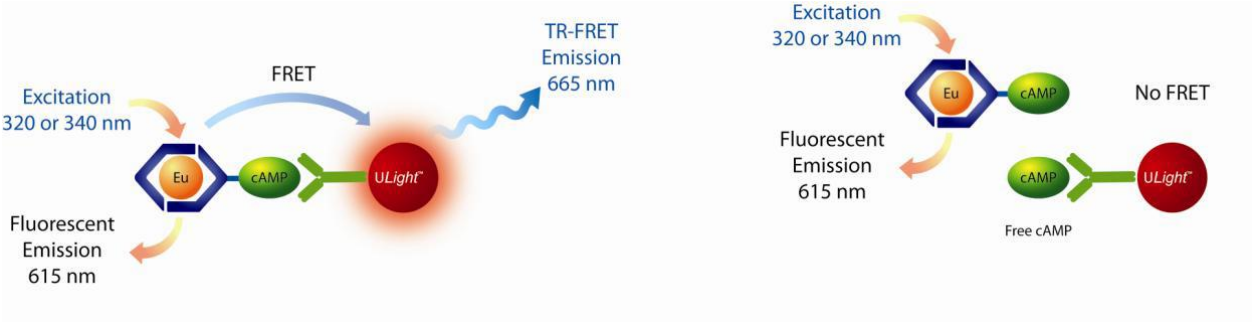

Figure 10: LANCE® Ultra cAMP assay principle[6]

The LANCE Ultra cAMP kit facilitates the measurement of modulation of adenylyl cyclase activity by G-protein coupled receptors by measuring cAMP via homogenous time-resolved fluorescence resonance energy transfer. Cellular produced cAMP and europium chelate-labeled cAMP (FRET donor) compete for binding of a cAMP specific monoclonal antibody (FRET acceptor). The change of the HTRF ratio (665 nm/615 nm) is therefore inversely proportional to the concentration of cAMP in the sample.

Table 12 materials for cAMP assay

	Manufacturer
LANCE *Ultra* cAMP assay kit	Perkin Elmer
384 well plate	Greiner
Flexstation 3	Molecular Devices

[6] ©Perkin Elmer

HEPES	Lonza
IBMX	BPS Bioscience
DMSO	Roth/Sigma Aldrich
Hank's Balanced Salt Solution (HBSS)	Gibco, Thermo Fisher

Media from transfected cells was changed 4 hours after transfection, the cells split 1:1 and incubated in 5 mL media. The next day the assay was performed as follows. 15 mL of fresh stimulation buffer was prepared using 14 mL of 1x HBSS, 75 µL of 1 M HEPES, 30 µL of 250 mM IBMX dissolved in DMSO, 200 µL of 7.5% BSA Stabilizer and the pH adjusted with 0.1 N NaOH and filled up to the final volume with 1x HBSS. Cells were washed off their wells with PBS EDTA, transferred into 15 mL centrifuge tubes, centrifuged at 350 rcf for 4 min, resuspended in 1 mL stimulation buffer, counted using a hemocytometer and diluted to a final concentration of 300 cells per 5 µL. Per well of a 384 well plate, 5 µL of peptide dilutions in stimulation buffer were added. Then 5 µL cell dilution was added to each well. The plate was incubated for 30 min at room temperature before 5 µL of 4x Eu-cAMP tracer solution and 5 µL of 4x U*Light*™-anti-cAMP solution was added to each well. The plate was then incubated again for an hour and read using a Flexstation 3.

2.4 **Microscopy**

Microscopy was carried out using a Nikon ECLIPSE Ts2 inverted routine microscope with 10X magnification in the eyepiece and a 40X magnification in the objective. The resulting 400X

magnification allows for detection of GFP down at the level of sub-cellular compartments. Images were captured with a Progres Gryphax Arktur microscope camera from Jenoptik mounted on the microscope. Cells were generally imaged 24 hours after transfection.

2.5 Bioinformatics

2.5.1 BLAST

Database searches were carried out using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990), which "compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches"[7]. BLAST is available on the website of the National Center for Biotechnology Information (NCBI)[8].

2.5.2 Sequence alignments

Sequences were aligned using Clustal Ω (version 1.2.4), a multiple sequence alignment tool for nucleic acid and protein sequences provided by the European Bioinformatics Institute[9], that is based on an algorithm developed by Higgins and Sharp (Higgins and Sharp 1988).

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[7] https://blast.ncbi.nlm.nih.gov/Blast.cgi, 04.04.2018

[8] National Center for Biotechnology Information, U.S. National Library of Medicine
8600 Rockville Pike, Bethesda MD, 20894 USA, 04.04.2018

[9] https://www.ebi.ac.uk/Tools/msa/clustalo/ 14.11.2017

3 Results

3.1 Cloning of a *Varroa destructor* Arachnotocin Receptor

3.1.1 Overview

This chapter describes the rationale and process behind the cloning of two different *Varroa destructor* arachnotocin receptor constructs as well as the results of various pharmacological experiments performed with these constructs.

3.1.2 Arachnotocin polypeptide precursor

A putative arachnotocin precursor was identified via BLAST (Basic Local Alignment Search Tool) and cloned from *Varroa destructor* cDNA provided by Dr. Alan Bowman (University of Aberdeen, King's College). Subsequent sequencing by LGC Genomics[10] and in silico translation using ExPASy translate (Gasteiger et al. 2003) elucidated the following precursor.

H_2N-MKLHVLVLANIVGLSLT**C**FITN**C**PIGGKRSDTGFGLVQFSSDFRQ

CPP**C**GPGSTGQ**C**FGPNI**CC**NSES**C**LIDAGDSPHLRS**C**KREALKLKP**C**TN

TGMR**C**GSENKGH**C**ALNRF**CC**TSEG**C**MVDEA**C**NGKDHDVIRESLMII-OH

Figure 11: Arachnotocin precursor sequence

The endoplasmic reticulum signal peptide is highlighted in blue, arachnotocin is underlined, the dibasic amidation signal GKR is highlighted in red and the conserved cysteine residues of

[10] LGC Genomics GmbH, Ostendstraße 25, 12459 Berlin, Germany

the neurophysin domain are highlighted in yellow. All these sequences are highly conserved across arthropods (Liutkeviciute et al. 2016).

3.1.3 Receptor identification

Considering the high degree of conservation of oxytocin/vasopressin-like peptide receptors across the animal kingdom, receptors in yet uninvestigated species can be identified by performing a BLAST search on their genome against related receptors. It has previously been established, that this approach even works across different phyla (Donaldson and Young 2008).

Starting from already published partial sequence information of *Varroa* destructor oxytocin/vasopressin-like peptide receptors (Liutkeviciute et al. 2016), a BLAST search for these receptors was performed using whole genome shotgun contig sequences. This resulted in two hits (GenBank entry ADDG01054890.1 and ADDG01034892.1 (Clark et al. 2016)) against the receptors of the red flour beetle (*Tribolium castaneum*) and various Daphnia species (*Daphnia magna, Daphnia pulex*). This indicates that there are two putative distinct arachnotocin receptors in *Varroa* destructor. Both sequences can be found in the appendix (6.1 *Varroa destructor* BLAST , page 99).

However, one of the two sequences, referred to as ANTR 2, was found to be shortened, just consisting of an approximately 70 amino acid long sequence at the N-terminus and was lacking most of the C-terminus when compared to known receptors from *Daphnia spp.* and *Tribolium castaneum* (Appendix 6.16.1, page 99). Due to this limited size, no further attempts were made to clone this receptor.

The other sequence displayed a full C-terminus including a STOP-codon but was truncated at the N-terminus when compared to the *Daphnia spp.* and the *Tribolium castaneum* receptors.

This difference in length lead to the investigation of additional in-frame start codons as alternate beginnings of this specific Open Reading Frame upstream of the first start codon in the aligned sequence, but none were found on the corresponding shotgun contig.

Using Protter, an interactive protein feature visualization and integration tool (Omasits et al. 2014) this receptor sequence was visualized as a 2D schematic (**Figure 12**). This sequence only displays six of the canonical seven transmembrane domains found in Seven Transmembrane Receptors (Pierce, Premont, and Lefkowitz 2002). Additionally, the N-terminus of the receptor, which generally plays a role in ligand recognition (Rosenbaum, Rasmussen, and Kobilka 2009), is not found on the extracellular, but on the intracellular side of the plasma membrane, which prevents an interaction with signal molecules with limited membrane permeability. This receptor will therefore be referred to as six transmembrane arachnotocin receptor (6T-ANTR) and is most likely a sequencing artefact without biological relevance.

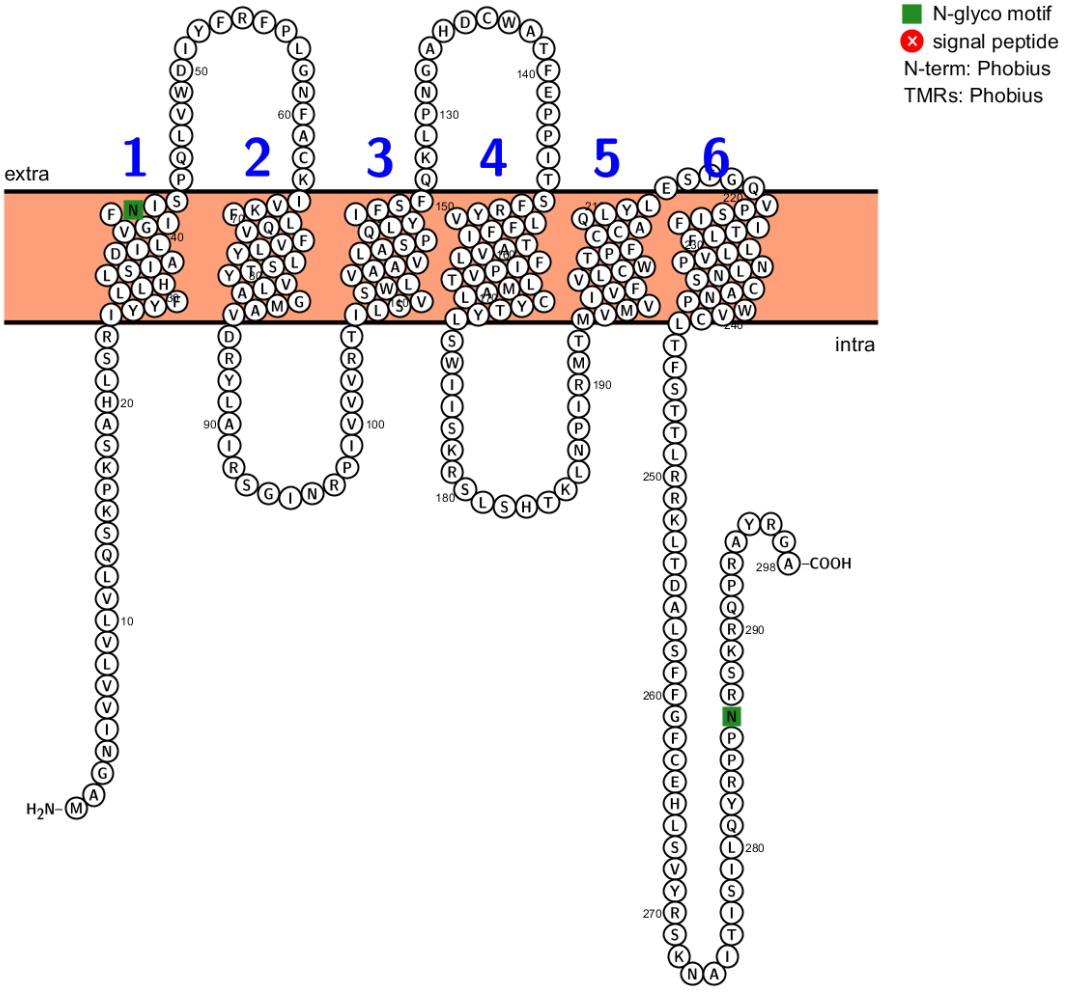

Figure 12: Protter visualization of 6T-ANTR

2D visualization of 6T-ANTR using Protter[11] (Omasits, Ahrens, Müller, & Wollscheid, 2014). One transmembrane domain (labelled 1-6) in the orange bar between extra and intracellular region is missing. The N-terminus, which is canonically important for ligand binding in the extracellular space (Pierce, Premont, and Lefkowitz 2002) is located in the intracellular space.

[11] http://wlab.ethz.ch/protter/start/ 2017

Due to this missing transmembrane domain, a larger scale database search for was conducted across additional *Varroa* species. This resulted in multiple (BLAST) hits in available Transcriptome Shotgun Assembly sequences of *Varroa jacobsoni*. Most interesting, these hits displayed a 100% sequence identity to 6T-ANTR plus an upstream part of the *Varroa jacobsoni* receptor not found in available *Varroa destructor* sequences at the time. Considering the general conservation of oxytocin/vasopressin-like receptors, the evolutionary proximity of these two species and the extreme sequence similarity, it was assumed, that an identical or near identical version of the *Varroa jacobsoni* receptor would be present in *Varroa destructor*. A Protter (Omasits et al. 2014) visualization of this putative receptor (**Figure 13**), referred to as ANTR, displays all seven canonical transmembrane domains as well as an approximately forty amino acid long extra cellular domain at the N-terminus. This prediction is consistent with known GPCRs. It was therefore assumed, that this receptor was also present in *Varroa destructor* and primers for cloning were designed using this sequence.

