Role of sulfakinin signaling in feeding of the red flour beetle *Tribolium castaneum*

Na YU

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) of Applied Biological Sciences

Promoter:	**Prof. dr. ir. Guy Smagghe** Department of Crop Protection Faculty of Bioscience Engineering Ghent University, Belgium
Chair of the examination committee:	**Prof. dr. Els Van Damme** Department of Molecular Biotechnology Faculty of Bioscience Engineering Ghent University, Belgium
Members of the examination committee:	**Prof. dr. ir. John Van Camp** Department of Food Safety and Food Quality Faculty of Bioscience Engineering Ghent University, Belgium
	Prof. dr. ir. Peter Bossier Department of Animal Production Faculty of Bioscience Engineering Ghent University, Belgium
	Prof. dr. ir. Jozef Vanden Broeck Department of Biology Faculty of Science Catholic University of Leuven, Belgium
	Dr. Ronald J. Nachman Agricultural Research Center, USDA, USA
Dean:	**Prof. dr. ir. Guido Van Huylenbroeck**
Rector:	**Prof. dr. Anne De Paepe**

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Rol van sulfakinine signalisatie bij de voedingsregulering van de kastanjebruine rijstmeelkever *Tribolium castaneum*

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Left: a *Tribolium castaneum* under a magnifying glass. Top right: primary structure of a sulfakinin peptide of nine amino acids. Bottom right: a predicted three-dimensional structure of the transmembrane sulfakinin receptor.

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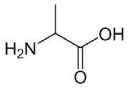
List of abbreviations

AA	amino acid
AC	adenylate cyclase
cAMP	cyclic adenosine 3',5'-cyclic monophosphate
CCK	cholecystokinin
CioR	cionin receptor
C-terminal	carboxyl-terminal
dsRNA	double-stranded RNA
G-protein	guanine nucleotide binding protein
GPCR	G-protein coupled receptor
IP3	inositol 1,4,5-trisphosphate
nsDSK	nonsulfated *Drosophila* sulfakinin
NFAT	nuclear factor of activated T-cells
N-terminal	amino-terminal
PLC	phospholipase C
RE	response element
R.H.	relative humidity
RNAi	RNA interference
SAR	structure-activity relationship
sDSK	sulfated *Drosophila* sulfakinin
SNS	stomatogastric nervous system
SK	sulfakinin
SKR	sulfakinin receptor
sSK	sulfated SK
nsSK	nonsulfated SK
$\underline{Y}$	$Y(SO_3H)$, sulfated tyrosine

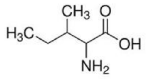
Overview of amino acid

Aliphatic

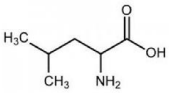
Alanine (Ala, A)
MW: 89.10



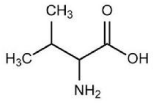
Isoleucine (Ile, I)
MW: 131.18



Leucine (Leu, L)
MW: 131.18

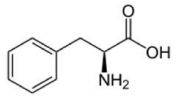


Valine (Val, V)
MW: 117.15

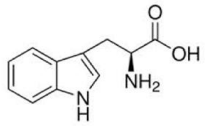


Aromatic

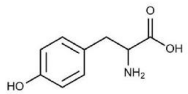
Phenylalanine (Phe, F)
MW: 165.19



Tryptophan (Trp, W)
MW: 204.23

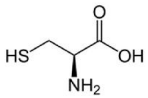


Tyrosine (Tyr, Y)
MW: 181.19

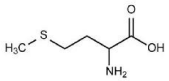


Neutral

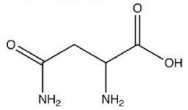
Cysteine (Cys, C)
MW: 121.16



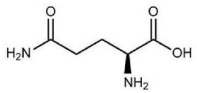
Methionine (Met, M)
MW: 149.21



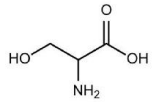
Asparagine (Asn, N)
MW: 132.12



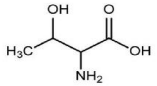
Glutamine (Gln, Q)
MW: 146.15



Serine (Ser, S)
MW: 105.09

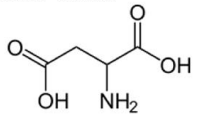


Threonine (Thr, T)
MW: 119.12

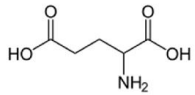


Acidic

Aspartic acid (Asp, D)
MW: 133.11

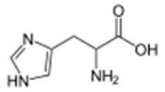


Glutamic acid (Glu, E)
MW: 147.13

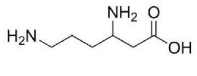


Basic

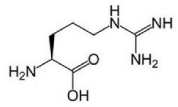
Histidine (His, H)
MW: 155.16



Lysine (Lys, K)
MW: 146.19

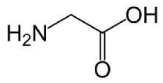


Arginine (Arg, R)
MW: 174.20

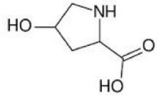


Unique

Glycine (Gly, G)
MW: 75.07

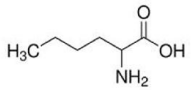


Proline (Pro, P)
MW: 115.13

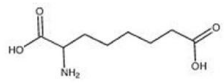


Unnatural

Norleucine (Nle)
MW: 131.17



α-aminosuberic acid (Asu)
MW: 189.21



(Molecular structures of amino acids were collected from http://en.wikipedia.org/wiki/Amino_acid)

Objectives and outline

Feeding in insects is a complex process that is regulated by many factors. One biological factor is neuropeptides. Neuropeptides and their corresponding receptors constitute signaling systems through the organism, regulating feeding in an either stimulatory or inhibitory manner.

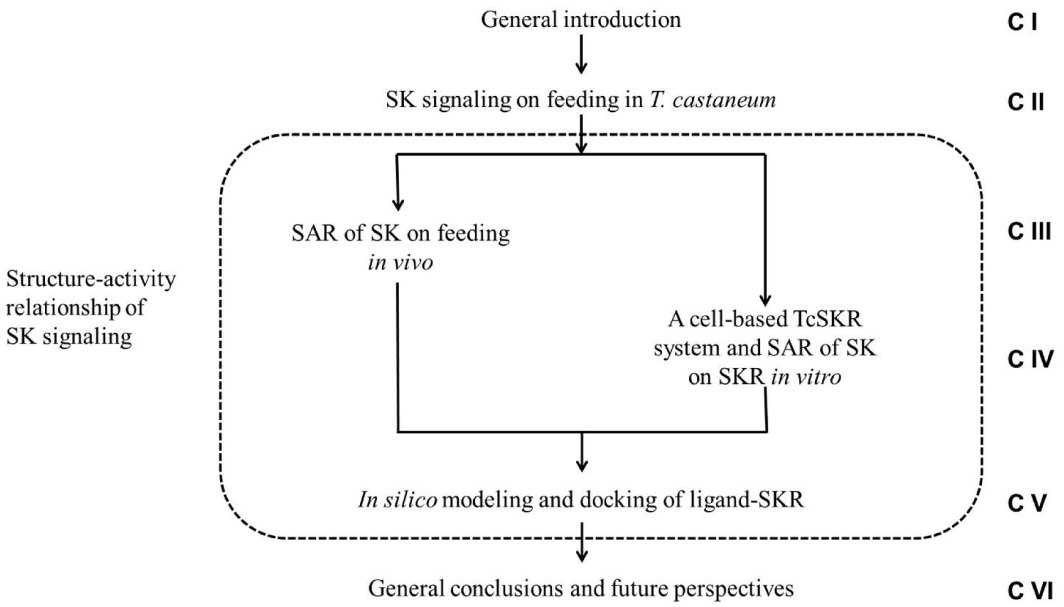
The neuropeptide cholecystokinin (CCK) signaling induces satiety in mammals. The insect neuropeptide sulfakinin (SK) is homologous to CCK and it is reported to be active in the regulation of feeding in a few insects. However, our knowledge on SK signaling in insects is much limited. Several questions should be addressed before we can go further with this feeding-regulatory system. How important is the SK signaling for insects and in which way? What is the mechanism of SK signaling? How could we take advantage of the SK signaling in practice such as pest control? Therefore, it is in our interest to expand the knowledge of SK signaling with respect to its role in the regulation of feeding in insects. In addition, study on SK signaling will pave the way for the studies of other feeding-regulatory neuropeptides and eventually the regulation of feeding in insects. Moreover, understanding the regulatory mechanisms underlying insect feeding is a prerequisite for the development of more effective and environmentally safe control methods of pest insects.

This study is conducted with the red flour beetle *Tribolium castaneum*, one notorious pest insect that causes considerable damage for stored grain, but also a model insect with whole genome sequence available and robust response to RNA interference (RNAi). So far, not much about SK signaling is known in *T. castaneum*.

The goal of this study is to understand the role and mechanism of SK signaling in *T. castaneum*, with special respect to the regulation of feeding. SK signaling consists of two predominant elements: SKs and SK receptors (SKRs). SKs are neuropeptides and SKRs are proteins belonging to the G-protein coupled receptor (GPCR) family, which are transmembrane proteins. GPCRs convert the extracellular stimuli into intracellular signaling upon activation. With SKRs belonging to GPCR, SK signaling is expected to transduct signaling following the same principle. The efficient interaction of ligand and receptor is the starting point of the signaling transduction and it is affected by the sequence and structure of both ligand and receptor. The role of SK signaling in feeding is studied by measuring food

intake after reducing or enhancing the SK signaling in insects. The mechanism of SK signaling is explored from two aspects: signaling transduction via SKRs and structure-activity relationship (SAR) of SK.

The outline of this thesis is illustrated below.



(C, chapter; SAR, structure-activity relationship)

More specifically, the thesis consists of the following:

Chapter I is a general introduction of related literature. It starts with the regulation of feeding and the relevant neuropeptides. Followed is the special focus on the sequence and function of the SK signaling in insects and its counterparts in metazoans. Then, GPCR signaling and RNAi are briefly introduced for their machinery and efficiency. Lastly, the development and growth conditions of the insect *T. castaneum* are included.

Chapter II characterizes and functionally studies SK signaling in the regulation of feeding in *T. castaneum*. One SK precursor gene and two SKR genes are present in the *T. castaneum* genome. The SK precursor gene and SKR genes will be cloned and characterized with sequence analysis. Their expression patterns will be examined in terms of developmental stage, tissue and nutritional status. In addition, the evolution of SKR and its counterparts in animals will be discussed through phylogenetic analysis. The functional study of SK signaling will be fulfilled in larvae by two means: the dsRNA-mediated RNAi which reduces SK

signaling and the direct injection of a synthetic SK peptide which enhances SK signaling. The effects of these treatments on feeding will be evaluated by measuring the mass of food consumption.

Chapter III explores the structure-activity relationship of SK on feeding in adult *T. castaneum*. SKs contain a conserved C-terminal YGHM/LRF-NH_2 with diverse N-terminal extentions. In most cases, SKs are present in two forms: sulfated SK (sSK) and nonsulfated SK (nsSK), depending on the presence of a sulfate group (SO_3H) on the Y residue. A series of synthetic SK-related peptides with amino acid substitution or deletion from native SK peptide will be injected into adults and evaluated for their effect on feeding. The active core sequence of SK will be examined based on the *in vivo* data. Specifically, the role of sulfate moiety will be discussed.

In order to study the two TcSKRs (TcSKR1 and TcSKR2) individually, a cell-based bioassay is developed in **Chapter IV**. First, two cell lines, Sf9-TcSKR1 and Sf9-TcSKR2, will be constructed to stably express the two TcSKRs. Then, a reporter gene luciferase assay will be introduced to respond to the activation of the individual TcSKR. With this cell-based bioassay, the pharmacological properties of the two TcSKRs will be determined, such as their affinity to SK peptide as well as their response patterns. Moreover, the SK-related peptides will be screened with the bioassay to evaluate their activities on individual TcSKR. These *in vitro* data will provide information for the study of the SAR of SK.

Chapter V describes the modeling of the two TcSKRs and the docking of the SK (-related) peptides to TcSKRs. First, the three-dimensional structures of both TcSKRs will be modeled with existing information and the structural distinction between the two TcSKR models will be discussed. Then, the sSK and nsSK will be docked to the two TcSKRs, where the effect of the sulfate moiety on the interaction of SK to SKR will be analyzed. Lastly, the SK-related peptides with Alanine-substitution will also be docked to the two TcSKRs in order to obtain further information on the SAR of SK. In addition, the *in silico* docking data will be inspected together with previous *in vivo* (**Chapter II** and **Chapter III**) and *in vitro* (**Chapter IV**) assays, which is expected to provide an integrated insight on the SAR of SK.

Lastly, the general conclusions and future perspectives are presented in **Chapter VI**.

Chapter I. General introduction

Parts of this chapter are published in:

Yu, N., Christiaens, O., et al. (2013) Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Science, 20: 4-14.

Yu, N., Smagghe, G. (2014) CCK (-like) and receptors: structure and phylogeny in a comparative perspective. General and Comparative Endocrinology, in press.

1. Feeding in insects

Feeding is the process by which food is obtained to provide energy and essential nutrients. It is one of the most basic behaviors for the survival of animals. Animals ingest nutrients necessary for energy production, maintenance of biochemical processes, growth and development. About 75% of all animal species on the Earth are insects (Strong et al., 1984). Insects feed on a great variety of food sources such as animals, plant tissues and dead organic matters. But, most insect species restrict themselves to a particular category of food. Insects are categorized based on four main classes of feeding habits: plant feeders, predators (e.g., feeding on aphids and mites), scavengers (e.g., feeding on dead and decaying organic matters) and parasites. Within each of these classes, various types of feeding can be found such as biting and chewing on leaves or animal tissues and sucking from plant or animal tissues. The physiological need for food by insects varies constantly due to changing factors such as nutritional status, food deprivation, developmental stage and internal regulation (Chapman, 1998).

Feeding in insects is initiated with the mouthpart and followed with food proceeded into the alimentary canal. The alimentary canal is divided into three main regions: foregut, midgut and hindgut (Figure I-1). Food is pushed back from the pharynx by the muscles of the pharyngeal pump and subsequently passed along the gut by peristaltic movements. The alimentary canal and its associated glands triturate, lubricate, store, digest and absorb the food material.

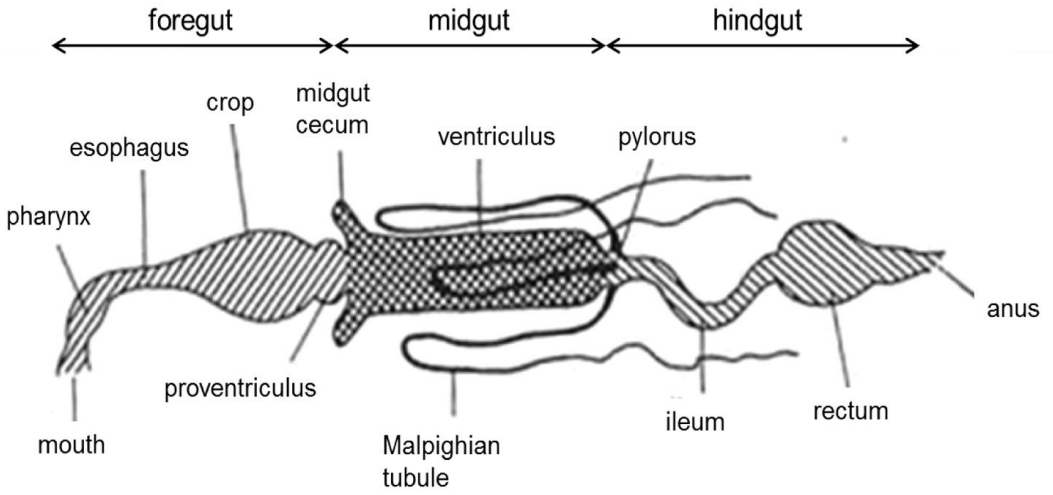


Figure I-1 Alimentary canal of insect (after Chapman, 1998).

2. The regulation of feeding in insects

Pest insects are expected to be controlled either by deterring or stopping feeding on natural food or by stimulating feeding on pesticide-treated materials, due to the development of resistance to current insecticides and the restrictions in insecticide usage by environmental concerns. Therefore, understanding the regulatory mechanisms underlying insect feeding is a prerequisite for the development of more effective and environmentally safe control methods of pest insects. The insights into the regulation of insect feeding will provide the proper background for an in-depth discussion of improving the existing pest control methods and the development of new methods (Chapman, 1998).

The regulation of feeding is highly complex and involves numerous external and internal factors (Geiselman, 1996). The regulation of feeding in mammalian vertebrates has been intensively studied. A current model describes a central feeding system, which has the overall control of feeding (Jensen, 2001). This system involves specific nuclei of the hypothalamus and receives input from two major peripheral systems. One short-term system (also called the peripheral satiety system), which transmits meal-related signals to the central system, and one long-term system giving information to the central feeding system on the amount of adipose tissue and the overall energy balance of the body.

Considerable progress has been made in understanding putative signals for hunger, satiation and satiety. These putative physiological controls of feeding include positive and negative sensory feedback, gastric and intestinal distension, effects of nutrients, nutrient reserves, and the release of peptides and hormones in the gastrointestinal tract or in the brain. In vertebrates, especially mammals, a group of small regulatory peptides that are produced by the brain-gut axis plays a major role in the endocrine regulation of feeding and the control of energy homeostasis (Figure I-2). These peptide hormones are divided into two groups based on their final effect on feeding: orexigenic peptides that stimulate appetite and induce food intake, and anorexigenic peptides that cause loss of appetite, reduce food consumption and increase energy expenditure (Cardoso et al., 2012). Neuropeptide Y (NPY) is one example of the orexigenic peptides. NPY administration into the paraventricular nucleus (PVN) or the perifornical hypothalamus (PFH) elicits eating, whereas injection in the PVN but not the PFH evokes concomitant hypothermia (Currie and Coscina, 1995) and increased energy substrate utilization. This suggests that the NPY action of the PVN modulates integrative and

regulatory mechanisms of feeding, thermogenesis and energy metabolism. Moreover, neuroanatomic evidence suggests an interaction between NPY and the anorexigenic melanocyte–stimulating hormone (MSH), suggesting that the control of food intake and energy expenditure is extremely complex (Eduardo et al., 2005). The anorexigenic peptide Cholecystokinin (CCK) has also received considerable attention in respect to the short term regulation of food intake (Raybould, 2007). Administration of CCK induces satiation, leading to a reduction in meal size (Lal et al., 2004). More findings suggest that CCK is required for the regulation of caloric intake within a meal, but that other factors such as leptin or insulin are involved for the regulation over multiple meals (Donovan et al., 2007; Whited et al., 2006). The involvement of neuropeptides in metazoan feeding behavior is suggested to be conserved via comparative sequence approaches and in functional studies (Mirabeau and Joly, 2013). Therefore, the knowledge obtained from mammals is often used as a base for the study of neuropeptides in the regulation of feeding in insects.

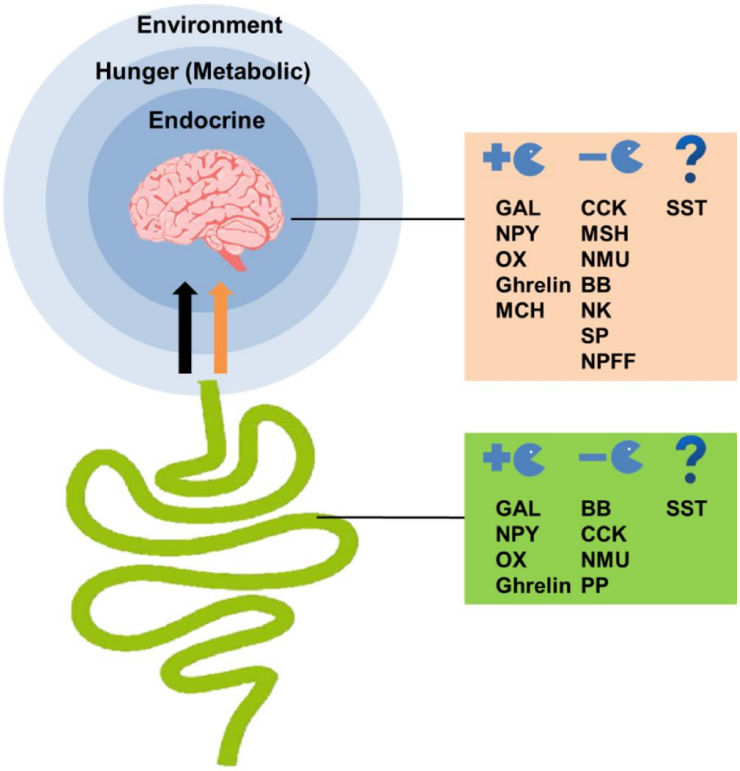


Figure I-2 Overview of endocrine factors that regulate feeding behavior in the human brain-gut axis. In humans and other vertebrates, feeding is regulated by signals from the environment (odor and taste), hunger (metabolic signals), and endocrine signals produced by the gut and brain. The orange arrow represents the blood connection

between gut and brain and the black arrow the nervous connection via the vagal afferent terminals through which peptides produced by the gut modulate the feeding response in the brain. Galanin (GAL), neuropeptide Y (NPY), orexin (OX), ghrelin and melanin concentrating hormone (MCH) are orexigenic peptides and promote appetite and feeding. Cholecystokinin (CCK), melanocortin peptides (MSH), neuromedin U (NMU), bombesin (BB), neuromedin K (NK), substance P (SP) and neuropeptide FF (NPFF) are anorexigenic. The role of somatostatin (SST) peptides in feeding is unclear (after Cardoso et al., 2012).

The regulation of feeding in insects is not so different from that in mammals (Simpson and Bernays, 1983). Central excitation is influenced positively by food as well as non-food stimuli, both from within and outside the animal; negatively by deterrent stimuli and by feedback from peripheral systems such as stretch receptors on the gut wall, hormones and blood composition (Wei et al., 2000). As food enters the gut during feeding, it stimulates stretch receptors located on the anterior wall of the crop. It may also enter the anterior part of the midgut and shunt food already there backwards to distend the ileum, wherein lie other stretch receptors. Additionally, stretching of the crop elicits the release of hormones from the storage lobes of the corpora cardiaca. Such hormones have a range of effects, including stimulating diuresis, increasing gut motility, reducing locomotor activity and increasing the electrical resistance of the palp tips (Maestro et al., 2001; Wei et al., 2000). Many of the identified neuropeptide hormones have an effect on the regulation of feeding given their influence on the contractility of the alimentary tract (Audsley and Weaver, 2009).