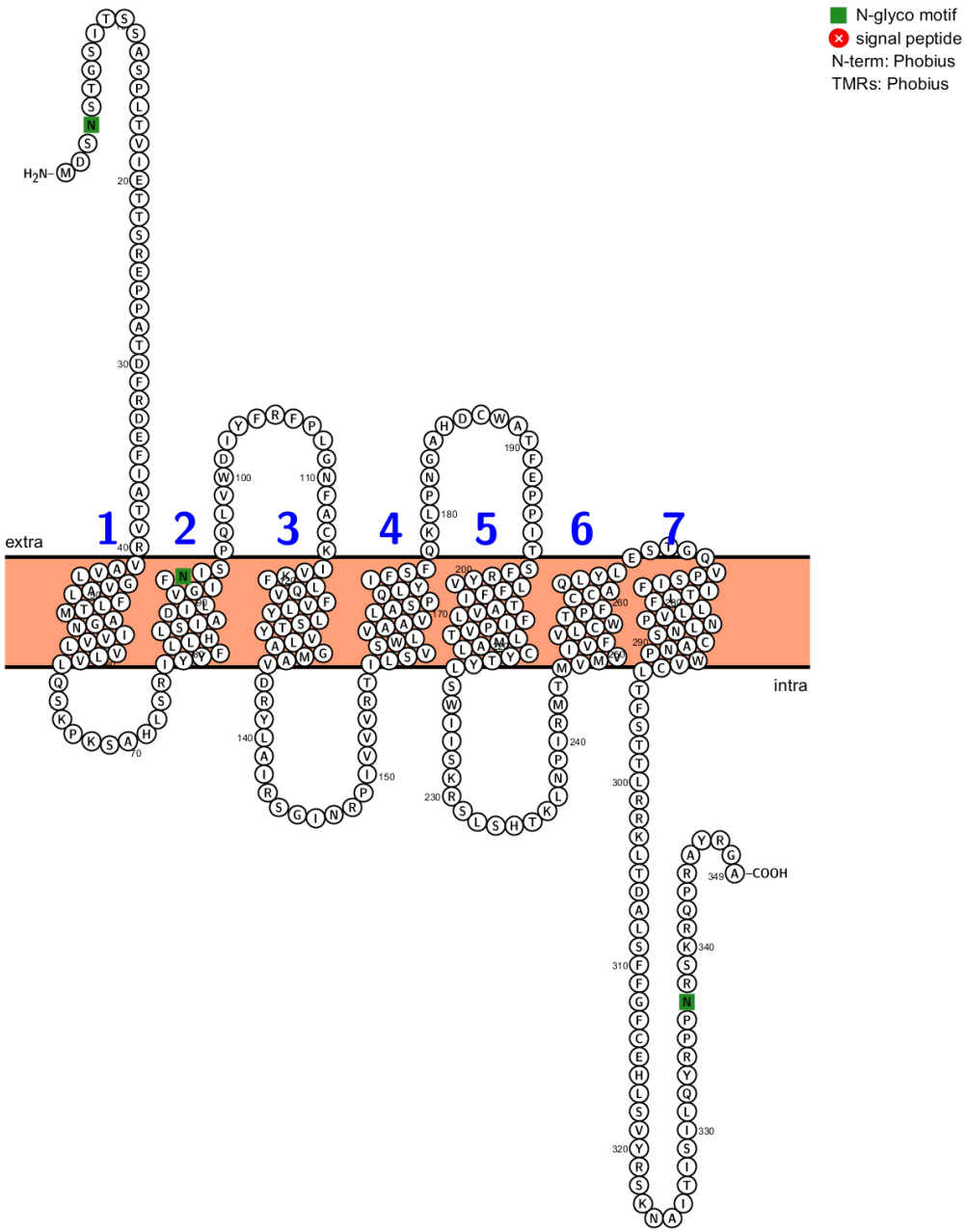

Figure 13: Protter prediction of ANTR

2D visualization of 6T-ANTR using Protter[12] (Omasits, Ahrens, Müller, & Wollscheid, 2014). All canonical seven transmembrane regions and the N-terminus are located correctly.

[12] http://wlab.ethz.ch/protter/start/ 2017

3.1.4 Cloning

Both 6T-ANTR and ANTR were successfully cloned as GFP tagged constructions into pEGFP-N1 from *Varroa destructor* cDNA provided by Dr. Alan Bowman (University of Aberdeen, King's College), as described in the Materials and Methods section. 6T-ANTR was intended to be used as a control and to be biologically inactive. The constructs were verified through external sequencing.[13] Both receptor constructs exactly matched the predicted sequences. For the full receptor sequences, please refer to the appendix (6.2 ANTR clones, page 104).

3.1.5 Receptor Pharmacology

The functionality of receptor constructs is usually verified by measuring its activation by the corresponding endogenous ligand. Because no *Varroa destructor* receptors have yet been pharmacologically described, it was unclear what downstream effects to measure while quantifying the response of this GPCR. Therefore, agonist activity assays with 6T-ANTR-GFP and ANTR-GFP were performed using both myo-inositol-1-phosphare and cAMP accumulation as readout, as these are two of the most commonly used downstream metabolites in oxytocin/vasopressin dependent receptor signalling.

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[13] AGRF
Gehrmann Laboratories, Research Rd, University of Queensland, Brisbane QLD 4072
ABN - 63 097 086 292

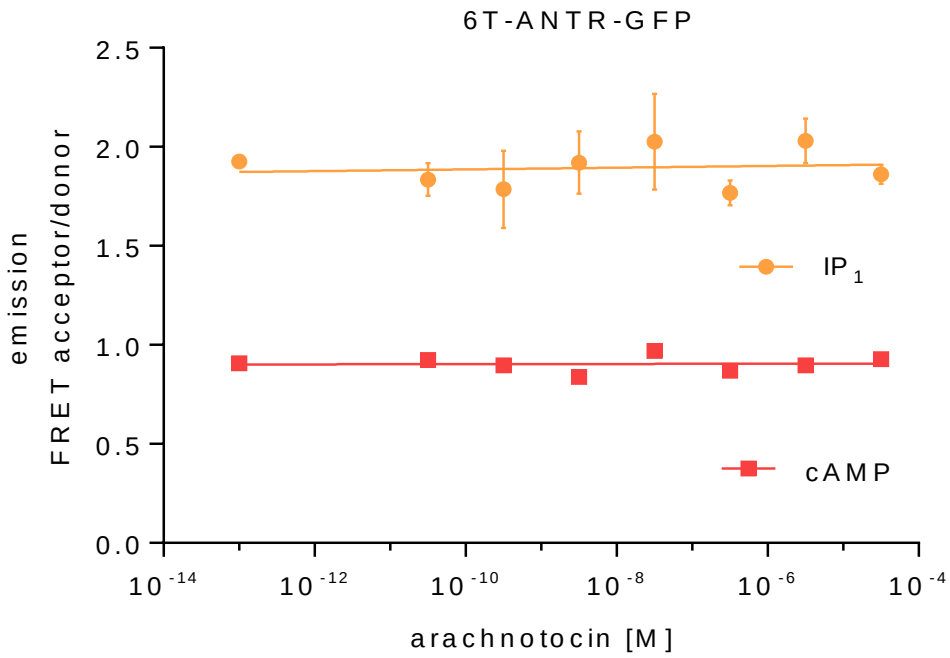

Figure 14: 6T-ANTR concentration response curve

Concentration response curve of ANT (30pM to 30µM) at 6T-ANTR-GFP expressed in HEK-293 cells as measured via cAMP and IP_1 assays. Data points were not normalized due to the lack of a concentration dependent effect and are therefore displayed as the ratio of FRET acceptor to donor emission. Error bars depict standard deviation. n = 3

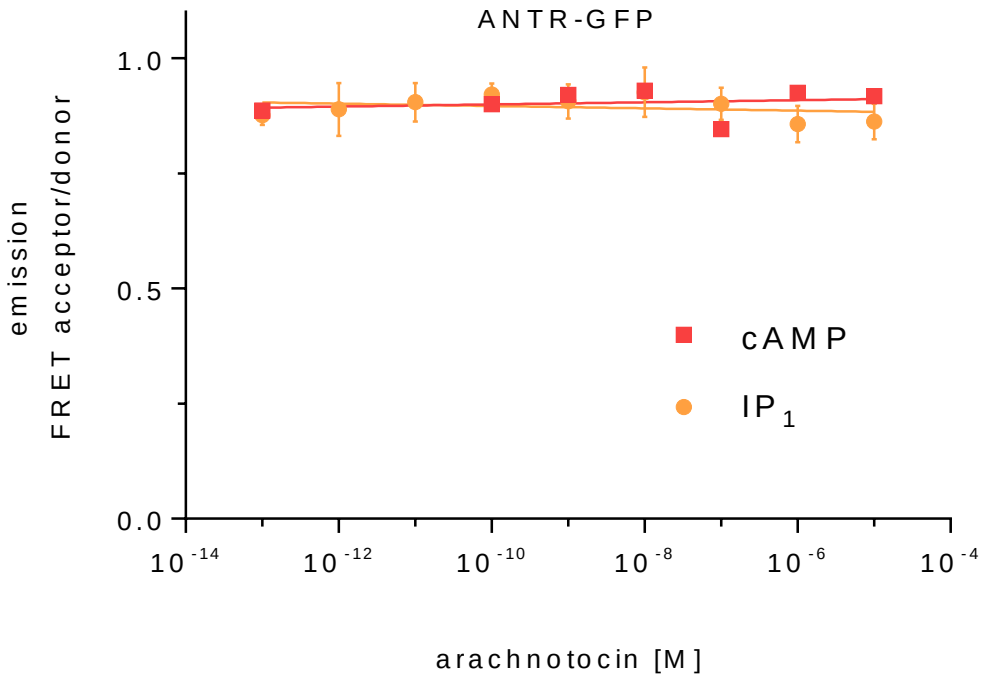

Figure 15: ANTR-GFP concentration response curve

Concentration response curve of ANT (30 pM to 30 µM) at ANTR-GFP expressed in HEK-293 cells as measured via cAMP and IP_1 assays. Data points could not be normalized due to the lack of a concentration dependent effect and are therefore displayed as the ratio of FRET acceptor to donor emission. Error bars depict standard deviation. n = 2

Figure 14 and **Figure 15** put the ratio of FRET acceptor emission to FRET donor emission (y-axis) measured in IP_1 and cAMP assays in relation to the concentration of arachnotocin (x-axis) used for stimulation of 6T-ANTR1-GFP-expressing HEK-293 cells. No concentration dependent response was detected for either receptor.

3.1.6 Receptor Localization

The above results prompted further investigation into the expression and localization of ANTR-GFP. Expression of the construct was examined 24 h after transfection in HEK-293 cells via fluorescence microscopy. As can be seen in **Figure 16**, the ANTR-GFP fusion protein did not primarily localize at the plasma membrane but appeared to be compartmentalized within the cell. In contrast, GFP on its own spreads evenly throughout the cell **Figure 17**.

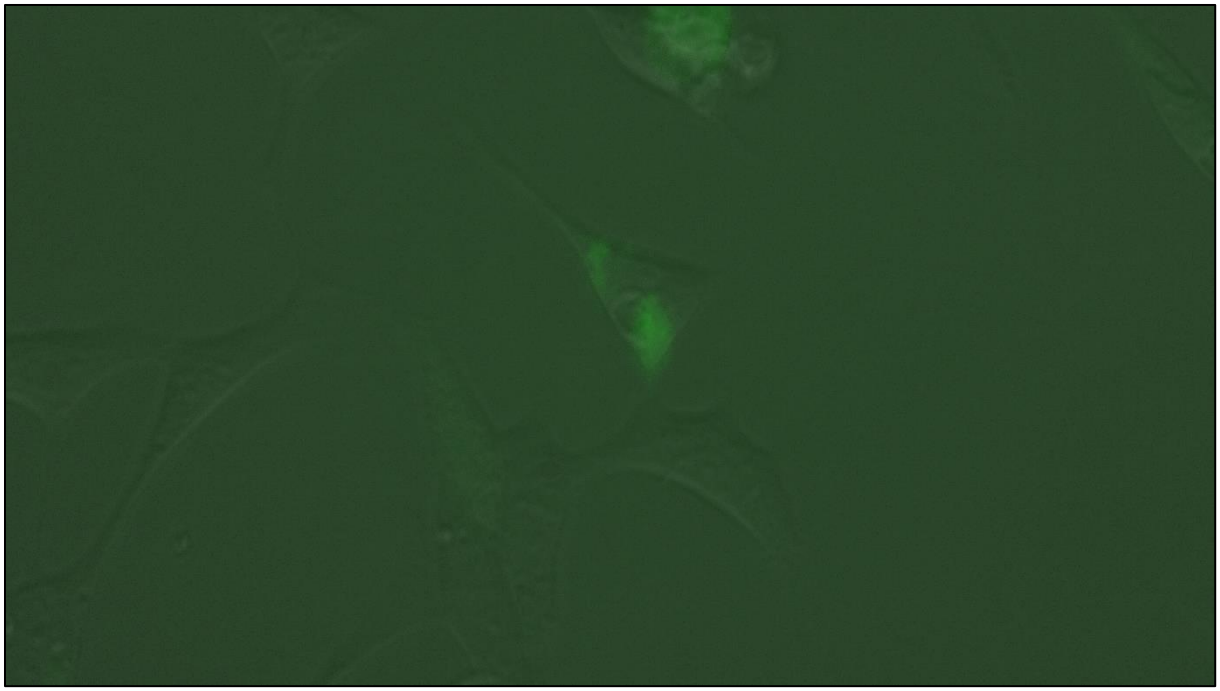

Figure 16: ANTR-GFP localization

Composed epifluorescence microscopy photography of HEK-293 cells two days after transient transfection with a ANTR-GFP expressing plasmid. Note that the fusion protein is located in specific parts of the cell. This indicates that the receptor is not being localized correctly on the cell membrane. Not all presented cells express the construct.