3. Neuropeptides associated with the regulation of feeding in insects

Neuropeptides[1] are ubiquitous in the nervous system at all levels of organization from hydrozoans to man and they are by far the most diverse signaling substances, both structurally and functionally (Nässel, 2002). Several insect neuropeptides have been reported to act at different levels in the regulation of feeding, such as FMRF-NH_2-like peptides, neuropeptide F,

[1] In this thesis, amino acids are presented with their single-letter code except where three-letter code is necessary. Amino acids and their letter codes are listed in the "overview of amino acid" on page vi. Position of an amino acid in a neuropeptide sequence is counted from the conserved C-terminus. An amino acid residue is often written as a single-letter code with a position number superscript. For instance, Y^6 represents that Y is at the position 6 from the C-terminus of a neuropeptide. A neuropeptide is named following the neuropeptide nomenclature proposed in Coast, G.M., Schooley, D.A., 2011. Toward a consensus nomenclature for insect neuropeptides and peptide hormones. Peptides 32, 620-631.

short neuropeptide F, allatostatins and allatotropin. More neuropeptides can be found in relevant reviews (Audsley and Weaver, 2009; Spit et al., 2012).

3.1. FMRF-NH_2-like peptides

FMRF-NH_2-like peptides represent the largest group of neuropeptides known in invertebrates. The story of FMRF-NH_2-like peptides began when Price and Greenberg (1977) isolated and identified the tetrapeptide FMRF-NH_2 from the bivalve mollusc, *Macrocallista nimbosa*. The family of FMRF-NH_2-like peptides consists extensively of FMRF-NH_2, FLRF-NH_2 and HMRF-NH_2 peptides. They have strong inhibitory effect on visceral muscles such as the cockroach gut (Aguilar et al., 2004; Predel et al., 2001), and the foregut of the blood sucking bug *Rhodnius prolixus* (Tsang and Orchard, 1991) and locust *Schistocerca gregaria* (Banner and Osborne, 1989), which suggests that they play a role in the regulation of feeding. Among these peptides, leucomyosuppressin inhibits gut motility and hence reduces food intake in the German cockroach *Blattella germanica* (Aguilar et al., 2004) and cotton leafworm *Spodoptera littoralis* (Vilaplana et al., 2008); sulfakinin inhibits food intake in locust *S. gregaria* (Wei et al., 2000), cricket *Gryllus bimaculatus* (Meyering-Vos and Müller, 2007a), cockroaches *B. germanica* and *Periplaneta americana* (Maestro et al., 2001; Veenstra, 1989) and flies *Phormia regina* and *Calliphora vomitoria* (Downer et al., 2007; Duve et al., 1995), and stimulates the release of digestive enzymes into the gut of *Rhynchophorus ferrugineus* (Nachman et al., 1997).

3.2. Neuropeptide F

Neuropeptide F (NPF) was first identified in a tapeworm, *Monieza expansa* (Maule et al., 1991). NPFs are neuropeptides with more than 28 residues (commonly 28–45 amino acids) and a consensus C-terminal RXRF-NH_2 (commonly RPRF-NH_2, but also RVRF-NH_2). The known regulatory roles of NPFs in *Drosophila* are in foraging, feeding and motivation, ethanol sensitivity, stress responses including nociception, aggression, reproduction, clock function and learning (Nässel and Wegener, 2011). NPFs stimulate feeding in insects such as the desert locust *S.gregaria* (Van Wielendaele et al., 2013).

3.3. Short neuropeptide F

The insect short neuropeptide F is a group of peptides with the C-terminal sequence XPXLRLRF-NH_2, which is important for receptor activation (Nässel and Wegener, 2011). Short neuropeptide F stimulates food intake and body weight gain in *Drosophila* (Lee et al., 2004). However, an opposite effect was observed in the desert locust *S. gregaria*, where injection of short neuropeptide F caused an inhibitory effect on food intake and knocking down of the short neuropeptide F receptor gene resulted in an increase of total food intake (Dillen et al., 2013).

3.4. Allatostatin

Allatostatins (ASs) are recognized in three types: A, B and C. The three types of ASs were first identified from the cockroach *Diploptera punctata* (Pratt et al., 1989; Woodhead et al., 1989), the cricket *G. bimaculatus* (Lorenz et al., 1995) and the moth *Manduca sexta* (Kramer et al., 1991), respectively. ASs inhibit hindgut motility and food consumption, and activate midgut α-amylase secretion in the cockroach *B. germanica* (Aguilar et al., 2003). In adult *Drosophila*, activation of neurons (or neuroendocrine cells) expressing the neuropeptide AS-A inhibits or limits several starvation-induced changes in feeding behavior, including increased food intake and enhanced behavioral responsiveness to sugar (Hergarden et al., 2012).

3.5. Allatotropin

The first allatotropin (AT) was characterized in *M. sexta* (Kataoka et al., 1989). It is a neuropeptide originally described as a regulator of juvenile hormone (JH) synthesis. The mRNA level of AT is specifically increased in the nerve cord of the last instar *M. sexta* larvae associated with the treatments which result in reduced feeding and increased levels of JH (Lee and Horodyski, 2002). It also inhibits ion transport in the midgut of *M. sexta* larvae (Lee et al., 1998). In addition, AT controls the release of digestive enzymes in the midgut of *S. frugiperda* (Lwalaba et al., 2010).

4. Sulfakinin signaling

Among the FMRF-NH_2-like peptides, sulfakinins (SKs) constitute an important group that has attracted considerable interest. SKs contain a consensus C-terminal YGHM/LRF-NH_2

hexapeptide, with a sulfate group (SO_3H) on the tyrosin (Y) residue in most cases. SKs exert physiological effects via the activation of sulfakinin receptors (SKRs). SKs and SKRs together with other molecules compose the SK signaling in insects.

4.1. Sulfakinin in insects

SKs were first isolated from the head extract of the cockroach *Leucopaea maderae* for their myoactivity on hindgut (Nachman et al., 1986b, 1986c). Similar peptides have been identified in the cockroach *P. americana* (Veenstra, 1989), the locust *Locusta migratoria* (Schoofs et al., 1991), the flesh fly *Neobellieria bullata* (Fónagy et al., 1992) etc. Genes encoding SK prepropeptide have been cloned in the fruit fly *Drosophila melanogaster* (Nichols et al., 1988) and the cricket *G. bimaculatus* (Meyering-Vos and Müller, 2007b). SK prepropeptide genes have also been annotated extensively in metazoans (Christie, 2008b; Hauser et al., 2010; Ons et al., 2011), thanks to the developing peptidomics, transcriptomics and genomics. Table I-1 summarizes the amino acid sequences of SKs that are determined either from isolated peptides or from predicted/cloned DNA.

Table I-1 Amino acid sequences of SK, CCK, gastrin and related peptides.

Class	Order	Species	SK (homolog)	Sequence [#]	Reference
Insecta	Blattodea	*Leucophaea maderae*	Leuma-SK-I	EQFEDYGHMRF *	Nachman et al., 1986c
			Leuma-SK-II	pQSDDYGHMRF *	Nachman et al., 1986b
		Periplaneta americana	Peram-SK-I	EQFDDYGHMRF *	Veenstra, 1989
			Peram-SK-II	pQSDDYGHMRF *	
		Blattella germanica	Blage-SK-I	EQFDDYGHMRF *	Maestro et al., 2001
	Coleoptera	*Zophobas atratus*	Zopat-SK-I	pETSDDYGHLRF	Marciniak et al., 2011
		Tribolium castaneum	Trica-SK-I	pQTSDDYGHLRF *+	this study; Li et al., 2008
			Trica-SK-II	GEEPFDDYGHMRF *+	
	Diptera	*Drosophila melanogaster*	Drome-SK-I	FDDYGHMRF *+	Nichols, 1992; Nichols et al., 1988
			Drome-SK-II	GGDDQFDDYGHMRF +	
		Calliphora vomitoria	Calvo-SK-I	FDDYGHMRF	Duve et al., 1995
			Calvo-SK-II	GGEEFDDYGHMRF	
		Lucilia cuprina	Luccu-SK-I	FDDYGHMRF *	Rahman et al., 2013
			Luccu-SK-II	GGEEQDDYGHMRF *	
		Neobellieria bullata	Neobu-SK-I	FDDYGHMRF *	Fónagy et al., 1992
			Neobu-SK-II	XXEEQFDDYGHMRF *	

(Table I-1 continued)

Class	Order	Species	SK (homolog)	Sequence [#]	Reference
Insecta	Diptera	*Anopheles maculatus*	Anoma-SK-I	FDDYGHMRF +	GenBank: AY341429
			Anoma-SK-II	GGEDGQFDDYGHMRF +	
		Anopheles gambiae	Anoga-SK-I	FDDYGHMRF +	GenBank: AY758365
			Anoga-SK-II	GGEDGQFDDYGHMRF +	
		Delia radicum	Delra-SK-I	FDDYGHMRF *	Audsley et al., 2011; Zoephel et al., 2012
			Delra-SK-II	GGEEQFDDYGHMRF *	
	Hemiptera	*Rhodnius prolixus*	Rhopr-SK-I	pQFNEYGHMRF *+	Ons et al., 2009, 2011
			Rhopr-SK-II	NSDEQFDDYGHMRF *+	
	Hymenoptera	*Apis mellifera*	Apime-SK-I	pQQFDDYGHLRF *+	Hummon et al., 2006
			Apime-SK-II	EQFEDYGHMRF +	
	Orthoptera	*Gryllus bimaculatus*	Grybi-SK-I	QSDDYGHMRF +	Meyering-Vos and Müller, 2007b
			Grybi-SK-II	EPFDDYGHMRF +	
		Locusta migratoria	Leumi-SK-I	pQLASDDYGHMRF *	Schoofs et al., 1991
Arachnida	Ixodida	*Ixodes scapularis*	Ixosc-SK-I	QDDDYGHMRF *+	Neupert et al., 2009
			Ixosc-SK-II	SDDYGHMRF *+	

(Table I-1 continued)

Class	Order	Species	SK (homolog)	Sequence [#]	Reference
Malacostraca	Decapoda	*Penaeus monodon*	Penmo-SK-I	pQFDEYGHMRF +	Johnsen et al., 2000
			Penmo-SK-II	VGGEYDDYGHLRF +	
			Penmo-SK-III	AGGSGGVGGEYDDYGHLRF +	
		Homarus americanus	Homam-SK-I	pEFDEYGHMRF +	Dickinson et al., 2007
			Homam-SK-II	GGGEYDDYGHLRF +	
		Litopenaeus vannamei	Litva-SK-I	pQFDEYDDYGHL/IRF *	Torfs et al., 2002
			Litva-SK-II	pQFDEYGHMRF *	
Remipedia	Nectipoda	*Speleonectes cf. tulumensis*	Spetu-SK-I	pQFDDYGHMRF +	Christie, 2014
			Spetu-SK-II	DFDDYGHMRF +	
Chromadorea	Rhabditida	*Caenorhabditis elegans*	NLP-12a	DYRPLQF +	McVeigh et al., 2006
			NLP-12b	DGYRPLQF +	
			NLP-120	SYRPLQF +	
Amphibia	Anura	*Xenopus laevis*	caerulein	pQQDYTGWMDF *	Bertaccini, 1976
Ascidiacea	Enterogona	*Ciona intestinalis*	cionin	NYYGWMDF *	Johnsen and Rehfeld, 1990
Mammalia	Primates	*Homo sapiens*	CCK-8	DYMGWDF *	Dockray et al., 1978
			gastrin-II	pQGPWLEEEEEAYGWMDF *	Bentley et al., 1966

#, p represents a pyroglutamyl moiety; *, sequences identified via peptide isolation; +, sequences deduced from cDNA/DNA. The C-terminal (-NH_2) and the sulfate moiety (-SO_3H) on the residue Y are emitted.