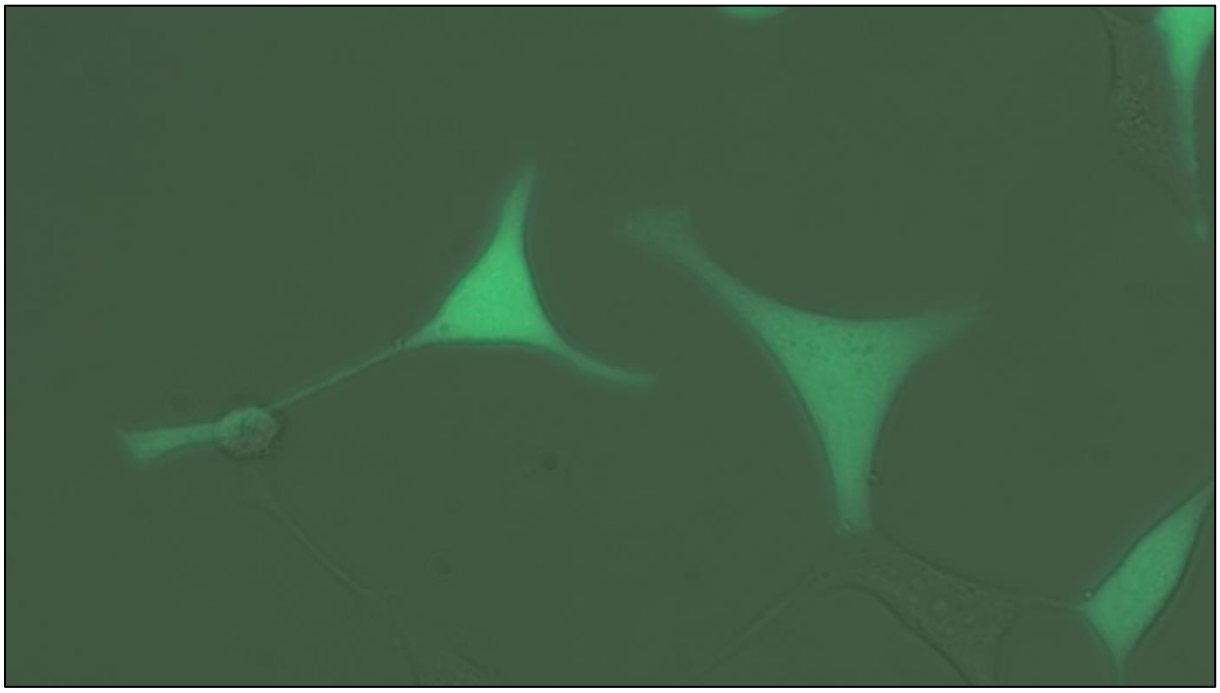

Figure 17: GFP localization

Composed epifluorescence microscopy photography of HEK-293 cells two days after transient transfection with a GFP expressing plasmid. The GFP signal (green) is spread out evenly throughout the cells. This strongly infers a cytosolic localization of the protein. Not all presented cells express the construct.

3.2 Peptide Pharmacology

3.2.1 Overview

This chapter presents the results of all practical work done on the pharmacological properties of *Varroa destructor* and *Asterias rubens* derived peptides on the human oxytocin/vasopressin GPCRs. Five different peptides were examined for their pharmacological properties: arachnotocin, asterotocin, [D-Glu8]-asterotocin, oxytocin and vasopressin. [D-Glu8]-asterotocin was designed due to promising results in a previous study substituting an amino acid in position eight in an oxytocin/vasopressin-like peptide with its respective D-amino acids (Di Giglio et al. 2017). The glutamate residue in position eight of this asterotocin analog was therefore substituted with D-glutamate.

Agonist activity assays with secondary messenger quantification kits were carried out to construct concentration response curves and to determine potency and efficacy of these peptides on the above-mentioned receptors. Additionally, the peptides were screened for antagonistic activity, followed by a closer investigation of arachnotocin antagonism on $hV_{1a}R$ via Schild regression analysis, to determine, whether this antagonism is mediated through an allosteric or a competitive mechanism.

3.2.2 hV$_{1a}$R

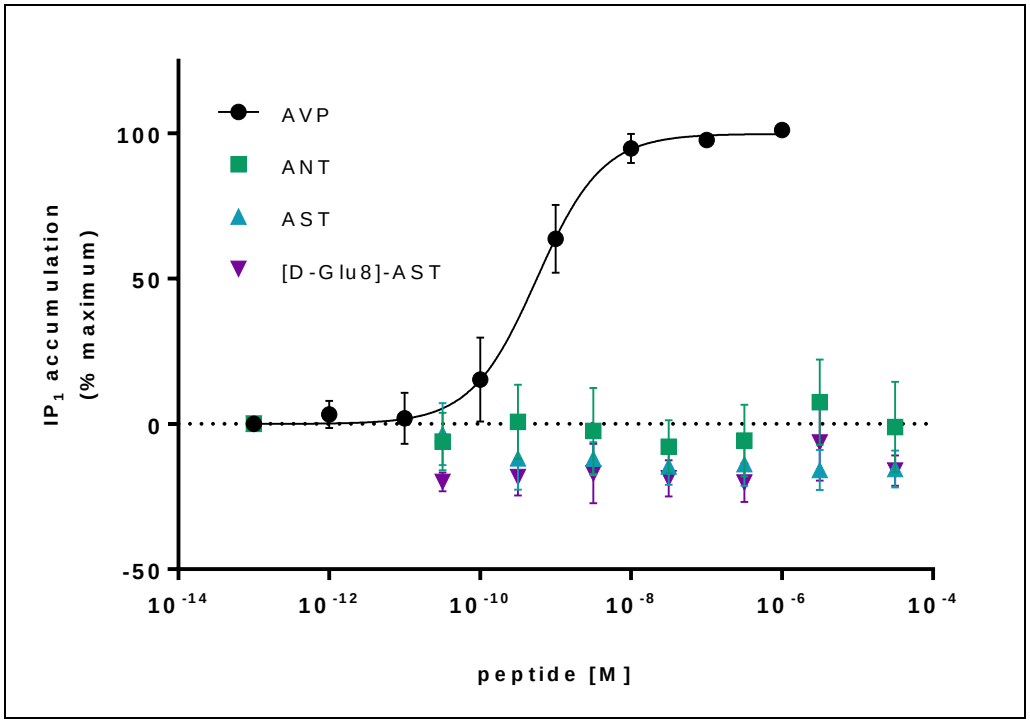

Figure 18: hV$_{1a}$R, concentration response curves

Concentration response curve of ANT, AST and [D-Glu8]-AST (30 pM to 30 µM) at the human V$_{1a}$R. Receptor induction was normalized to accumulation of IP$_1$ above baseline. Data points were fitted by nonlinear regression curves (sigmoidal, slope =1). Error bars depict standard deviation. n=3

As demonstrated in **Figure 18**, no IP$_1$ response could be detected for ANT, AST and [D-Glu8]-AST at the human V$_{1a}$R up to a concentration of 30 µM peptide.

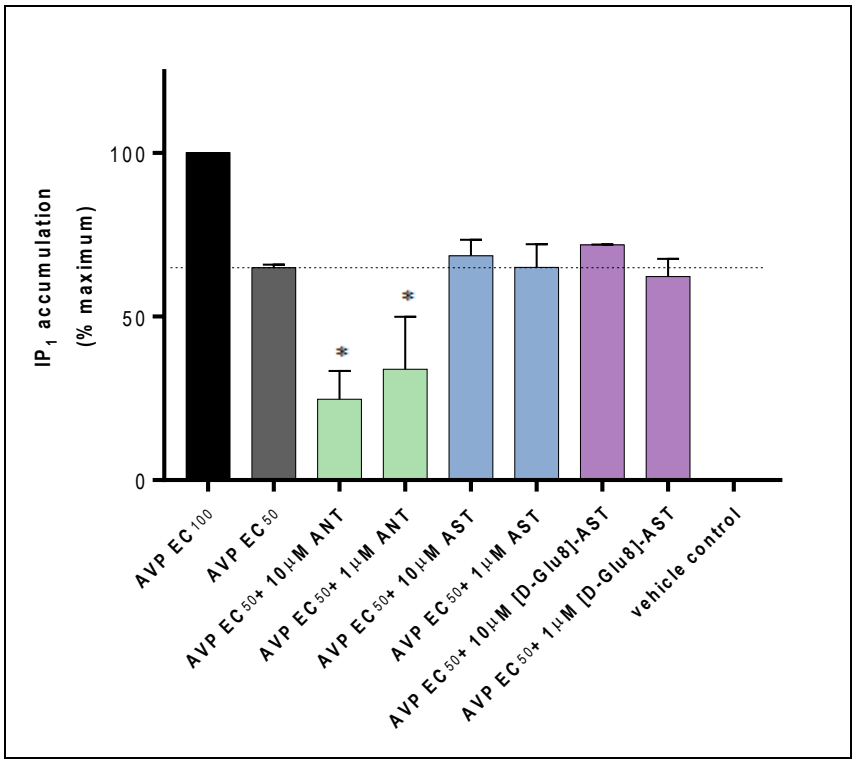

Figure 19: hV$_{1a}$R, antagonist activity screen

*Accumulation of IP$_1$ by stimulation of human V$_{1a}$R with AVP (EC$_{50}$) in the presence of 10 µM and 1µM ANT, AST and [D-Glu8]-AST. Data was normalized to accumulation of IP$_1$ above baseline (=0%) and accumulation of IP$_1$ in the presence of AVP EC$_{100}$ (=100%). Error bars depict standard deviation. *: significant antagonistic activity (p-value < 0.05), n=2*

As depicted in **Figure 19**, no antagonistic activity of AST and [D-Glu8]-AST was detected at hV$_{1a}$R in the preliminary screen up to a concentration of 10µM. However, arachnotocin partially inhibited the IP$_1$ response to activation of hV$_{1a}$R at a concentration of 1 and 10 µM in a seemingly concentration depended manner. To gain further insight into the nature of this effect, this interaction was therefore further investigated utilizing a Schild regression analysis (3.3 Schild regression analysis of arachnotocin on hV$_{1a}$R, page 71).

3.2.3 hV$_{1b}$R

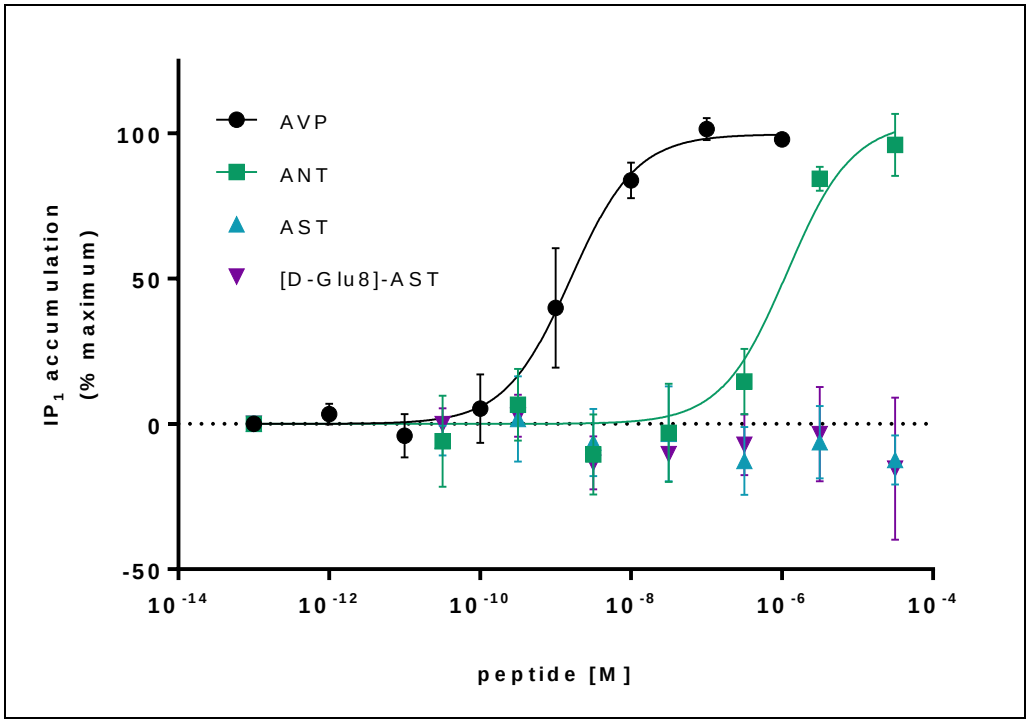

Figure 20: hV$_{1b}$R, concentration response curves

Concentration response curve of ANT, AST and [D-Glu8]-AST (30pM to 30µM) at the human V$_{1b}$R. Receptor induction was normalized to accumulation of IP$_1$ above baseline. Data points were fitted by nonlinear regression curves (sigmoidal, slope =1). Error bars depict standard deviation. n=3

As depicted in **Figure 20**, ANT is a full agonist at the human V$_{1b}$R with a potency of ~1 µM (EC$_{50}$), which is a decrease of about three orders of magnitude in potency compared to AVP (EC$_{50}$ ~1.5 nM). No IP$_1$ response could be detected for AST and [D-Glu8]-AST at a concentration up to 30 µM.