The distribution of SKs has been studied by means of immunochemistry. In addition to head, SKs have been detected in the central and sympathetic nervous system of the American cockroach *P. americana* and the field cricket *Teleogryllus commodus* (East et al., 1997) and in the suboesophageal ganglion of the blow fly *P. regina* (Downer et al., 2007). However, data also show that the expression of SKs is restricted to the brain in the field cricket *G. bimaculatus* (Meyering-Vos and Müller, 2007b) and the blow fly *C. vomitoria* (Duve et al., 1994, 1995).

SKs are involved in various physiological processes as a general neurotransmitter. First, they influence the frequency and amplitude of foregut and hindgut visceral muscle contractions and heart contraction (Maestro et al., 2001; Nachman et al., 1986b, 1986c; Nichols, 2007; Nichols et al., 2008b). Second, they significantly inhibit food intake in the locust *S. gregaria* (Wei et al., 2000), the cockroach *B. germanica* (Maestro et al., 2001) and the cricket *G. bimaculatus* (Meyering-Vos and Müller, 2007a). In the blow fly *P. regina*, SKs decrease carbohydrate ingestion (Downer et al., 2007). In *D. melanogaster*, SK is released to regulate feeding with insulin-like peptides in a coordinated fashion (Söderberg et al., 2012). Moreover, SKs stimulate the release of the digestive enzyme α-amylase in the red palm weevil *R. ferrugineus* (Nachman et al., 1997) and the coconut pest *Opisina arenosella* (Harshini et al., 2002).

4.2. Sulfakinin homologs in Metazoa

Homologs of insect SKs have been identified as cholecystokinin (CCK)/gastrin in vertebrates (Johnsen, 1998), cionin in tunicates (Johnsen and Rehfeld, 1990; Sekiguchi et al., 2012) and neuropeptide-like protein 12 (NLP-12) in nematodes (Janssen et al., 2008), based on their structural and functional similarity.

4.2.1. Sequence

CCK and gastrin have been studied widely in mammalian vertebrates, especially in human. They share a common amidated C-terminal tetrapeptide sequence, WMDF-NH_2 (Figure I-3), which constitutes the minimal structure necessary for receptor binding and biological activity, although potencies of both peptides depend upon their N-terminal extensions (Dufresne et al., 2006). CCKs are expressed as peptides of various lengths including 58, 39, 33 and eight residues, each containing a sulfated Y residue (Eysselein et al., 1990). Gastrins are processed

to mature products with 34 and 17 amino acid residues (Dockray et al., 2001). Structural and functional similarities of CCK and gastrin suggest that they have evolved from a common ancestor (Dimaline and Dockray, 1994; Dockray, 1977; Larsson and Rehfeld, 1977; Vigna, 1986). In addition to mammals, CCK and gastrin peptides have been identified in many non-mammalian species representing the major vertebrate classes, including fish, amphibians, reptiles and birds (Johnsen, 1998).

Cionin in the tunicate *Ciona intestinalis* (Johnsen and Rehfeld, 1990; Thorndyke and Dockray, 1986) and caerulein in the frog *Xenopus laevis* (Hoffmann et al., 1983) were identified as the homologs of CCK/gastrin in vertebrates. They both terminate with the same WMDF-NH_2 sequence. However, caerulein is only found in frog skin and displays functions distinct from CCK/gastrin. Therefore, it is considered as a side branch in the evolution of CCK family (Vigna, 2000; Wechselberger and Kreil, 1995) and is not included in the study here. Cionin possesses two (sulfated) Ys at positions 6 and 7 (Johnsen and Rehfeld, 1990), which is unique in the series of CCK (-like) peptides (Figure I-3).

In nematodes, distant relatives of SKs are identified as neuropeptide-like protein 12 (NLP-12) with the C-terminal sequence YRPLQF-NH_2 (Figure I-3). In *Caenorhabditis elegans*, NLP-12 (also called CK) proves to be the endogenous ligand of the "CCK receptor" (Janssen et al., 2008; McVeigh et al., 2006). So far, NLP-12-like peptides have been identified in different species of nematodes (Janssen et al., 2009).

Figure I-3 depicts the amino acid sequences of six representative SK (-like) prepropeptides including CCK, gastrin, cionin, SK and NLP-12. Notably, an insect SK prepropeptide contains two SK peptides with YGHM/LRF-NH_2 in the C-terminus and a nematode prepropeptide three NLP-12 peptides. The predominant Y (sulfated or nonsulfated *in vivo*) is mostly preceded by a D or a DD/DG motif and followed by an R or G (Figure I-3). In the vertebrate gastrin, the Y is preceded by an EA or AA motif. The consistent C-terminal motif is HM/LRF-NH_2 in insect SKs, PLQF-NH_2 in nematode NLP-12-like peptides, and WMDF-NH_2 in vertebrate CCK/gastrin and *C. intestinalis* cionin. M and L are very similar hydrophobic amino acids that are conserved between all clades at the C-terminal position 3. An M → L substitution in SK or CCK has been used previously to improve the chemical stability and did not influence the functional activity of these peptides (Nachman et al., 1988;

Ron et al., 1992). The amino acid at position 2 (Q, R or D) is only weakly conserved (Figure I-3).

Phylogenetic analysis by Janssen et al. (2008) demonstrated that arthropod SKs, nematode NLP-12s and the vertebrate CCK/gastrin form separate clades, with cionin the most closely related to CCK/gastrin. The authors also hypothesized that the CCK/gastrin signaling system was already well established prior to the divergence of protostomes and deuterostomes. Sekiguchi et al. (2012) proposed that the CCK/gastrin family is essentially conserved in both invertebrates and vertebrates and that cionin and vertebrate CCK/gastrin are derived from a common ancestor. For more evolutionary information, please refer to the review by Johnsen (1998).

4.2.2. Functions

CCK is produced in enteroendocrine I cells of the upper small intestine and brain neurons. It can exert an endocrine effect via receptors in the brain or a paracrine effect via receptors in the gut (Bi and Moran, 2002; Kennedy et al., 1999). In humans, CCK is reported to induce satiety, slow down gastrointestinal motility, stimulate secretion of pepsinogen, inhibit gastric acid secretion by stimulating the production of fundic somatostatin, stimulate gallbladder contraction, and induce endocrine and exocrine pancreatic secretion (Jensen, 2002; Owyang and Logsdon, 2004; Singh and Webster, 1978). CCK can also stimulate nociception, memory and learning processes, panic and anxiety (Kennedy et al., 1999). Extensive reviews about the functions of CCK and its receptors in humans can be found in the relevant medical literature (De Tullio et al., 2000; Dufresne et al., 2006; Rehfeld et al., 2007).

Cionin from *C. intestinalis* stimulates the contraction of rainbow trout gallbladders and the release of histamine and gastric acid in the rat stomach, implying that cionin exerts activities similar to CCK/gastrin in vertebrates (Mårvik et al., 1994; Schjoldager et al., 1995). Interestingly, the sulfated Y^7 of cionin is shown to play a more important role in activation of cionin receptors than sulfated Y^6 (Sekiguchi et al., 2012). Given the fact that Y is present at the position 7 of CCK and the position 6 of gastrin, it is speculated that chordate ancestors might have possessed a CCK-like original peptide with a sulfated Y^7, and the hybrid feature of cionin might have arisen in the *Ciona* evolutionary lineage (Sekiguchi et al., 2012).

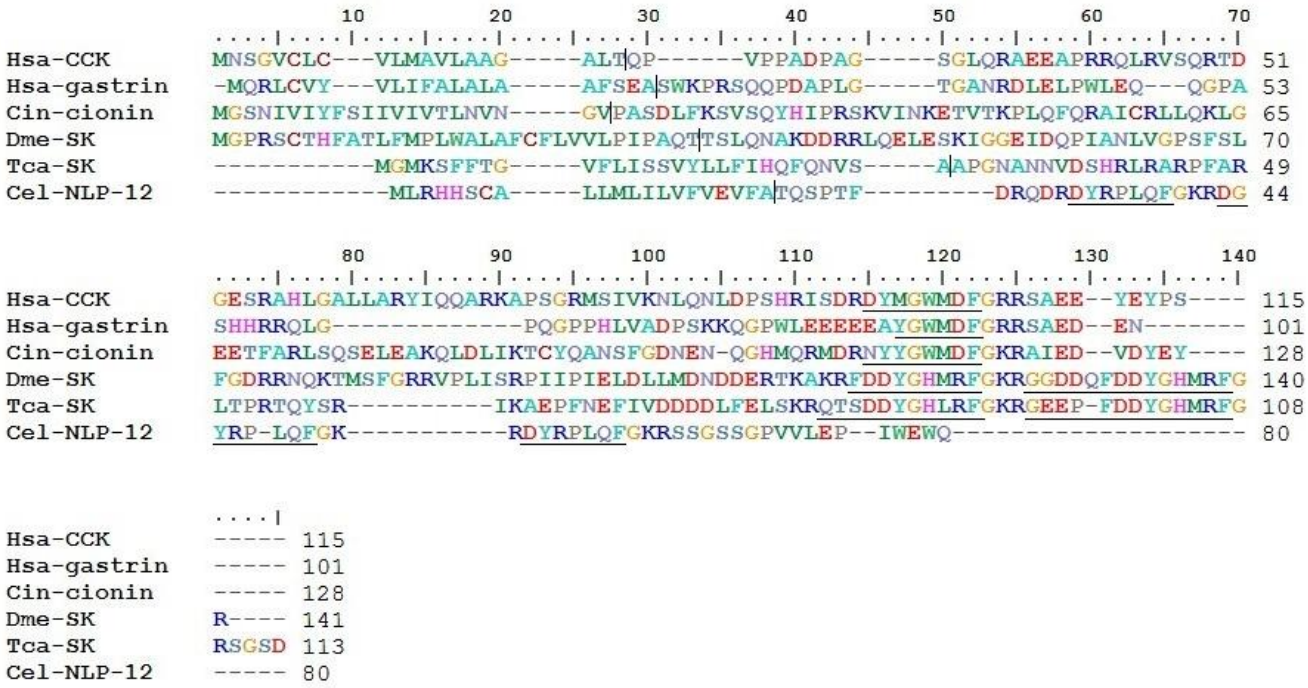


Figure I-3 Alignment of six SK (-like) prepropeptides. Sequences are human CCK precursor (Hsa-CCK, CAG47022.1), human gastrin precursor (Hsa-gastrin, NP_000796.1), *Ciona intestinalis* cionin precursor (Cin-cioin, CAA48884.1), *Drosophila melanogaster* SK precursor (Dme-SK, AAF52173.2), *Tribolium castaneum* SK precursor (Tca-SK, EFA04708.1) and *Caenorhabditis elegans* NLP-12 precursor (Cel-NLP-12, CCD67953.1). The alignment was conducted using ClustalW multiple alignment in BioEdit version 7.0.0 (Hall, 1999). The color of amino acid residue is default color in BioEdit. Dash represents a gap. The putative cleavage site of the signal peptide is indicated by a black vertical bar. The amino acids of biologically active peptides are underlined. CCK-8 is underlined and other CCK variants are with N-terminal extensions from CCK-8.

To investigate the function of NLP-12 (or CK) in nematodes, Janssen et al. (2008) carried out several tests using NLP-12 mutant strain and CK receptor (CKR) mutant strain. The CKR-2 mutants displayed a decrease in intestinal amylase activity, although the development, locomotion and reproduction of both NLP-12 mutant and CKR mutant strains were not affected. However, neither sulfated nor nonsulfated NLP-12 peptides displayed any myotropic activity on increasing the frequency and amplitude of cockroach hindgut contraction, which was dissimilar to the function of CCK and SK (Janssen et al., 2008). Nevertheless, NLP-12 displayed similar biological activities as CCK/gastrin with respect to digestive enzyme secretion and fat storage (Janssen et al., 2008). As a result, more research is required to elicit its functions in nematodes and other invertebrates.

4.3. Sulfakinin (-like) receptor in Metazoa

The action of neuropeptides involves the activation of specific receptors[2], which promotes the activation of intracellular signaling mechanisms that ultimately leads to a cellular response (Cardoso et al., 2012).

4.3.1. Structures and ligand-specificity

CCK and gastrin exert their effects through their transmembrane receptors, CCK receptors (CCKRs). CCKRs are members of the rhodopsin GPCR superfamily (see section 5 below) (Dufresne et al., 2006). The properties of the receptors have been extensively characterized in many species, both physiologically and pharmacologically, by agonist and antagonist binding studies, as well as by the effects of these agents on isolated tissue preparations (Deweerth et al., 1993; Noble and Roques, 1999).

Two CCKRs are present in humans, namely CCK1R and CCK2R. CCK1R is mainly localized in peripheral organs and discrete areas of the brain whereas CCK2R is primarily expressed in brain and stomach (Dufresne et al., 2006). Both receptor types are highly conserved, showing 70-80% amino acid identity between receptors in different species. The CCK1R and CCK2R also show a high degree of mutual similarity with around 50% amino acid identity, suggesting that they share a common ancestor. Both CCKRs contain seven

[2] In this thesis, amino acids are presented with their single-letter code except where three-letter code is necessary. The amino acids and their letter code are listed in the "overview of amino acid" on page vi. Position of an amino acid in a receptor protein is counted from the N-terminus and the amino acid residue is written as single-letter code with a position number behind. For instance, R219 represents that R is at the position 219 from the N-terminus of a receptor protein.

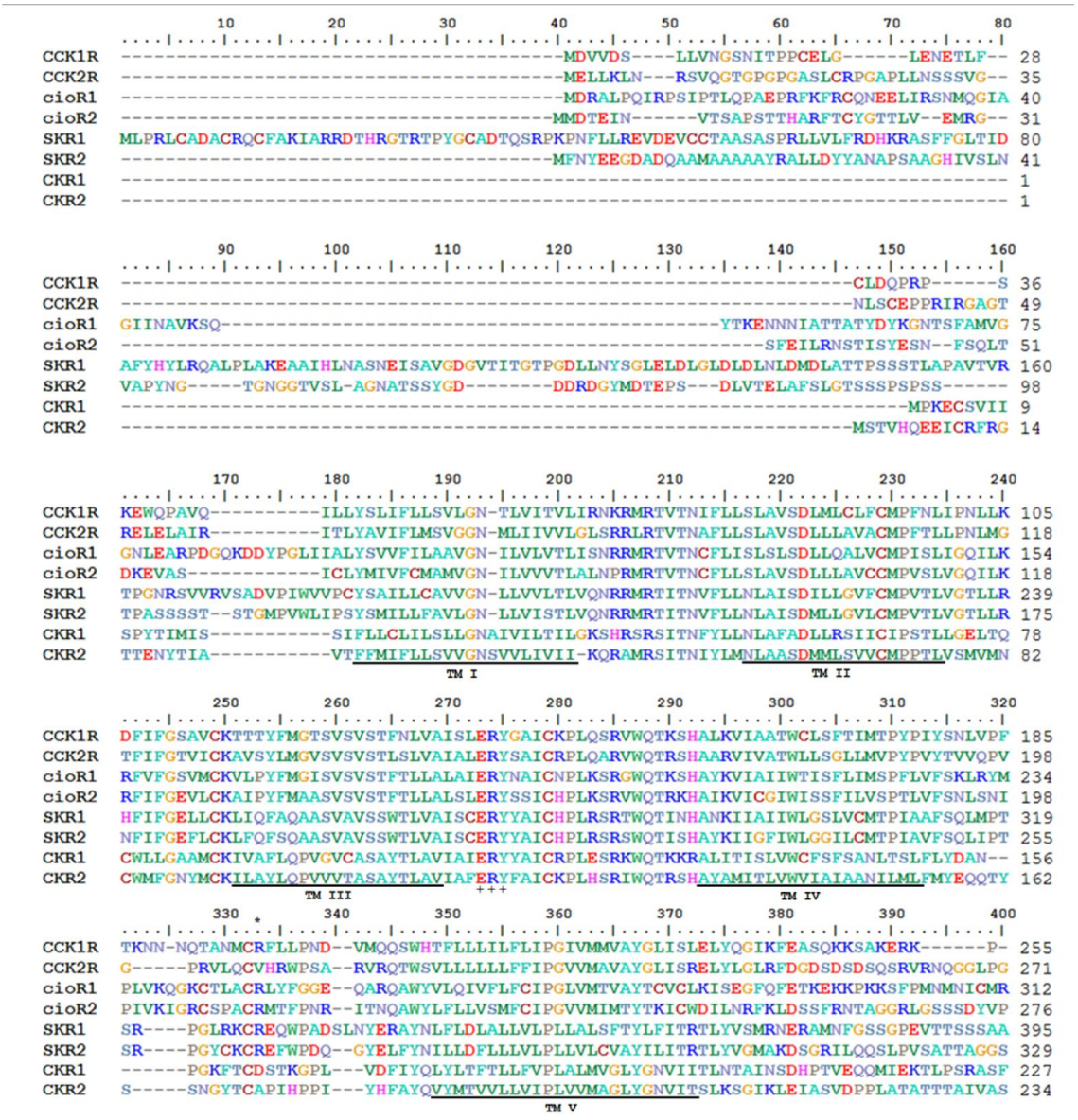
transmembrane domains, with extracellular N-terminal and intracellular C-terminal ends, which is a typical characteristic of GPCRs. Other sequence signatures essential for receptor activation are also present in the CCKRs, such as an E/DRY motif at the bottom of the transmembrane domain III, and NPXXY motif (X represents any amino acid) within transmembrane domain VII (Figure I-4; Dufresne et al., 2006; Galés, C., 2000). While the CCK1R shows high specificity for sulfated CCK, CCK2R is only marginally dependent on sulfation of the ligands and shows little discrimination between gastrin and CCK. CCK1R binds and responds to sulfated CCK with a 500- to 1000-fold higher affinity than sulfated gastrin or nonsulfated CCK. Dufresne et al. (2006) covered CCK and gastrin receptors in detail in their review.

Cionin receptors (CioRs) are cloned and characterized as CioR1 and CioR2 (Figure I-4; Nilsson et al., 2003; Sekiguchi et al., 2012) in *C. intestinalis*. CioR1 shows 35-40% sequence similarity with human CCKRs and for CioR2 the similarity is 59-60%. Moreover, an R residue in the extracellular loop II, which is responsible for CCK1R binding to sulfated CCK, is conserved in CioRs and CCK1R but not in CCK2R (Figure I-4; Sekiguchi et al., 2012). This is likely an explanation for the two sulfated Ys in cionin. To activate CioRs, di-sulfated cionin is much more potent than mono-sulfated cionin derivatives, and sulfation on Y^7 more potent than Y^6. In contrast, nonsulfated cionin exhibits no activity on either CioR1 or CioR2. Thus, it is conclusive that sulfated Y of cionin is required for the activation of CioRs at physiological concentrations (Sekiguchi et al., 2012).

Before the discovery of NLP-12 peptides, two genes encoding CCK-like peptide receptors (CKRs) were found in the *C. elegans* genome with high identity to CCKRs and SKRs (McKay et al., 2007). CKR1 is localized in the nerve ring neurons (McKay et al., 2007) and CKR2 is cloned as two splice isoforms, CKR2a and CKR2b (Janssen et al., 2008). The two isoforms only differ from each other at the C-terminal region. CKR2 was studied for their receptor characteristic since CKR1 was not cloned successfully according to Janssen et al. (2008). CKR2 shows a slightly higher affinity to the nonsulfated NLP-12 peptides than to the sulfated NLP-12. The natural NLP-12 peptides also perfectly co-eluted with the synthetic nonsulfated peptides, which suggests that native NLP-12 peptides are likely to be nonsulfated and thereby, sulfate moiety is not essential and might even slightly impede the activation of CKR2 in *C. elegans*.

The SK (-like) receptors display their ligand-specificity not only to the sulfated/nonsulfated peptides, but also to the peptides from different phyla. Human CCK-8 could not activate the *Drosophila* SKR1 (Kubiak et al., 2002), which involves the difference in the basic R^2 residue in SKs versus the acidic D^2 residue in CCK-8, because replacement of the D^2 residue with R^2 transformed the inactive CCK into an active analog on the cockroach hindgut (Nachman et al., 1988). Consistently, *C. elegans* CKRs could be activated by neither the human CCK-8 nor the insect SKs, which may also involve the different properties of the neutral Q^2 residue in NLP-12 (CK), the acidic D^2 in CCK-8 and the basic R^2 in SK (Janssen et al., 2008).

Genes encoding sulfakinin receptors (SKRs) have also been predicted in many insects thanks to the accumulating number of sequenced genomes (Hauser et al., 2006a, 2006b, 2008; Tanaka et al., 2013). However, to our knowledge, SKRs have only been cloned and characterized in *D. melanogaster* (Chen et al., 2012; Kubiak et al., 2002). Therefore, it is of interest to functionally and pharmacologically characterize SKRs in other insect species.