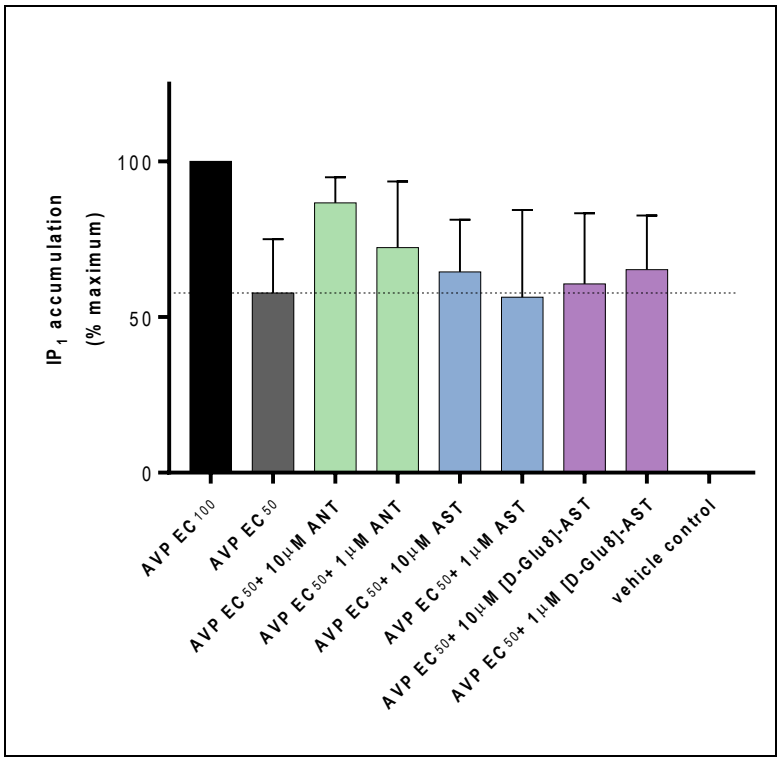

Figure 21: hV$_{1b}$R, antagonist activity screen

*Accumulation of IP$_1$ by stimulation of human V$_{1b}$R with AVP (EC$_{50}$) in the presence of 10µM and 1µM ANT, AST and [D-Glu8]-AST. Data was normalized to accumulation of IP$_1$ above baseline (=0%) and accumulation of IP$_1$ in the presence of AVP EC$_{100}$ (=100%). Error bars depict standard deviation. *: significant antagonistic activity (p-value < 0.05), n=3*

As depicted in **Figure 21**, no significant antagonistic activity was detected of and [D-Glu8]-AST at hV$_{1b}$R up to a concentration of 10 µM. As would be expected from previous results (**Figure 20**), ANT is an agonist at this receptor at 10 µM, whereas no significant agonism could be detected at 1 µM (P-value 0.3292).

3.2.4 hV$_2$R

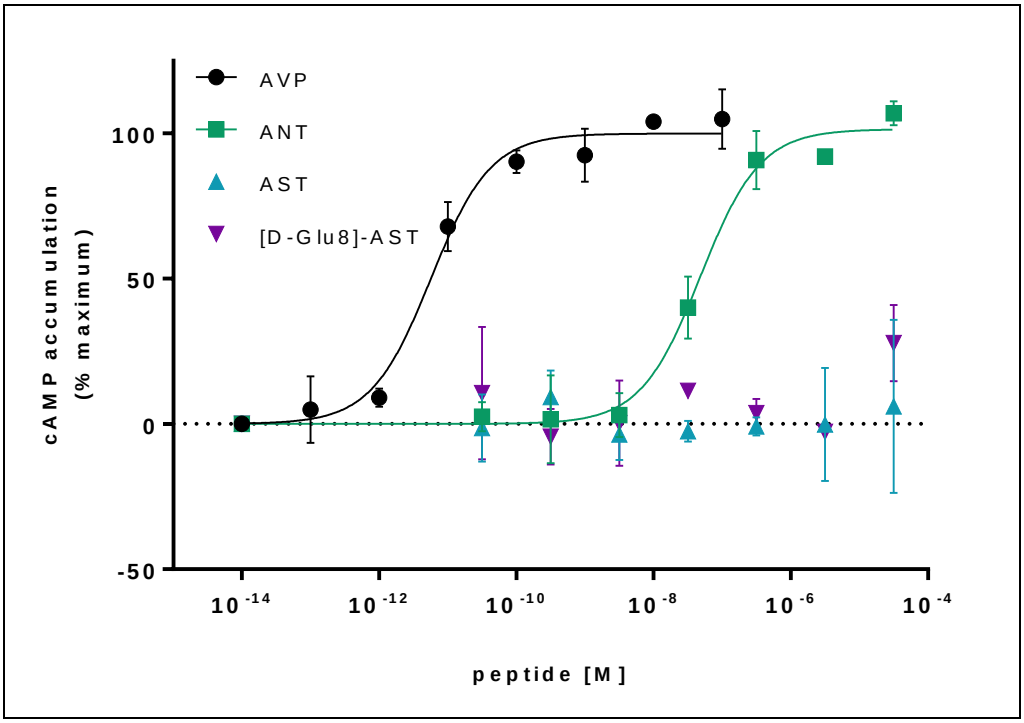

Figure 22: hV$_2$R, concentration response curves

Concentration response curve of ANT, AST and [D-Glu8]-AST (30pM to 30µM) at the human V$_2$R. Receptor induction was normalized to accumulation of cAMP above baseline. Data points were fitted by nonlinear regression curves (sigmoidal, slope =1). Error bars depict standard deviation. n=2

ANT is a full agonist at the human V2R (**Figure 22**) with a potency of 50 nM, which is a decrease of roughly four orders of magnitude in potency compared to AVP (EC$_{50}$ ~5.5 pM). No significant agonist activity was found for AST and [D-Glu8]-AST.

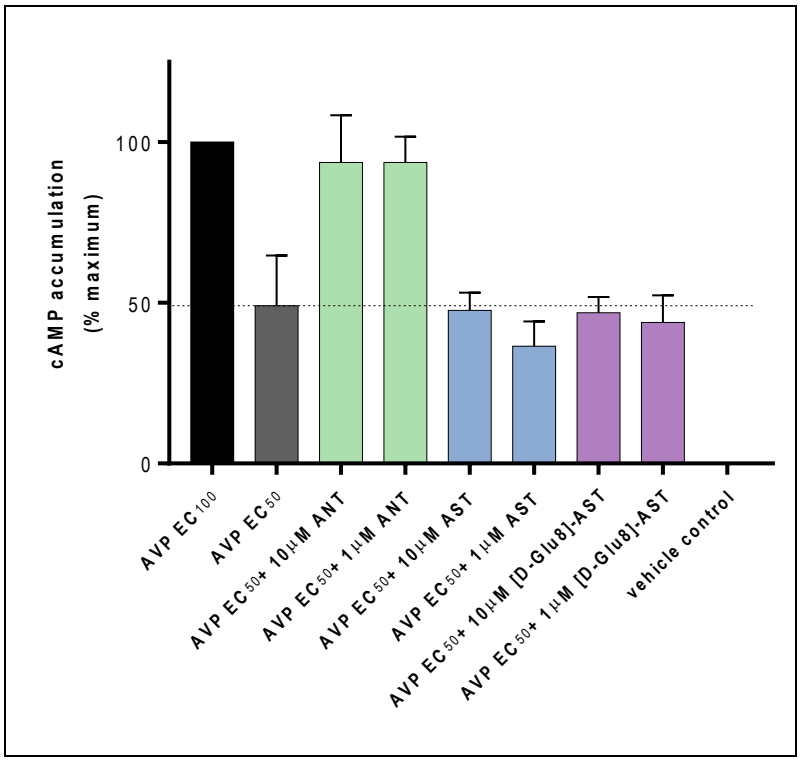

Figure 23: hV$_2$R, antagonist activity screen

*Accumulation of cAMP by stimulation of human V$_2$R with AVP (EC$_{50}$) in the presence of 10µM and 1µM ANT, AST and [D-Glu8]-AST. Data was normalized to accumulation of cAMP above baseline (=0%) and accumulation of cAMP in the presence of AVP EC$_{100}$ (=100%). Error bars depict standard deviation. *: significant antagonistic activity (p-value < 0.05), n=3*

Again, no antagonistic activity of AST and [D-Glu8]-AST was detected at hV$_2$R up to a concentration of 10µM (**Figure 23**). In accordance with previous results (**Figure 22**), ANT is a full agonist at hV$_2$R at 1 and 10M, thereby increasing the response of hV$_2$R in presence of AVP (EC$_{50}$) compared to hV$_2$R stimulation with AVP (EC$_{50}$) on its own.

3.2.5 OTR

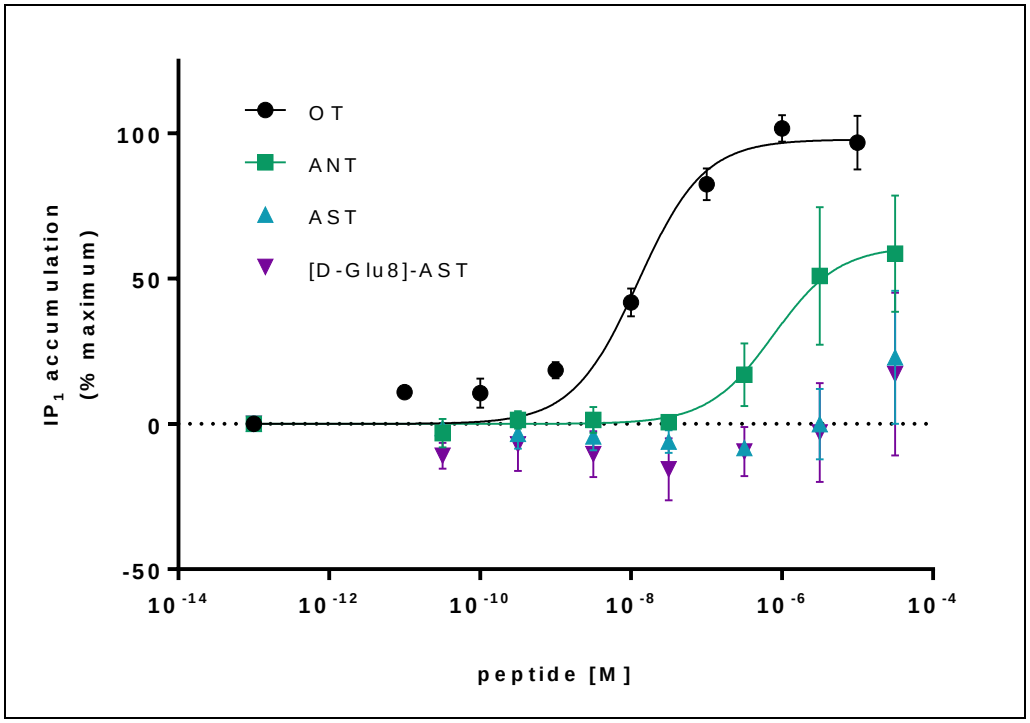

Figure 24: OTR, concentration response curves

Concentration response curve of ANT, AST and [D-Glu8]-AST (30 pM to 30 µM) at the human OTR. Receptor induction was normalized to accumulation of IP_1 above baseline. Data points were fitted by nonlinear regression curves (sigmoidal, slope =1). Error bars depict standard deviation. n=3

ANT is a partial agonist with an apparent E_{max} of 60% at the human OTR and a potency of ~770 nM, which is a decrease of approximately two orders of magnitude in potency compared to the endogenous ligand (EC_{50} ~13 nM). No notable agonist activity was found for AST and [D-Glu8]-AST at concentrations below 30 µM.

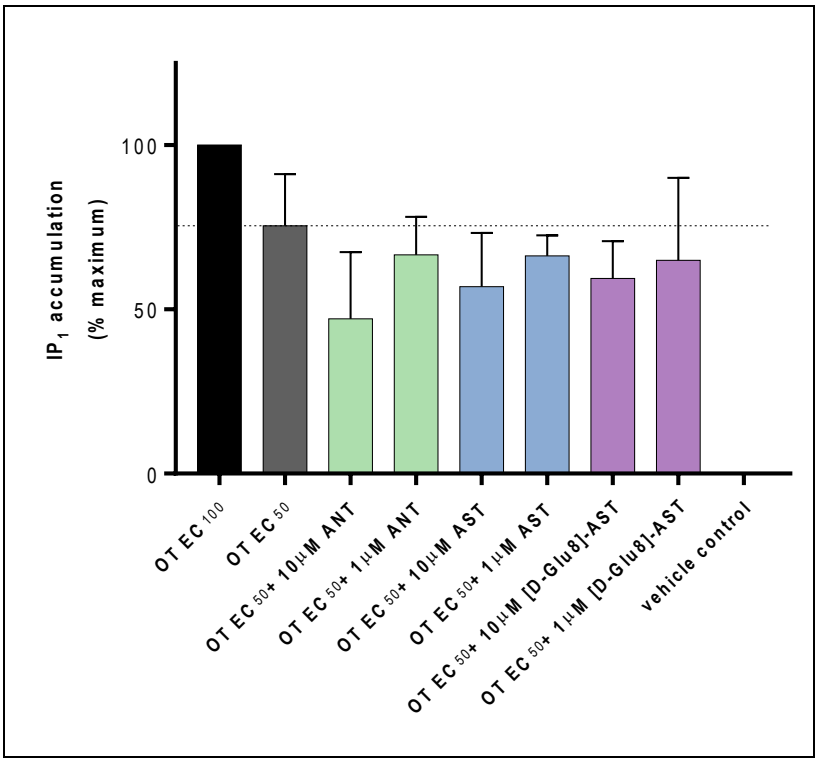

Figure 25: OTR, antagonist activity screen

*Accumulation of IP_1 by stimulation of human OTR with AVP (EC_{50}) in the presence of 10µM and 1µM ANT, AST and [D-Glu8]-AST. Data was normalized to accumulation of IP_1 above baseline (=0%) and accumulation of IP_1 in the presence of AVP EC_{100} (=100%). Error bars depict standard deviation. *: significant antagonistic activity (p-value < 0.05), n=3*

AST (P-value 0.1526 and 0.3178) and [D-Glu8]-AST (P-value 0.1489 and 0.3178) fail to exhibit any antagonistic effect up to a concentration of 10 µM at the human oxytocin receptor (**Figure 25**). In presence of oxytocin, ANT appears to act as a weak antagonist, which is to be expected from a partial agonist, however this result is not significant (P-value 0.0693 and 0.3978).