CCK1R
CCK2R
cioR1
cioR2
SKR1
SKR2
CKR1
CKR2
TM I
TM II
TM III
TM IV
TM V

(Figure I-4)

(Figure I-4 continued)

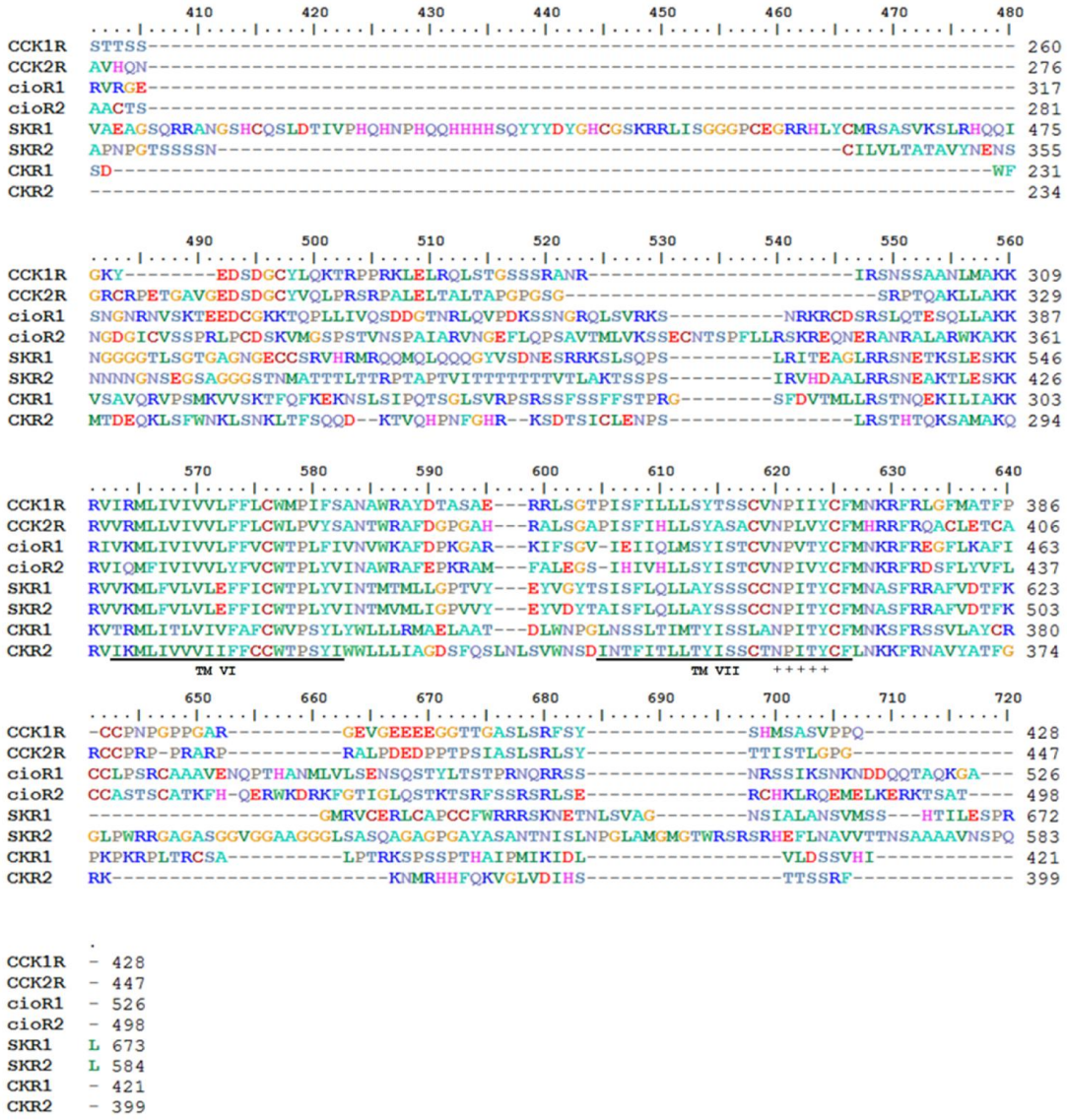


Figure I-4 Alignment of amino acid sequences of eight representatives of SK (-like) receptors. Sequences are *Homo sapiens* CCK1R and CCK2R, *Ciona intestinalis* CioR1 and CioR2, *Drosophila melanogaster* SKR1 and SKR2 and *Caenorhabditis elegans* CKR1 and CKR2 (Table II-2). The alignment was conducted using ClustalW multiple alignment in BioEdit version 7.0.0 (Hall, 1999). The color of amino acid residue is default color in BioEdit. Seven putative transmembrane domains are underlined. The conserved R residue in the extracellular loop II is marked with asterisk. The significant sequence E/DRY motif at the bottom of the transmembrane domain III and the NPXXY motif within the transmembrane domain VII are marked with "+".

5. GPCR signaling

5.1. Mechanism of GPCR signaling

SKRs belong to the protein family of G-protein coupled receptors (GPCRs), which are transmembrane proteins. GPCRs are named so because of their ability to recruit and regulate the activity of intracellular heterotrimeric G-proteins. G-protein composes Gα, Gβ and Gγ subunits. Gα-protein mediates the G-protein-dependent signaling pathways of GPCRs. Figure I-5 shows the classic GPCR signaling pathways. Once bound with ligand, the receptor is activated and induces a conformational change in the Gα protein, followed by the binding of GTP to Gα-protein. Four main classes of Gα-proteins are known as $G\alpha_i$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12}$ (Simon et al., 1991).

In a classic GPCR signaling complex, both the GTP-bound Gα and the released Gβγ-dimer can modulate several cellular signaling pathways. These include, among others, the stimulation of adenylate cyclase (AC) via $G\alpha_s$ and the inhibition of AC via $G\alpha_i$, as well as the activation of phospholipase C (PLC) via $G\alpha_q$ (Oldham and Hamm, 2008). The stimulation of AC results in an increase in the intracellular cyclic AMP (cAMP); the activation of PLC produces inositol triphosphate (IP3), which stimulates the intracellular calcium mobilization (Oldham and Hamm, 2008).

The signaling cascades modulate gene transcription via the interaction of activated transcription factor with specific response element (RE), which are located upstream of the regulated gene. By fusing these REs with reporter genes, we can couple the activation of a GPCR to the regulation of the transcription of the reporter gene (Figure I-6). Thus, this affords a feasible way to detect GPCR modulation (Cheng et al., 2010; Fan et al., 2005). The two reporter plasmids in Figure I-6b are commercially available reporter systems containing the cAMP response element (CRE) and the nuclear factor of activated T-cells response element (NFAT-RE), respectively. In mammalian cells, increased cAMP activates protein kinase A (PKA) by dissociating the regulatory subunit of PKA from the catalytic subunit (PKAC). This activated PKAC then recruits the Ca^{2+}/CalmK-IV (Calmodulin (Calm)-dependent Kinases), MEK (MAPK/ERK Kinases)/ ERK1/2 (Extracellular Signal-Regulated Kinases) and together they translocate to the nucleus (Ahmed and Frey, 2005; Choe and Wang, 2002). In the nucleus they activate the CRE binding protein (CREB) and CREB interacts with its nuclear partner CREB binding protein (CBP) and drives the transcription of

the downstream gene (Radhakrishnan et al., 1999). In another signaling pathway, increased Ca^{2+} binds to the calcium sensor protein calmodulin, which in turn activates calcineurin. Calcineurin dephosphorylates and activates NFAT transcription factors, which then translocate to the nucleus, where they can cooperate with multiple transcriptional partners to regulate gene expression. Although the two reporter system have been widely used for GPCR signaling study in mammalian cells such as HEK293 cells (Beggs and Mercer, 2009) and CHO-K1 cells (Schucht et al., 2011), few studies have been reported for their application in insect cells.

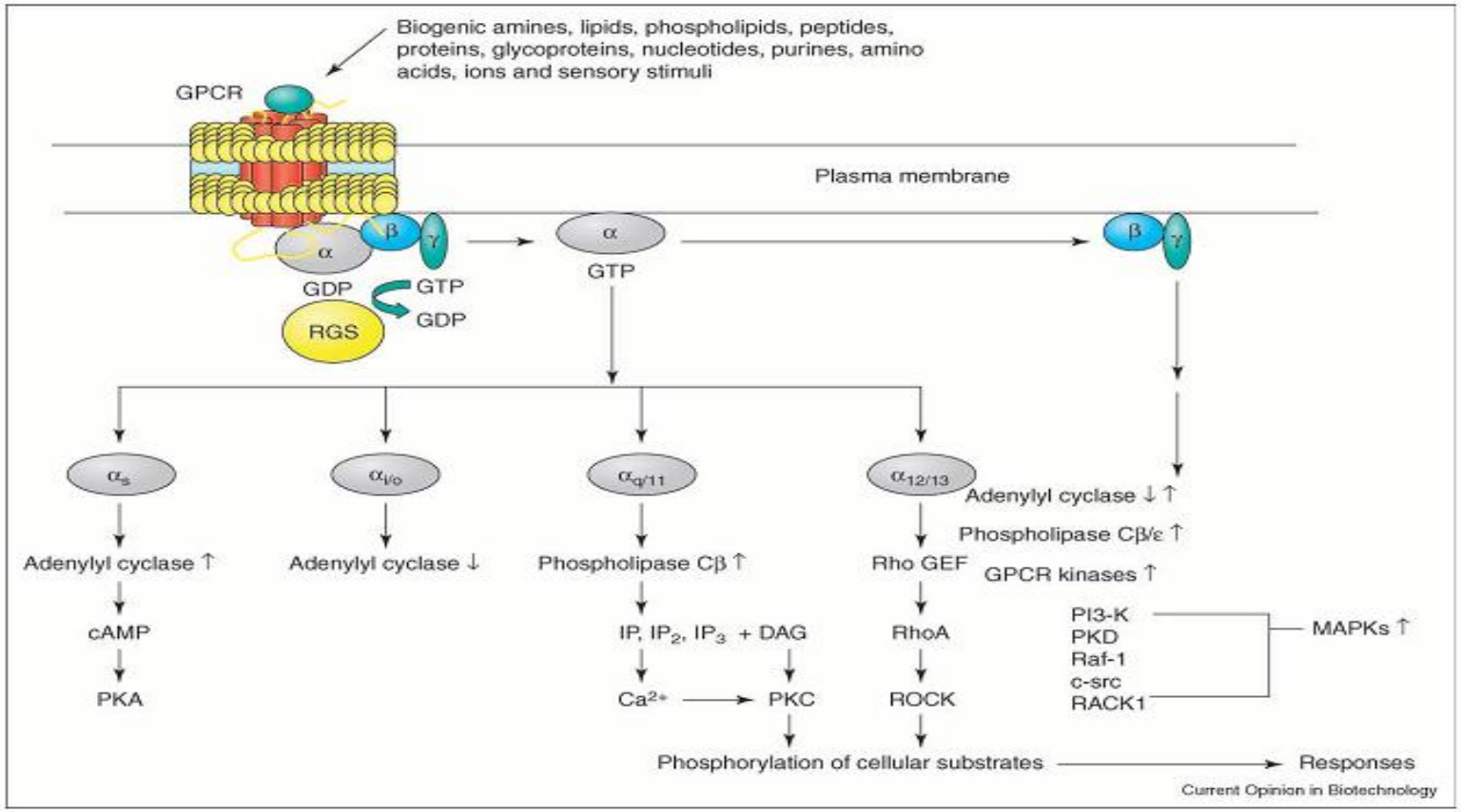


Figure I-5 Classic GPCR signaling pathways. Ligands of GPCRs are diverse from molecules as small as ions to as big as proteins. Ligands initiate the signaling pathway by binding and activating GPCRs. Inside of cells, several signaling pathways are present depending on the type of Gα protein that GPCR binds. Signaling is then transduced to nucleus and regulates gene expression, which elicits biological responses (after Thomsen et al., 2005).