3.3 Schild regression analysis of arachnotocin on $hV_{1a}R$

3.3.1 Overview

Schild regression analysis is a pharmacological method for the classification of the effects of agonists and antagonists on cellular responses mediated via ligand-receptor interactions (Arunlakshana and Schild 1959). Competitive antagonists compete with an agonist for the active site of the receptor, thus displacing the agonist in a manner that is depending on both the concentration of the agonist and the antagonist. Allosteric antagonists however modulate the receptor by binding at another site than the active site, thereby regulating the response in a manner that is independent of the agonist concentration. The Schild regression analysis facilitates the determination of the pA_2 value of a competitive antagonist, which is the measure of the functional affinity for its target receptor and therefore a measure of the inhibitory potency of a drug. More precisely, it is the negative logarithm of the required molar concentration of the antagonist to produce a two-fold shift to the right in an agonist concentration response curve. The pA_2 is quantified via concentration response curves of the endogenous ligand at a fixed concentration in absence and presence of the antagonist at various concentrations. For competitive antagonists, this results in a dextral shift of the concentration response curves, that is proportional to the antagonist concentration, while the E_{max} remains unaffected.

The Schild regression plot itself is a double logarithmic plot, relating the agonist dose-ratio of the EC_{50} of the agonist in presence (A´) and absence (A) of the antagonist as log(A´/A-1) against the logarithm of the antagonist concentration (-logB). The slope is hereby expected to be 1 for competitive antagonist, indicating that both agonist and antagonist are acting on the same site of the receptor. A slope >1 indicates inactivation or uptake of the antagonist, as the activity of the antagonist is reduced disproportionally compared to the concentration. A slope <1 on the

other hand indicates inactivation or uptake of the agonist. The pA_2 value is the intersection of a linear regression on this plot with the abscissa (-logB).

3.3.2 Schild results

Schild regression analysis was performed on the human $hV_{1a}R$ with AVP at an EC_{50} in presence of 1, 3 and 10 µM ANT using the Cisbio HTRF IP_1 kit. The corresponding concentration response curves on the Schild plot (**Figure 26**) were normalized to the maximum activation by the endogenous peptide (=100%) and a vehicle control (=0%).

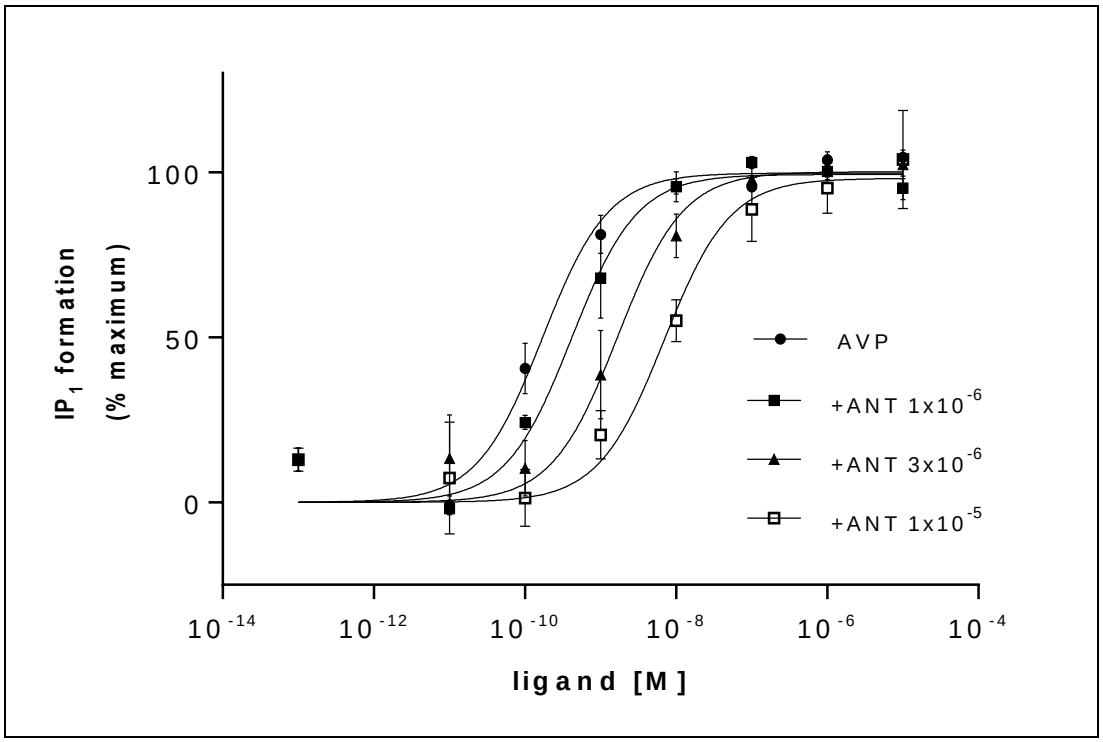

Figure 26: Schild plot of ANT at $hV_{1a}R$

Accumulation of IP_1 by stimulation of human $V_{1a}R$ with AVP (EC_{50}) in the presence of 10µM, 3µM, 1µM and 0µM ANT. Receptor induction was normalized to accumulation of IP_1 above baseline. Data points were normalized to the maximum (100%) and minimum (0%) response generated by AVP and fitted by nonlinear regression curves (sigmoidal, slope =1). Error bars depict standard deviation. n=3

The concentration response curves above are dextrally displaced in an ANT concentration dependent manner, whereas the E_{max} of all curves remains the same. This strongly suggest that ANT acts as a competitive antagonist to AVP on $hV_{1a}R$. This assumption is further substantiated by the structural similarities between both ligands and their shared evolutionary background. The regression in the Schild regression analysis below (**Figure 27**), is linear with a slope of ~1.5. A non-linear regression curve would indicate a not purely competitive antagonist, while a linear regression indicates a competitive antagonist. The pA_2 of ANT on $hV_{1a}R$ is approximately 800 nM.

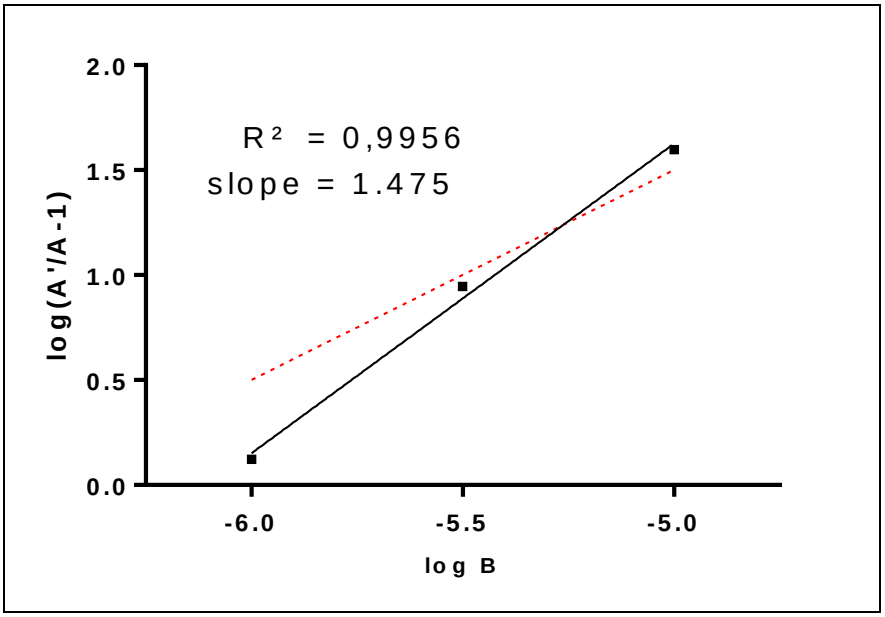

Figure 27: Schild regression plot of ANT at hV$_{1a}$R

This graph plots the agonist dose-ratio of the EC$_{50}$ of the agonist in presence (A') and absence (A) of the antagonist as log(A'/A-1) against the logarithm of the antagonist concentration (logB). The slope equals 1.475 indicating a disproportional loss of activity of the antagonist with reduced antagonist concentration (R^2 = 0.9956). The dotted red line represents a reference line with a slope of 1.

3.4 Structure-activity relationship of the investigated peptides

3.4.1 Overview

In the following chapter, I will compare the amino acid sequences and 2D-structures of the examined peptide hormones and put them into relationship with their biological activity. This is referred to as a structure-activity relationship and may serve as a starting point for the targeted modification of molecules to obtain specific, desired properties.

3.4.2 Multiple Sequence Alignment

Asterotocin, D-[Glu8]-asterotocin, arachnotocin, oxytocin and vasopressin were aligned using Clustal Ω as described in the methods section.

```
[D-Glu8]-Asterotocin     CLVQDCPeG
Asterotocin              CLVQDCPEG
Arachnotocin             CFITNCPIG
Oxytocin                 CYIQNCPLG
Vasopressin              CYFQNCPRG
                         *  .  :** *
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Figure 28: Multiple sequence alignment of the examined neuropeptides

Multiple sequence alignment of [D-Glu8]-asterotocin, asterotocin, arachnotocin, oxytocin and vasopressin using Clustal Ω.Green letters represent glycine-, amine-, sulfhydryl- and hydroxyl residues, red letters represent small and hydrophobic residues, blue letters represent acidic residues, while magenta letters represent basic residues. Lower case letters represent D-amino acids. Asterisks indicate single, fully conserved residues, colons indicate strongly conserved residue, whereas periods indicate residues with weakly similar properties (Gonnet PAM 250 matrix score >0.5 and =< 0.5 respectively).

3.4.3 Commonalities

All peptides display the canonical features of oxytocin/vasopressin-like peptides. They are nonapeptides with a disulfide bond between the cysteine residues and an amidated three residue C-terminal tail. Furthermore, proline and glycine, in position seven and nine respectively, are conserved across all investigated peptides as well.

3.4.4 Asterotocin and [D-Glu8]-asterotocin

The complete absence of any effect, agonistic or antagonistic, of asterotocin and [D-Glu8]-asterotocin on the human oxytocin/vasopressin receptors, indicates, that the discrepancies between these peptides and the endogenous neuroendocrine peptide hormones prevent any interaction with the human receptors. Meanwhile, both asterotocin and [D-Glu8]-asterotocin are full agonists at the *Asterias rubens* asterotocin receptor, with [D-Glu8]-asterotocin displaying a lower potency (EC_{50} ~1 µM) compared to the endogenous ligand (EC_{50} ~75 nM).

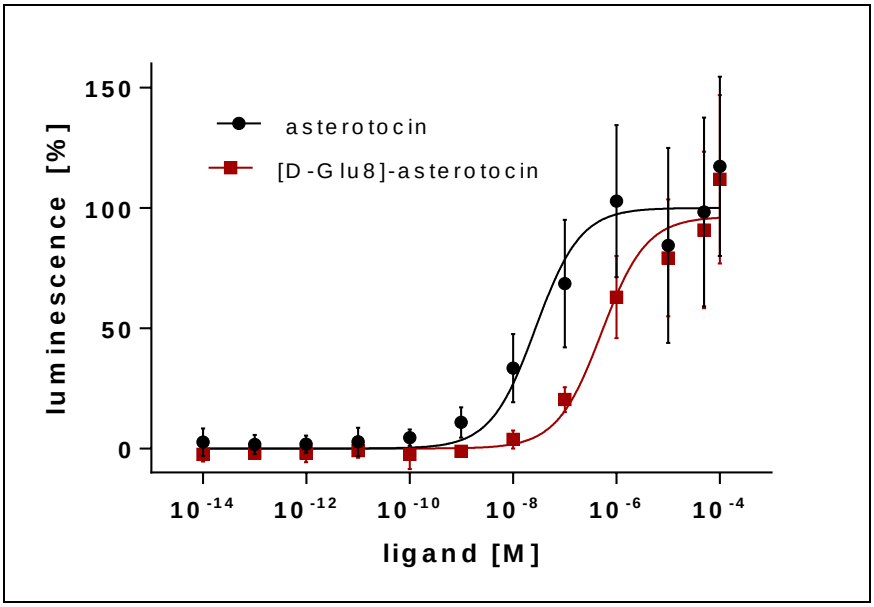

Figure 29: asterotocin receptor, concentration response curve

Concentration response curve of asterotocin and [D-Glu8]-asterotocin on the Asterias rubens asterotocin receptor. Data was normalized to luminescence above baseline (=0%) and maximum luminescence in presence of asterotocin (=100%). [D-Glu8]-asterotocin is a full agonist on the asterotocin receptor with a reduced potency (EC_{50} ~1 µM) compared to the endogenous ligand (EC~74 nM). Kindly provided by Esther Adeiye Odekunle. And Maurice Elphick, Queen Mary University of London, Mile End Road, London E1 4NS.

The most notable difference of asterotocin when compared to the human peptides is the reversal of charge in position eight, replacing a positively charged arginine (vasopressin) or neutrally charged leucine (oxytocin) with a negatively glutamic acid. Another change in charge is an aspartic acid residue in position five, replacing an uncharged asparagine. Positively charged residues in the binding pocket of $V_{1a}R$ (Glu1x35and Asp2x65)[14] strongly interact with the

[14] Generic residue numbers from the GPCR database, GPCRdb.org (Isberg & et al, 2015)

arginine residue in position eight of vasopressin (Di Giglio et al. 2017). Accordingly, the positive charge of asterotocin in position eight results in a dramatically reduced interaction between ligand and receptor. This effect seems to be independent of the exact stereochemical configuration of this residue.

Furthermore, asterotocin includes a leucin instead of a tyrosine in position two. The same substitution can be observed in inotocin. The replacement of the polar amino acid in position two can also be found in annetocin, phenypressin, nematocin, octopressin and arachnotocin.

3.4.5 Arachnotocin

Arachnotocin is a partial agonist at the human oxytocin receptor with reduced potency and efficacy compared to oxytocin (**Figure 24**). Additionally, it is a full agonist at the human vasopressin receptor 1b (**Figure 20**) and 2 (**Figure 22**) with reduced potency but an antagonist at the human vasopressin receptor 1a (**Figure 18**). Arachnotocin deviates from oxytocin in three positions and from vasopressin in four.

Table 13 comparison of the potency of ANT and native ligands on human receptors

	EC_{50} endogenous ligand	**EC_{50} ANT**
$V_{1a}R$	~22 nM	-
$V_{1b}R$	~1.5 nM	~1 µM
V_2R	~5.5 pM	~50 nM
OTR	~13 nM	~770 nM

```
Arachnotocin            CFITNCPIG
Oxytocin                CYIQNCPLG
Vasopressin             CYFQNCPRG
                        *  .  :** *
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Figure 30: Multiple sequence alignment of the examined neuropeptides

Multiple sequence alignment of arachnotocin, oxytocin and vasopressin using Clustal Ω.Green letters represent glycine-, amine-, sulfhydryl- and hydroxyl residues, red letters represent small and hydrophobic residues, blue letters represent acidic residues, while magenta letters represent basic residues. Lower case letters represent D-amino acids. Asterisks indicate single, fully conserved residues, colons indicate strongly conserved residue, whereas periods indicate residues with weakly similar properties (Gonnet PAM 250 matrix score >0.5 and =< 0.5 respectively).