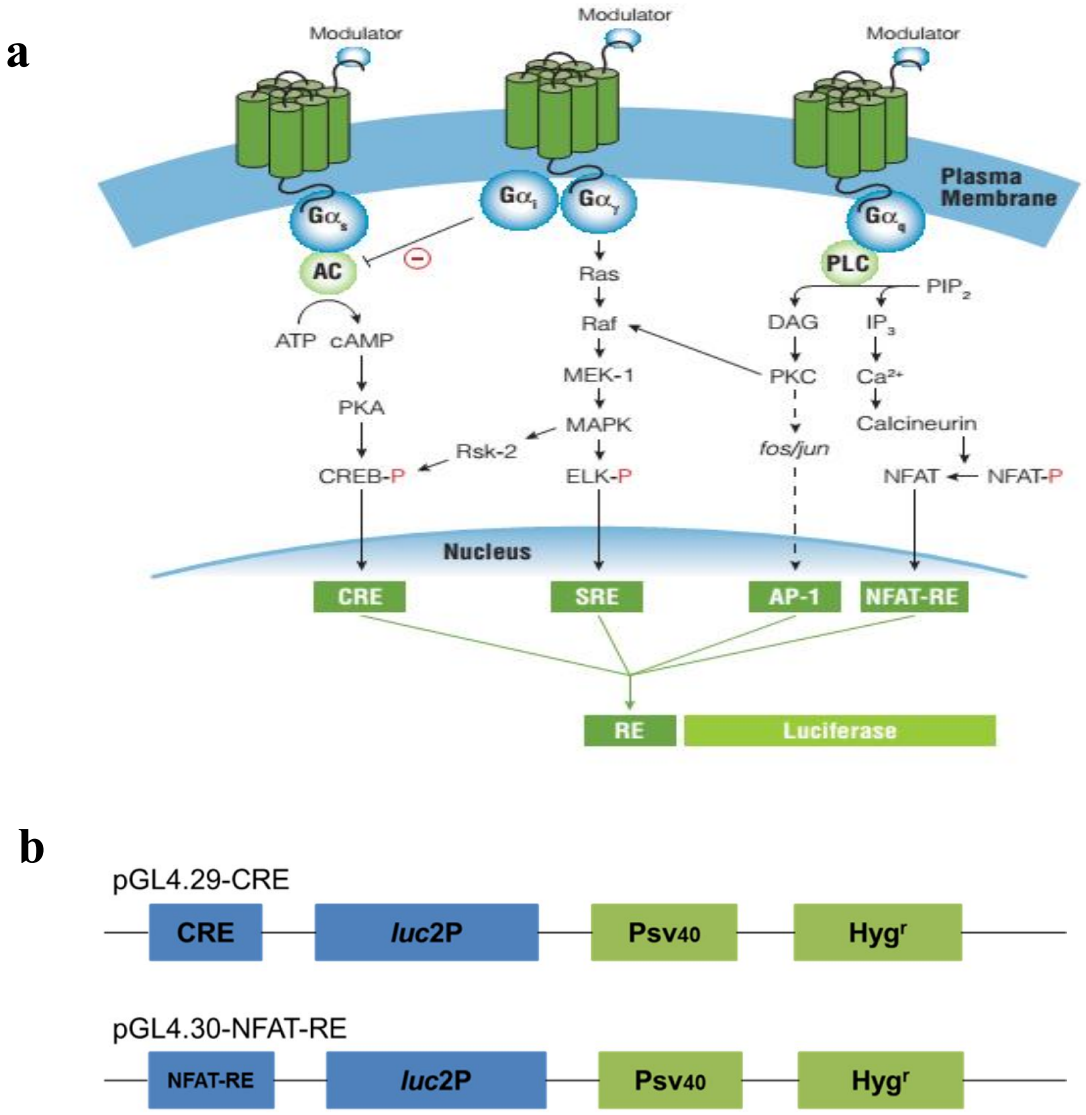


Figure I-6 Schematic diagram showing GPCR signaling pathways (a) and two plasmids involved in the GPCR signaling assay (b). As a consequence of the signaling pathways, corresponding response element (RE) is activated, which in turn activates the transcription of the adjacent reporter gene. Two plamids pGL4.29-CRE and pGL4.30-NFAT-RE contain the same sequence for reporter gene *luc*2P and selective Hyg^r beside the cAMP response element (CRE) and nuclear factor of activated T-cells response element (NFAT-RE), respectively. *luc*2P is an enhance luciferase gene. Psv40 is SV40 late poly(A) signal.

5.2. Platform for insect GPCR signaling study

Most of the insect neuropeptide receptors are GPCRs. They have attracted lots of attention because of their important roles in insect physiology. The neuropeptide receptors are relatively conserved during evolution and this has facilitated the prediction of neuropeptide GPCR genes in the sequenced insect genomes such as *D. melanogaster*, *Tribolium castaneum* and *Apis mellifera* (Hauser et al., 2006a, 2006b, 2008). Meanwhile, insect neuropeptide GPCRs are functionally and pharmacologically studied (Caers et al., 2012). A neuropeptide GPCR is often deorpharized in a cell-based assay, where the GPCR of interest is exogenously expressed in host cells. Fluorescence and bioluminescence are common observable parameters in these assays, where molecules such as luminescent photoprotein aequorin and the promiscuous $G\alpha_{16}$-protein are often employed (Douris et al., 2006; Knight et al., 2003). The activation of a GPCR is quantitated by measuring fluorescence or luminescence output relating to the intracellular calcium mobilization or the intracellular cAMP accumulation.

Many of the fundamental properties of functional assay systems are determined by the nature of the host cells. These properties include 1) the ability to express the target GPCR with appropriate posttranslational modifications and in the required cellular location, 2) the presence (or absence) of endogenous GPCRs, G-proteins, effector proteins, scaffolding proteins and accessory molecules, and 3) the ease and cost of maintaining and storing cell lines. To date, the cells most commonly used in functional GPCR assays include mammalian (Jayawickreme and Kost, 1997), amphibian (McClintock and Lerner, 1997) and yeast cells (Pausch, 1997). All these cell platforms have their own advantages and disadvantages. For example, the high number of endogenous GPCRs commonly found in mammalian or amphibian cells can lead to false positive results in screening applications; yeast cells can fail to express post-translationally modification and/ or traffic receptors properly, which leads to false negative results (Knight et al., 2003). The maintenance of mammalian cell cultures requires carefully controlled conditions, fairly expensive media and CO_2. Last but not the least, the generation of mammalian cell clones often takes several months of subculture for that stably overexpress recombinant proteins, making mammalian systems rather labor intensive (Douris et al., 2006).

Insect GPCR signaling studies have been very often carried out in mammalian CHO or HEK cells, such as the characterization of the *R. prolixus* CAPA receptor (Paluzzi et al., 2010), the

short neuropeptide F receptor in the desert locust *S. gregaria* (Dillen et al., 2013) and the allatotropin receptor in the bumblebee *Bombus terrestris* (Verlinden et al., 2013). In *T. castaneum*, an allatotropin-like receptor and a 5-HT_1-type serotonin receptor are also characterized in the mammalian cell-based assay (Vleugels et al., 2013; Vuerinckx et al., 2011).

Insect cells provide an alternative to mammalian cells. Insect cell-based expression systems are prominent among expression platforms for their ability to express virtually all types of heterologous recombinant proteins. Stably transformed insect cell lines are especially suited for the production of secreted and membrane-anchored proteins (McCarroll and King, 1997), such as the *Drosophila* Schneider 2 (S2) cell line (Torfs et al., 2000; Vanden Broeck et al., 1995), the *Spodoptera* Sf9 cell line (Joyce et al., 1993), the *Spodoptera* Sf21 cell line and the *Bombyx* Bm5 cell line (Farrell et al., 1998, 1999). The Sf9 cell line, derived from *S. frugiperda* pupal ovarian tissue, is one of the most widely used insect cell lines. First, it is a suitable host for the expression of recombinant proteins with high yield, resulting in increased signals in binding assays. Second, three types of Gα-protein have been found in Sf9 cells: $G\alpha_i$, $G\alpha_q$ and $G\alpha_s$ proteins (Knight and Grigliatti, 2004), which makes it possible to transduce GPCR signaling. Therefore, Sf9 cell line is considered a versatile model system to investigate the pharmacological properties of GPCRs, not only for mammalian GPCRs but also for insect GPCRs (Douris et al., 2006; Schneider and Seifert, 2010). However, the major pitfall of insect cells for insect GPCR signaling study is that the potential endogenous GPCRs may respond to tested compounds and bring out false positive results.

6. RNA interference

RNA interference (RNAi) refers to the double-stranded RNA (dsRNA)-mediated gene silencing in eukaryotic cells (Hannon, 2002). It can be categorized into two pathways: the small interference RNA (siRNA) pathway and the microRNA (miRNA) pathway (Jinek and Doudna, 2009). The siRNA pathway is triggered by either endogenous or exogenous dsRNAs and silences endogenous genes carrying homologous sequences at both transcriptional and post-transcriptional levels. The miRNA pathway is triggered by miRNAs derived from hairpin-structured non-coding mRNAs. Since we use exogenous dsRNAs to silence target gene in our research, we mainly focus on the siRNA pathway in this section.

6.1. Mechanism of RNAi

The hallmark of RNAi is the discovery of dsRNAs as the trigger that initiate gene silencing, as dsRNA is substantially more effective at producing interference than is either strand individually (Fire et al., 1998). Soon afterwards, it is confirmed that the gene silencing phenomena known variously as post-transcriptional gene silencing, co-suppression, quelling and RNAi, share a common underlying mechanism in plants, fungi and animals (De Carvalho et al., 1992; Napoli et al., 1990; Romano and Macino, 1992).

Figure I-7 briefly depicts the mechanism of RNAi. In the initiation step, dsRNAs are processed by an RNase III ribonuclease, Dicer, into siRNAs with length of ~21 nucleotides. Following the production of siRNAs is the formation of a RNA-induced silencing complex (RISC), which is a protein-RNA effector nuclease complex that recognizes and destroys target mRNAs. In RISC, the most important elements are the siRNAs and the Argonaute protein. The double-stranded siRNAs are unwound in the RISC into a guide strand and a passenger strand. The passenger strand leaves the RISC and is degraded, while the guide strand leads the RISC to and pairs with the target mRNA. Thus, the mRNA is enzymatically degraded. The RNAi mechanism is covered in many reviews in detail (Hannon, 2002; Jinek and Doudna, 2009; Meister and Tuschl, 2004; Mello and Conte, 2004).

6.2. Systemic RNAi

A fascinating feature of RNAi is its ability to spread throughout the organism, the phenomenon called “systemic RNAi”. The RNAi effect is not only present in the cells directly exposed to dsRNAs but also triggered in the cells far from the initial administration site, and even in the progeny. This phenomenon has been found in plants and many animals (Aronstein et al., 2006; Dong and Friedrich, 2005; May and Plasterk, 2005; Mlotshwa et al., 2002; Voinnet, 2005; Xie and Guo, 2006).