In position two, arachnotocin has a phenylalanine instead of a tyrosine in oxytocin and vasopressin. In position four, a threonine is present instead of a glutamine in oxytocin and vasopressin. The last disparity to oxytocin is an isoleucine in position eight instead of a leucine.

Concerning the differences between vasopressin and arachnotocin, there are two additional substitutions to the two “common” changes in position two and four. Isoleucine residues replace the phenylalanine in position three and the arginine in position eight.

4 Discussion

4.1 Investigation of the *Varroa destructor* arachnotocin receptor

The initial bioinformatic search identified two putative arachnotocin receptors in *Varroa destructor*, however, only one of them was investigated within this thesis, as the other arachnotocin receptor sequence could not be identified at full length.

Concerning the other arachnotocin receptor, both the truncated 6T-ANTR-GFP as well as the ANTR-GFP construct were successfully cloned from *Varroa destructor* cDNA. However, ANTR-GFP failed to localize correctly to the plasma membrane when expressed in HEK-293 cells (**Figure 16**). This failure may well be a result of the introduced GFP tag, which may impair the correct folding of the receptor, resulting in a retention of the constructs in the endoplasmic reticulum. No additional experiments could be performed with the untagged receptor due to time constraints, thus the expected interaction between arachnotocin and this receptor could not be confirmed.

It should be noted that there is a significant difference in the IP_1 accumulation measured for 6T-ANTR-GFP and ANTR-GFP, even though no arachnotocin concentration dependent effect could be observed (**Figure 14 and Figure 15**). This is simply the result of assay optimization and cell count adjustment between the two experiments.

Further investigation into the function of this, as well as the second putative arachnotocin receptor, may yield valuable insight into the neuroendocrinology of the *Varroa* mite. This is of importance because of the sever economic impact on the bee-keeping industry in North America and Europe by the mite (Guzmán-Novoa et al. 2010; Cox-Foster et al. 2007; Abbo et al. 2017). Considering the absence of an oxytocin/vasopressin orthologue in bees, this may well act as a potential target for the treatment of *Varroa* infestations, as oxytocin/vasopressin like peptides are not only involved in water homeostasis (Gruber 2014) but also implied to play

important roles in regulating metabolic processes and locomotion (Liutkeviciute, et al, yet unpublished results). Recently, an already commercially available drug, atosiban was discovered to be an antagonist of the *Tribolium castaneum* receptor (Keov et al, yet unpublished results). Perhaps a similar approach may work for the rapid development of novel arachnotocin receptor ligands.

4.2 Human oxytocin/vasopressin receptor pharmacology

4.2.1 On the importance of novel ligands for the OT/AVP system

The oxytocin/vasopressin signaling system has been under investigation for almost a century (Dale 1906) and plays a crucial role in a vast number of physiological functions and psychological behaviors, such as attachment (Insel and Young 2001), anxiety-related behaviour (Landgraf 2006; Liebsch et al. 1996), fear conditioning (Eckstein et al. 2015), aggression (Bosch et al. 2005; Wersinger et al. 2002), social recognition (Bielsky and Young 2004; Stoop 2016), memory and learning (Alescio-Lautier and Soumireu-Mourat 1998). It is furthermore involved in various disorders and diseases including social anxiety disorder, autism, schizophrenia (Goldman et al. 2008; Keri, Kiss, and Kelemen 2008), borderline personality disorder, stress, depression (Scantamburlo et al. 2007; McQuaid et al. 2014), preterm-labor, cardiovascular diseases, and cancer (Meyer-Lindenberg et al. 2011). Additionally, discriminating between the involved receptors when it comes to elucidating their functions is still challenging, due to selectivity issues (Gruber, Koehbach, and Muttenthaler 2012; Manning et al. 2012). Consequently there is still an unsatisfied need for the development of novel, highly selective ligands for the four GPCRs involved in this system.

In most cases of drug development, small molecule drugs are favored over peptide drugs, due to their low production cost, more efficient drug delivery and oral bioavailability. In case of the oxytocin/vasopressin signaling system however, the ability of peptides to interact with proteins on a large surface area as well as their structural and chiral complexity may put them at an advantage over small molecule drugs as these features increase specificity and limit side effects (Gruber, Koehbach, and Muttenthaler 2012). Several big companies such as Pfizer and Merck have already halted their efforts to develop non-peptide antagonists for the involved receptors

due to selectivity issues (Manning et al. 2012). Furthermore, the strong evolutionary conservation of oxytocin/vasopressin-like peptides and their receptors can be leveraged to provide starting points for the development of novel ligands without the need to screen thousands of different compounds from synthetic libraries. The recent development of a selective $hV_{1a}R$ antagonist based on the oxytocin/vasopressin orthologue inotocin from *Lasius niger* highlights the potential of highly conserved homologous neuropeptides as a starting point for drug development (Di Giglio et al. 2017).

Inspired by these findings, this thesis set out to examine whether this strategy would be transferable to other evolutionarily related oxytocin/vasopressin-like peptides. *Lasius niger* is a member of the Insecta class in the Arthropod phylum, it was therefore decided to investigate the oxytocin/vasopressin-like peptide arachnotocin form *Varroa destructor*, as this species is a still a member of the Arthropod phylum but of the Arachnida class. Additionally, a neuropeptide from the Echinodermata phyla, asterotocin and a derivative thereof ([D-Glu8]-AST) were investigated. These three peptides were probed for their pharmacological properties on all four GPCRs of the human oxytocin/vasopressin signaling system (3.2 Peptide Pharmacology, page 62).

4.2.2 Arachnotocin is a competitive inhibitor of $hV_{1a}R$

Concentration response curves and antagonist screens revealed that arachnotocin acts as an antagonist at the $hV_{1a}R$ (**Figure 18** and **Figure 19**). A subsequent Schild regression analysis indicated that arachnotocin is a competitive antagonist at this receptor, as the performed Schild regression analysis was linear (**Figure 27**). However, the slope of this analysis did not equal 1, but ~1.5. A slope greater than 1implies that the activity of the antagonist is reduced disproportionally strongly compared to its concentration via update or inactivation. It should be noted

though, that this slope may also be a consequence of insufficient data quality and future experiments should be undertaken with an increased concentration range, a higher number of data points per curve and a larger variety of antagonist concentrations. The pA_2 of ANT on $hV_{1a}R$ as determined in this thesis is approximately 800 nM.

Arachnotocin deviates from vasopressin in four positions: In position two arachnotocin contains phenylalanine instead of a tyrosine, threonine replaces a glutamine in position four, and isoleucine residues replace the phenylalanine in position three and the arginine in position eight. The change from tyrosine to phenylalanine in position two conserves a relatively hydrophobic side change, whereas the substitution of glutamine to threonine conserves the polar uncharged side chains in this position. The first of these isoleucines conserves the non-polarity of the phenylalanine. All three of these changes may however introduce minor changes to the stereochemistry. The second isoleucine substitution at position eight is more significant, as it removes charge and polarity provided by arginine and replaces them with another non-polar isoleucine.

The selective antagonistic effect of arachnotocin on $hV_{1a}R$ is presumably caused by the isoleucine residue in position eight, as vasopressin contains a arginine residue in this position that strongly interacts with two positively charged residues in the binding pocket of $V_{1a}R$: Glu1x35[15] and Asp2x65[16] (Di Giglio et al. 2017). This once again underlines the importance of residue eight for receptor recognition, which has already been shown to be a strong contributor to receptor selectivity and high affinity ligand binding (Chini, 1997).

[15] Generic residue numbers from the GPCR database, GPCRdb.org (Isberg & et al, 2015)

[16] Generic residue numbers from the GPCR database, GPCRdb.org (Isberg & et al, 2015)

4.2.3 Arachnotocin activates $V_{1b}R$, V_2R and OTR

In contrast to its effect on $V_{1a}R$, arachnotocin activates the other three receptors of the human oxytocin/vasopressin signaling system to varying degrees. On $hV_{1b}R$ (**Figure 20**) and hV_2R (**Figure 22**) arachnotocin is a full agonist (EC_{50} ~1 µM and ~50nM) with a one hundred or a thousand fold reduction in potency respectively when compared to vasopressin.

At OTR arachnotocin acts as a partial agonist with an E_{max} of ~60% and a reduced potency of ~800nM when compared to oxytocin (EC_{50} ~10 nM) (**Figure 24**). Partial agonists are agonists that elicit activity below maximum level, though they can appear as full agonists when a receptor reserve is present (Wacker, Stevens, and Roth 2017). This finding is consistent with **Figure 25**, which depicts ANT acting as an antagonist in the presence of oxytocin, as partial agonists generally behave like competitive antagonists in presence of full agonist. This effect can be explained through the concept of fractional occupancy: The partial agonist competes with the full agonist for receptor binding. An increase in partial agonist concentration increases the fraction of receptor bound to the partial agonist and decreases the fraction of receptor bound to the full agonist. Since the partial agonist has a lower efficacy than the full agonist, the activity of the receptor therefore decreases.

Compared to oxytocin, arachnotocin contains a phenylalanine in position two (instead of a tyrosine), threonine in position four (instead of glutamine) and isoleucine in position eight (instead of a leucine). Together these three changes result in a potency loss of about two orders of magnitude and about 50% reduced efficacy at the human oxytocin receptor. The arguable most dramatic of those changes is the change in polarity, replacing a polar tyrosine with a less polar phenylalanine residue. The other two substitutions conserve charge and polarity but may result in a less then optimal stereochemical properties for receptor binding.

4.2.4 Asterotocin does not interact with human OT/AVP receptors

Both asterotocin and [D-Glu8]-asterotocin failed to elicit a quantifiable response in secondary messengers (IP_1 and cAMP) when investigated for agonistic or antagonistic properties on any of the four receptors up to a concentration of 10 µM (3.2 Peptide Pharmacology, page 62). A closer look at the structure of these two peptides (3.4 Structure-activity relationship of the investigated peptides, page 7562) revealed that the positive charge in the critical position eight probably disrupts the interaction of the peptides with important residues in the binding pocket, as it has previously been demonstrated, that positively charged residues in the binding pocket of $V_{1a}R$ (Glu1x35and Asp2x65)[17] interact with the arginine residue in position eight of vasopressin (Di Giglio et al. 2017). This effect can be observed independent of the stereochemical configuration of the glutamic acid residue in position eight. The presence of leucin in position two as compared to tyrosine in OT and AVP may to have a much smaller effect on the interaction with the human receptors, as this substitution is also contained in inotocin.

[17] Generic residue numbers from the GPCR database, GPCRdb.org (Isberg & et al, 2015)

4.2.5 The future of peptide compounds for the OT/AVP system

Despite progress in the development of probes for the modulation of oxytocin/vasopressin receptor signaling, there is a continuing need for selective ligands to facilitate the differentiation of the biological functions of the involved receptors and provide highly targeted therapeutics for diseases associated with the oxytocin/vasopressin system (Manning et al. 2008, 2012; Lacivita et al. 2017; Guzmán-Novoa et al. 2010; Cataldo, Azhari, and Esposito 2018; Gruber, Koehbach, and Muttenthaler 2012). This thesis presents yet another orthologous oxytocin/vasopressin-like neuropeptide (Di Giglio et al. 2017) with a functional dichotomy on the human oxytocin/vasopressin receptors. Considering the ubiquity of oxytocin/vasopressin-like peptides, additional orthologues are bound to be discovered in the future (Gruber 2014; Hoyle 1999; Koehbach et al. 2013; Liutkeviciute et al. 2016). Screenings of these might discover antagonists for receptors other than $V_{1a}R$, which may serve as a steppingstone towards the development of novel, highly selective ligands for research purposes and the novel treatments for autism (Lacivita et al. 2017), depression (Scantamburlo et al. 2007; McQuaid et al. 2014), schizophrenia (Keri, Kiss, and Kelemen 2008; Goldman et al. 2008) and preterm-labor (Meyer-Lindenberg et al. 2011).

5 References

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6 Appendix

6.1 *Varroa destructor* BLAST and alignments

6.1.1 6T-ANTR WGS sequence

>gi|283709968|gb|ADDG01054890.1| Varroa destructor strain Korean VDK00055186-1868, whole genome shotgun sequence
GGGGTCGGGCGTTGAGATAAGTAGAAATGGTAAATCCTCATGGGTAAACGTAAACACTGGTCCTTATCCG
CCGTCGATTAATGCGTGTATATACGCGTTATGAAATATTCCAATCCAGTCGCGAAAAGTCGTGTGTCCTT
TTACTTGCTCGTGGTTACTGTTTTGTCTCTAGTCACGCACCTCTATACGCTCTGGGCTGCCTTTTCGAGC
GATTCGGTGGTCGGTACTGAAGGATAGATATTGTAATGGCATTTTTTGAACGGTAAACTGATAGATGTTC
ACAGAAGCCGAAAAATGACAGCGCGTCGGTGAGTTTTCGACGCAGAGTAGTGCTGAATGTCAAACACACC
CATGGGTTCGCGCATGAATTGAGATTAGGCACAAGCAGAAATAGTGTGATGAATATGGAGGGAACCTGGC
CGGTCGATTCGAGATAGAGTTGGGCACAGCAGAAGGGTGTCCAACAGAGTACGAAGACAATCACCATAAC
CATTGTCATCCGTATCGGGTTCAGCTTTGTGTGACTCAATGATCGTTTTGAGATGATCCAAGAGAGATAT
GTATAGCACAAAGCCATCAGCGTCACAGGAATGAAAAGAACTGCTGTTATGAAGAATAGGACGTAACGAA
AGCTAGTTATCGGCGGCTCGAAAGTCGCCCAGCAGTCGTGTGCCCCATTGGGCAGCTTCTGGAATGAAAA
TATGTACAGTTGTGGTGAAGCTAAGACAGCCGCTACCAGCCAGCTAACGCTTAGAATTGTTCTGACAACA
ACAATTGGCCTGTTTATGCCAGATCTGATGGCGAGGTATCGATCGACAGCCATTCCAGCTAACACGTACG
TAGACAGATAGAGTACAAACACTTGGAGAAATTTCACAATCTTACAAGCGAAATTGCCGAGCGGGAACCT
GAAATAGATATCCCACACGAGCTGAGGCGAGATGTTAAATATACCCACGAGGATGTCAGCAATCGAGAGG
TGCAGCAGGAAGTAGTAGATTCGTGAGAGGTGGGCCGATTTCGGTTTGCTTTGAAGAACTAGGACGAGCA
CAACGATGTTGCCGGCCATCGTCAGGAACAACGCTACGCCTAACACTGCAACACGAACTGTCGCTATAAA
CTCGTCGCGAAAATCGGTAGCTGGTGGTTCTCGACTGGTCGTCTCAATCACTGTAAGAGGCGACGCGCTA
GAAGTAATCGAACCCTGAAATAAAGATAAACAACGACAATAGGGTATCCGAAATGTTAACAGTTAACGTT
TCGGATAATTATTAATTGTAAAGCAACGAAATCTTCGTATCCATCCGACAAGCGTATAACTATTTGCATT
CCCCGGTATATTTGGAGGTGCATGACACTTCATCGATGTTTAGGTAATAAACGTTGAAAATGAAATTGAT
GATGTTATCATTTTTAAATATACCACCTGAGATCGTATTGAAATACATGTAGCTGGAAATTTAATTTTCA
AGTTATATTTGGCTTCGTATTTGTTACACAGCTAACCTACAATAACTTGTTGATTTATCTAAACTTAAGC
TGGAAATCACCTAATCCTTAGCATCCAGTCTAAGAGAGTTGATACAAATTCATGAACGGAGTAATATTCT
AATTTGGTGAAAAATAACATGATTTATTATAGTAAGAGCGTCGTTAACGGATCAGATTTATTATAACTAT
ATGCTGGATGATCAGGGTGCTTCAGCTAAATACGTCGCTTTAGTCCTAGCACTTATCGTATGATCCCAGA
GAAATCCGCTGAAAATCTAACGGTATCATCCTCGTTCTTGTTATGTTCTACTATAACTGCATTTATTATT
AGTTGTTCCTTTTTGGTAGGTTTGTACACAATGCAATTTGTTAGGAT

6.1.2 Presumed 6T-ANTR peptide sequence

>putative_inotocin_receptor1_Vdestructor_ADDG01054890.1
MAGNIVVLVLVLQSKPKSAHLSRIYYFLLHLSIADILVGIFNISPQLVWDIYFRFPLGNF
ACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSGINRPIVVVRTILSVSWLVAAVLASPQ
LYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFITAVLFIPVTLMALCYTYLSWIISKRS
LSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLESTGQVPSIFITLFLLVPNLNSCANPW
VCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNAITISILQYRPPNRSKRQPRAYRGA

6.1.3 Alignment of presumed 6T-ANTR with D6WPA3_TRICA

```
CLUSTAL 2.1 multiple sequence alignment

putative_inotocin_receptor1_Vd      --------------------------------------------------M
tr|D6WPA3|D6WPA3_TRICA              MYTPKLSQMDISENSTYLFDKHEDRNNTDRDENLARVEVATLAIIFLVTV
                                                                                      :

putative_inotocin_receptor1_Vd      AGNIVVLVLVLQSKPKS--AHLSRIYYFLLHLSIADILVGIFNISPQLVW
tr|D6WPA3|D6WPA3_TRICA              IGNSTVLLALWTRRRYAGRKKLSRMYFFILHLSIADLITAFLSVLPQLAW
                                     ** .**: :   :  :   :***:*:*:*******::..::.: ***.*

putative_inotocin_receptor1_Vd      DIYFRFPLGNFACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSGINRPI
tr|D6WPA3|D6WPA3_TRICA              DITYRFYGGFLLCKVVKYGQTLGPYLSSYVLMATAIDRHQAICYPLTYCS
                                    ** :**  * : **:**: *.:  ***:*** . *:**: **   :.

putative_inotocin_receptor1_Vd      VVVR---TILSVSWLVAAVLASPQLYIFSFQKLPNGAHDCWATFEPPITS
tr|D6WPA3|D6WPA3_TRICA              WTSRRSKVMVYLAWVASLAFCIPQLTIFTYTSVGEDEYDCWATFQEPWGK
                                     . *   .:: ::*:.: .:. *** **:: .: :. :******: *  .

putative_inotocin_receptor1_Vd      FRYVLFFITAVLFIPVTLMALCYTYLSWIISKRS----------------
tr|D6WPA3|D6WPA3_TRICA              RAYVTWYSISVFMVPLVVLIFTYTSICIEIWQSSESSLRPRSSQKSAPGK
                                      ** ::  :*:::*:.:: : ** :.  * : *

putative_inotocin_receptor1_Vd      ----LSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLESTGQVPSIF---
tr|D6WPA3|D6WPA3_TRICA              RTPLISRAKINTVKQTIAVIVMYIACSTPFILAQLWATWDPQSPFIDGPV
                                        :*::*:*.:: *:.::::::: * ***  ***:     * * *

putative_inotocin_receptor1_Vd      ITLFLLVPNLNSCANPWVCLTFSTTLRRKLTDALSFFG------------
tr|D6WPA3|D6WPA3_TRICA              FVILTLLYSLNSCVNPWIYLAFNRELPRLLLRHYTASSKNYRSATGGNSA
                                    :.:: *: .****.***: *:*.  * * *    :  .

putative_inotocin_receptor1_Vd      ----------FCEHLSVYRSKNAITISILQYRPPNRSKRQPRAYRGA---
tr|D6WPA3|D6WPA3_TRICA              SNSSGDAQSTSLRPFSRWSLCNSARSNKYPTRVPHRPYVAQYNARRWIVT
                                               . :* :   *:   .    * *:*.       *

putative_inotocin_receptor1_Vd      ---
tr|D6WPA3|D6WPA3_TRICA              TTT
```

6.1.4 Putative ANTR 2 WGS sequence

>gi|283729967|gb|ADDG01034892.1| Varroa destructor strain Korean VDK00035060-2474, whole genome shotgun sequence
ATTTTGATCAGTTGATAAGTTGAACATGCGTGTATAAGCATTAAATTCATTTGGCTCGATCAGTTCCATT
CGGTATGAACTTGCTGAACTGTCAGTTGTCCGATACGTTCAGACATAAGCATTGTGCATCCGAAAACGAG
CCATTTACGTGGCCCTCTATTTGATCTTCTTGAGGCTGCTAATTAAGTGATATTAGCAAATCGATAAGCC
AGCTTTCAGCTGCCCCGTGCGGCTTTATGCGGAAAAATTGCATGTATTGATTATTCGTTATTTCCTAGCC
AACGTTACAAGCTAATTTGTGGAAAAAATCGTTCCTACTTCGAACGTTTGACCGTCAGGAGAAGCAAGCG
ATAATTCCGCGTTCAGGAATCGAACGAAACTGCGTAGAGGTTGAGCAGACGTCTCACAACGTTTGTTTGG
TGGGGTCTCAATGGAAAGACGCCTGGATCTCATTCCAAAATTTTGCTAGAAATAACTTTTTAGAGCACTT
AGTAATTTCTTTCTGAGATCTTGTGCATGATCACGTTCCTAATGTATGTTATAGGCTTCCGCATTAATTG
GCCTTAGAATCTCTGCACGGTACGTCGTGCCAGATTGGCCCGTACTAGCAATAGGCAGTATGTTCTTTGT
ATTTCAGGGTCGTCATATGCATCTTCTCTTCTGCGGATGCACGATTTCGCCTATCTGGCTCGTTTGACAT
CTCGATAATTTCGGCGTCTCTGATCCGAAACGTCGCGGCTAGTTCAGCCCTGTTAGGAGATTTGACAAAG
TCTCTCTCTGCTTCGTCATCAACAATCACGTCACAGCTGTCTTCCCTATTACTTTCAATCAGTTATCTTT
TTCACGTGGTAAAATATAGCGGAAAACGTAACGCCGATTGAATCCTCACGTCATATTAAAACGCTGAATT
GTTCAGATTTTCAACTTTTGCGTATGCACAGTATTTACTGTTTGTTATTGATGTCAAGTCAAAGGTAGTT
CTTGATAAAATTAAGTTAAGGCCAGCCTGTAGCATAAATATTCTTCAATTTTACAGATATGTTGCGGACG
GATTTGCATAGAGGCTATTGACATTAAATAAATGTATAAGATTATGCTAACATACTAATGCAAAAGGTTT
TTATTTTTGAGCTCGTACAAATACAGGATTGAAAAAGCTAACGTTTTGTCTCAATTTACGATTATAATGA
TGTAGCATGTCGATGTTTAACAATGGATATTTTTCTGCAAGATAATTTTAGCCGAGCAAGGAACTTTTTC
AATAATTTCTACATTACAGGCCTGCAAACGACGCCATAGCACTAGTGTTCAGTTACATGGTATGTACTGT
TCACAGGCCAAGAGTTATGTAGCTCAAAAAGGCCGCAAAGGCGTGTATGTATGTATGTATGTAAGACCCC
TGAGAGCCAAAAATCCAAGTGTTTTAAAAAGATGATTCTTTACTGCAAACGAATACTATTTATAACCGAC
GGGCAAGAAACACCCACTAGCGTTTCCTTTTCTGCAACACGTATAAGACCCAAACGCACACATACACGCG
TATACGTCCTACATAGTTCTGGATTAAGCCACAGGTGGCGTTTTGATCATACGGAGCACTCCGCCATCGG
CGTATGCCGTCTATCTAGCTAGAGTAAAGTTCGAGAGCTCGGAGTTACTTCGGTCGATTGAAGAGAGTGA
TCGATGCTGGACGGTGCTCGCAAATCCTTATTTTCTAGATGGATACAATCAGAGAACACGTGAAAACTTT
GCAACAGCTTAACGCAATGTGGCTAACGAATAATTCGAGACGGCGCTAGCAATCGTAAACAATTCACTTG
ACTGACCAACAAATGGTTAGTTCTGAAGAGGTTACCGAAGATGAGATGGTGATGTTAGAACCCGATGACT
GGGTGTCGTCGGTGAAGTGTTCGACACTTCTGCTAATCTTTGTTATGACGTTGTCATCTAACATGTTTGT
GCTATGGGCGGTCTTTCTCCGGAGCCGGTCGTATCGCTTCTGCGCGCGTTCCGTGTACGGCAAAGTTTCT
AAGAGGCAAGACGCAGCAGCTCTCTATCCCGGGTGCAACTTTTTATGATGCACCTGTCAATAGCAGACAT
TCTCGTTGCTCTGCTAAACATACTGCCCCAGCTCGCCTGGGACATCACGGCTCGCTTCTACGGCGGTGCG
CTTTTATGTAAATTTGTCAAATATGCTCAAGTACTTGTTCTCTATCTAAGCACATACATTCTCACAGGTA
TGTCACTCGATCGACTAGTATCTATGCGTGCCATTCACACTCAATGGAAGGCGGGGATGCGCGCGAATCA
CCGTTCATCATCGACCCGGCGAACTGAGAAGGCAACTTCAACCGAAATGTCCTCATCGAAGTTAATATGC
AAAAGGAACGGCTCCCTTTCCGGTGGCAGTTCGGGTAAAACGAAGCGTTTTTCACACGGTTTCAATAGTC
ATCAATCTCGTGCCGGTTATCGG

6.1.5 Putative ANTR 2 peptide sequence

>Varroa_destructor_INTreceptor2o
EARRSSSLSRVQLFMMHLSIADILVALLNILPQLAWDITARFYGGALLCKFVKYAQVLVLYLSTYILTGMSLDRLVSM

6.1.6 Varroa jacobsoni ANTR transcriptome hit

TSA: Varroa jacobsoni GG11755_comp1_c0_seq11 transcribed RNA sequence
Sequence ID: GETO01129497.1Length: 886Number of Matches: 1
Related Information
Range 1: 95 to 886GraphicsNext MatchPrevious Match

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps	Frame
537 bits(1384)	0.0	Compositional matrix adjust.	264/264(100%)	264/264(100%)	0/264(0%)	-1

```
Query  35   DILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSG  94
            DILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSG
Sbjct  886  DILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSG  707

Query  95   INRPIVVVRTILSVSWLVAAVLASPQLYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFI  154
            INRPIVVVRTILSVSWLVAAVLASPQLYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFI
Sbjct  706  INRPIVVVRTILSVSWLVAAVLASPQLYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFI  527

Query  155  TAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLE  214
            TAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLE
Sbjct  526  TAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLE  347

Query  215  STGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNA  274
            STGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNA
Sbjct  346  STGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNA  167

Query  275  ITISILQYRPPNRSKRQPRAYRGA  298
            ITISILQYRPPNRSKRQPRAYRGA
Sbjct  166  ITISILQYRPPNRSKRQPRAYRGA  95
```

```
>gb|GETO01129497.1| TSA: Varroa jacobsoni GG11755_comp1_c0_seq11 transcribed
RNA sequence
TATTTCATAACGCGTATATACACGCATTAATCGACGGCGGATAAAGTCGTGTGTCCTTTTTACTTGCTCG
TGGTTACTGTTTTGTCTCTAGTCACGCACCTCTATACGCTCTGGGCTGCCTTTTCGAGCGATTCGGTGGT
CGGTACTGAAGGATAGATATTGTAATGGCATTTTTTGAACGGTAAACTGATAGATGTTCACAGAAGCCGA
AAAATGACAGCGCGTCGGTGAGTTTTCGACGCAGAGTAGTGCTGAATGTCAAACATACCCATGGGTTCGC
GCATGAATTGAGATTAGGCACAAGCAGAAATAGTGTGATGAATATGGAGGGAACCTGGCCGGTCGATTCG
AGATAGAGTTGGGCACAGCAGAAGGGTGTCCAACAGAGTACGAAGACAATCACCATAACCATTGTCATCC
GTATCGGGTTCAGCTTTGTGTGACTCAATGATCGTTTTGAGATGATCCAAGAGAGATATGTATAGCACAA
GGCCATCAGCGTCACAGGAATGAAAAGAACTGCTGTTATGAAGAATAGGACGTAACGAAAGCTAGTTATC
GGCGGCTCGAAAGTCGCCCAGCAGTCGTGTGCCCCATTGGGCAGCTTCTGGAATGAAAATATGTACAGTT
GTGGTGAAGCTAAGACAGCCGCTACCAGCCAGCTAACGCTTAGAATTGTTCTGACAACAACAATTGGCCT
GTTTATGCCAGATCTGATGGCGAGGTATCGATCGACAGCCATTCCAGCTAACACGTACGTAGACAGATAG
AGTACAAACACTTGGAGAAATTTCACAATCTTACAAGCGAAATTGCCGAGCGGGAACCTGAAATAGATAT
CCCACACGAGCTGAGGCGAGATGTTAAATATACCCACGAGGATGTC
```

6.1.7 Translation of *Varroa jacobsoni* ANTR transcriptome hit

```
>Varroa_jac_receptor2
DILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSG
INRPIVVVRTILSVSWLVAAVLASPQLYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFI
TAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLE
STGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNA
ITISILQYRPPNRSKRQPRAYRGA-
```

```
CLUSTAL 2.1 multiple sequence alignment


Varroa_jac_receptor1      MATFAIADDTLHNELNQGTSIPQELSFNKNNLVKVIMYITMFVIGVSGNVPVFLSLIRNR
Varroa_jac_receptor2      ------------------------------------------------------------


Varroa_jac_receptor1      HRKSRIKMMMLHLTIADLIVTFIMLPIEIAWNITVQWLAGNLTCKVLMFFRVFGIYLSST
Varroa_jac_receptor2      ----------------DILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQVFVLYLSTY
                                        *::* :: :. ::.*:* .::  **::**:: *::** :***:

Varroa_jac_receptor1      VLVCFSLDRYFAVLHPLQVNDAHRRGKMMLTLAWMVSFICSVPQTIIFSSLTHPDIEKFT
Varroa_jac_receptor2      VLAGMAVDRYLAIRS--GINRPIVVVRTILSVSWLVAAVLASPQLYIFS------FQKLP
                          **. :::***:*:     :* .    : :*:::*:*: : : **  ***      ::*:.

Varroa_jac_receptor1      QCVTFAFFSDNNPNEKKAYTIQFLLAIYWIPLMLIVWCYLKILREIFRRSGESSQQETIL
Varroa_jac_receptor2      NGAHDCWATFEPPITSFRYVLFFITAVLFIPVTLMALCYTYLSWIISKRS----------
                          : .  .: : : *  .  *.: *: *: :**: *:. **  :   * :**

Varroa_jac_receptor1      FRIELRRSDPKMMERHRNKTLRLSVVIVLAFLSCWTPFVIINLWLLFDPKGVDDRIEDHI
Varroa_jac_receptor2      ------------LSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLESTG-----QVPSIF
                                     :.: : :.:*:::*:*:.*: *****   :*:*         :: . :

Varroa_jac_receptor1      QTFMLVLAGGNSCVNPLIYGSLGHFNSAGGPLSRCRERQDLASRMNFLSRGNARDTDESS
Varroa_jac_receptor2      ITLFLLVPNLNSCANPWVCLTFSTT-----------LRRKLTDALSFFGFCEHLSVYRSK
                           *::*::.. ***.** :  ::.             *:.*:. :.*:.  :  .. .*.

Varroa_jac_receptor1      SSCSYVFTTHSHQMQSHHQRYHHRHGRSANTALIYTRDANTGGQFRNQHRQMSCIQTTGH
Varroa_jac_receptor2      NAITISILQYRPPNRSKRQPRAYRGA----------------------------------
                          .: :  :  :    :*::*   :* .

Varroa_jac_receptor1      NHDAF-
Varroa_jac_receptor2      ------
```

6.1.8 *Varroa jacobsoni* putative ANTR

Translated from GenBank: NJHO01004860.1

```
MAGNIVVLVLVLQSKPKSAHLSRIYYFLLHLSIADILVGIFNISPQLVWD
IYFRFPLGNFACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSGINRPIV
VVRTILSVSWLVAAVLASPQLYIFSFQKLPNGAHDCWATFEPPITSFRYV
LFFITAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPIRMTMVMVIVFV
LCWTPFCCAQLYLESTGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTLR
RKLTDALSFFGFCEHLSVYRSKNAITISILQYRPPNRSKRQPRAYRGA-
```

6.2 ANTR clones

6.2.1 6T-ANTR

MAGNIVVLVLVLQSKPKSAHLSRIYYFLLHLSIADILVGIFNISPQLVWDIYFRFPLGN
FACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSGINRPIVVVRTILSVSWLVAAVLAS
PQLYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFITAVLFIPVTLMALCYTYLSWIIS
KRSLSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLESTGQVPSIFITLFLLVPNLNSC
ANPWVCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNAITISILQYRPPNRSKRQPRAY
RGA-

6.2.2 6T-ANTR-GFP

MAGNIVVLVLVLQSKPKSAHLSRIYYFLLHLSIADILVGIFNISPQLVWDIYFRFPLGNF
ACKIVKFLQVFVLYLSTYVLAGMAVDRYLAIRSGINRPIVVVRTILSVSWLVAAVLASPQ
LYIFSFQKLPNGAHDCWATFEPPITSFRYVLFFITAVLFIPVTLMALCYTYLSWIISKRS
LSHTKLNPIRMTMVMVIVFVLCWTPFCCAQLYLESTGQVPSIFITLFLLVPNLNSCANPW
VCLTFSTTLRRKLTDALSFFGFCEHLSVYRSKNAITISILQYRPPNRSKRQPRAYRGAWV
PRARDPPVATMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICT
TGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTR
AEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRH
NIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGI
TLGMDELYK-

6.2.3 ANTR

MDSNSTGSITSSASPLTVIETTSREPPATDFRDEFIATVRVAVLGVALFLTMAGNIVVLV
LVLQSKPKSAHLSRIYYFLLHLSIADILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQ
VFVLYLSTYVLAGMAVDRYLAIRSGINRPIVVVRTILSVSWLVAAVLASPQLYIFSFQKL
PNGAHDCWATFEPPITSFRYVLFFITAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPI
RMTMVMVIVFVLCWTPFCCAQLYLESTGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTL
RRKLTDALSFFGFCEHLSVYRSKNAITISILQYRPPNRSKRQPRAYRGA-

6.2.4 ANTR-GFP

MDSNSTGSITSSASPLTVIETTSREPPATDFRDEFIATVRVAVLGVALFLTMAGNIVVLV
LVLQSKPKSAHLSRIYYFLLHLSIADILVGIFNISPQLVWDIYFRFPLGNFACKIVKFLQ
VFVLYLSTYVLAGMAVDRYLAIRSGINRPIVVVRTILSVSWLVAAVLASPQLYIFSFQKL
PNGAHDCWATFEPPITSFRYVLFFITAVLFIPVTLMALCYTYLSWIISKRSLSHTKLNPI
RMTMVMVIVFVLCWTPFCCAQLYLESTGQVPSIFITLFLLVPNLNSCANPWVCLTFSTTL
RRKLTDALSFFGFCEHLSVYRSKNAITISILQYRPPNRSKRQPRAYRGAWVPRARDPPVA
TMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDT

LVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQL
ADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK-

Abstract

The oxytocin/vasopressin signalling system is linked to a wide range of disorders and diseases, including social anxiety disorder, autism, schizophrenia, borderline personality disorder, stress, depression, preterm-labour, cardiovascular diseases, and cancer. It consists of two nonapeptide ligands, oxytocin and vasopressin, and four G protein-coupled receptors (OTR, $V_{1a}R$, $V_{1b}R$ and V_2R). The high similarity of the oxytocin/vasopressin-receptors, especially in the extracellular parts of the receptors, which display a homology of ~80%, is the main bottleneck in development of receptor specific ligands for this system. Consequently, the investigation into the physiological and pathological function of the involved receptors is severely limited. Recently a new approach to exploit the evolutionary conservation of receptors and their signalling peptides was used to develop a selective human vasopressin V_{1a}-receptor antagonist based on a black garden ant (*Lasius niger*) neuropeptide.

This thesis presents a possible vantage point for the development of a selective, competitive human V_{1a} receptor inhibitor based on ligand-receptor selectivity profiles generated via competitive immune assays of a honey bee mite (*Varroa destructor*) oxytocin/vasopressin-like peptide (arachnotocin) on the human oxytocin/vasopressin receptors. Additionally, a putative endogenous receptor for this peptide was cloned as a GFP construct, but failed to localize to the plasma membrane. This receptor may however eventually serve as a target for novel mite pesticides. Investigation into the molecular pharmacology of a neuropeptide and an analogue derived from the common starfish (*Asteria rubens*) did not display any interaction with the human oxytocin/vasopressin receptors, however the increased insight into the structure-activity relationship of these neuropeptides may provide valuable reference points for design of selective or biased ligands for this system.

Zusammenfassung

Das Oxytocin/Vasopressin System ist mit einer Vielzahl an Krankheiten und Funktionsstörungen assoziiert. Dazu gehören unter anderem Autismus, Borderline-Persönlichkeitsstörung, Schizophrenie, Soziale Phobie, Stress, Depression, Frühgeburt, Herz-Kreislauf-Erkrankungen und Krebs. Es besteht aus zwei Nonapeptid-Liganden, Oxytocin und Vasopressin, und vier G Protein-Gekoppelten Rezeptoren (OTR, $V_{1a}R$, $V_{1b}R$ und V_2R). Starke Ähnlichkeiten der Aminosäuresequenzen der involvierten Rezeptoren, besonders der extrazellulären Teile, die eine Homologie von ~80% aufweisen, ist der eigentliche Flaschenhals in der Entwicklung spezifischer Liganden für dieses System. Folglich ist die Erforschung der physiologischen und Pathologischen Funktionen dieses Systems stark limitiert. Kürzlich konnte ein neuer, selektiver humaner V_{1a} Rezeptor Antagonist basierend auf einem Neuropeptid der Schwarzen Wegameise (*Lasisus niger*) entwickelt werden, indem die Gruppe die evolutionäre Erhaltung der Rezeptoren und ihrer Liganden ausnutze.

Basierend auf diesem Konzept präsentiert diese These einen möglichen Ausgangspunkt für die Entwicklung eines selektiven, kompetitiven humanen V_{1a} Rezeptor Antagonisten basierend auf einem Oxytocin/Vasopressin-ähnlichem Peptid (Arachnotocin) aus der Varroa Milbe (*Varroa destructor*). Hierzu wurden Ligand-Rezeptor Selektivitätsprofile an den menschlichen Rezeptoren über kompetitive Sekundärmessenger-Immunoassays generiert. Ein möglicher endogener Rezeptor für Arachnotocin wurde als GFP Konstrukt aus *Varroa destructor* kloniert, lokalisierte allerdings nicht an der Plasmamembran. Auch wurde ein Neuropeptid aus *Asterias rubens* auf den humanen Rezeptoren gemessen, hier konnte jedoch kein Effekt nachgewiesen werden. Dennoch könnten die hierdurch generierten Daten wertvolle Hinweise für das Design weiterer peptidbasierter Liganden für das Oxytocin/Vasopressin System liefern.