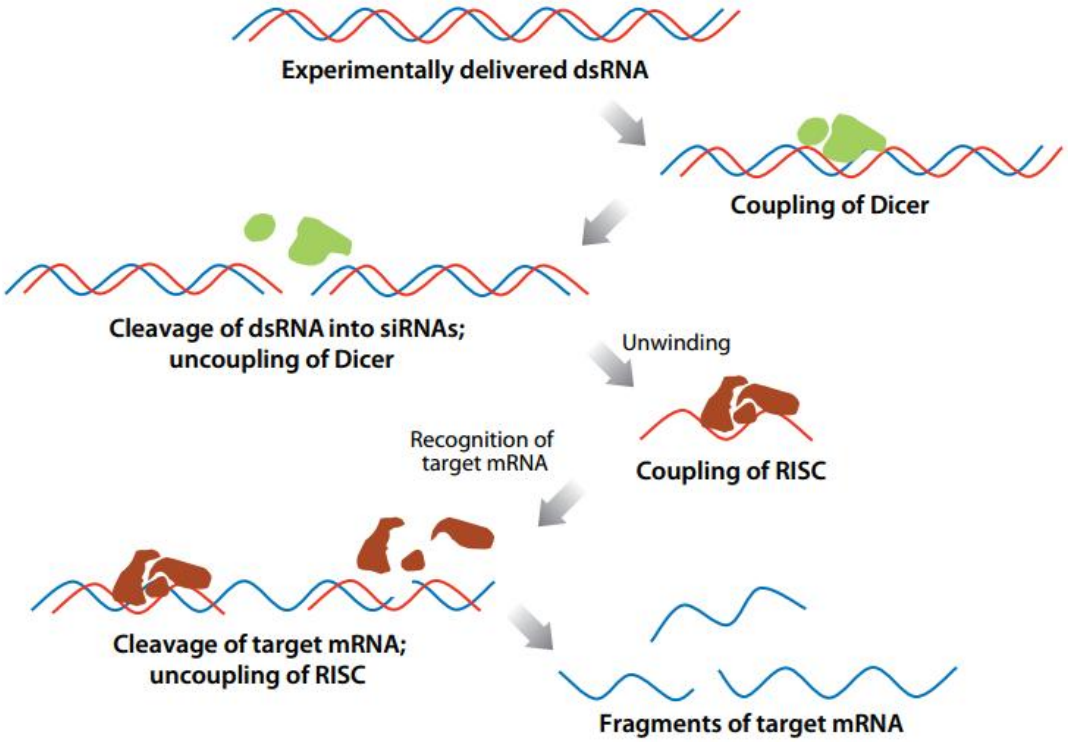


Figure I-7 Basic mechanism of double-stranded RNA (dsRNA)-mediated RNA interference (RNAi). The dsRNA is cleaved into fragments of ~21 nucleotides (small interference RNAs, siRNAs) by the enzyme Dicer. The siRNAs are unwound in the RNA-induced silencing complex (RISC), and the guide strand couples to the target mRNA. Then RISC blocks and degrades the target mRNA (after Bellés, 2010).

One important step of systemic RNAi is the uptake of dsRNA (environmental RNAi) (Figure I-8). The uptake of dsRNA in *C. elegans* has been investigated and several relevant genes have been identified. Among them, gene *sid-1* is regarded as a primary element in the transmembrane channel-mediated mechanism, facilitating the import of dsRNAs from environment into cells (Tijsterman et al., 2004; Winston et al., 2002). However, *sid-1* homologs in *T. castaneum* do not seem to be required for the robust systemic RNAi (Tomoyasu et al., 2008) and a gene initially identified as *sid-1*-like is not required for the systemic RNAi in *L. migratoria* (Luo et al., 2012).

Intriguingly, there is no robust systemic RNAi effect in the best known insect model *D. melanogaster*, because RNAi is only observed in haemocytes but no other tissues in dsRNA-injected larvae (Miller et al., 2008). It is speculated that failure in upstream events in the

systemic RNAi pathway, such as dsRNA cellular uptake, transport or maintenance, is responsible for the ineffective RNAi in most *Drosophila* larval tissues. However, several publications reported successful silencing by direct injection of dsRNAs in adult *Drosophila* (Dzitoyeva et al., 2001; Goto et al., 2003), suggesting the difference in tissue specificity and developmental stages. RNAi is exerted in *Drosophila* S2 cells when the cells are soaked in dsRNA-containing medium, which involves the endocytosis-mediated uptake mechanism (Saleh et al., 2006; Ulvila et al., 2006). For details about the two dsRNA uptake mechanisms, please refer to relevant reviews (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008).

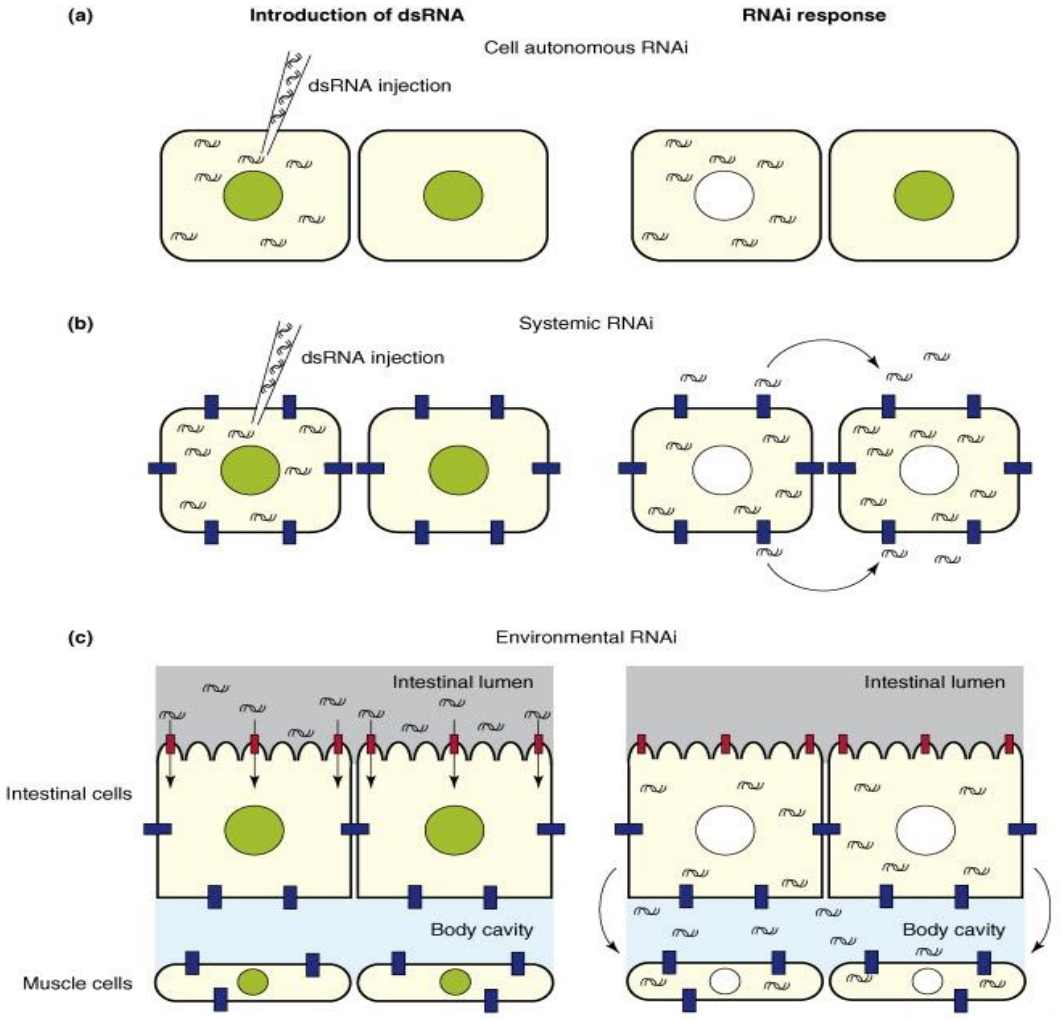


Figure I-8 Schematical representation of the cell autonomous, systemic and environmental RNA interference (RNAi). On the left are cells expressing green fluorescent protein (GFP). Theses cells are exposed to double-stranded RNA (dsRNA) targeting *gfp* expression. Cells on the right show an RNAi response to dsRNA targeting

***gfp* expression. (a) Cell autonomous RNAi: GFP expression is silenced only in the cell that is directly exposed to dsRNAs. The neighboring cell continues to express GFP. (b) Systemic RNAi: GFP expression is silenced in the cell that is directly exposed to dsRNAs. In addition, dsRNAs and dsRNA-derived silencing signals can spread by dsRNA-transporting channels (depicted by blue rectangles) and this leads to the silencing of GFP expression in the neighboring cell. (c) Environmental RNAi: dsRNA molecules present in the intestinal lumen are taken up at the apical membrane of intestinal cells by dsRNA importers (depicted by red rectangles) and lead to the silencing of GFP expression in the intestinal cells. The dsRNAs and dsRNA-derived silencing signals are exported from the intestinal cells and spread to cells throughout the animal by systemic RNAi and lead to the spread of GFP silencing in distant cells (after Whangbo and Hunter, 2008).**

The second element for successful systemic RNAi is the amplification of silencing signal. A RNA-dependent RNA polymerase (RdRP) catalyzes the synthesis of secondary siRNAs and amplifies the silencing response in *C. elegans* and plants (Meister and Tuschl, 2004). In contrast, RdRP homologs have not been found in any insect genome sequenced to date while systemic RNAi seems to exist in many insect species (Bucher et al., 2002; He et al., 2006; Shukla and Palli, 2012; Tomoyasu et al., 2008). Hence, there might be a different mechanism for insects to maintain and amplify silencing signals from even a minute amount of dsRNAs within the organism and even through generations.

The red flour beetle *T. castaneum* has been used as a model insect for RNAi because of its robust systemic RNAi (Tomoyasu et al., 2008). In addition, larval RNAi and parental RNAi benefit the analysis of adult development and early embryogenesis (Bucher et al., 2002; Tomoyasu and Denell, 2004). Therefore, gene functional studies have been widely carried out in different tissues in *T. castaneum* at different developmental stages (Fraga et al., 2013; Knorr et al., 2013; Shukla and Palli, 2013; Xu et al., 2013). In the ongoing large scale RNAi screen project “iBeetle”, a quantity of genes are being systematically knocked down and functionally studied (iBeetle-Base, http://ibeetle-base.uni-goettingen.de/).

6.3. Delivery of dsRNA

DsRNAs can be delivered into the organism by injection (Fire et al., 1998), feeding (Patel et al., 2007; Turner et al., 2006) or soaking (Alvarado and Newmark, 1999; Tabara et al., 1998;

Timmons et al., 2001). The choice of dsRNA delivery method depends on many factors, such as the 6target gene, the species, and the purpose and scale of the application.

The most widely used routes for administering RNAi in insects are the injection of dsRNAs into the hemolymph and feeding. Injection straightforwardly sends a certain amount of dsRNAs into a specific site, while it is time-consuming and trauma-causing. In contrast, feeding is a less-invasive and potentially a high-throughput method. But it is limited in precise estimation of dsRNA utility and can be more prone to degradation of dsRNA before uptake. The other methods exploited in a minority of studies include soaking in dsRNA-containing solution, electroporation, incorporation of nanoparticle, dsRNA-expressed transgene plant etc. A recent review (Yu et al., 2013) compared the different dsRNA-delivery approaches. So far, most cases of successful RNAi in *T. castaneum* are carried out via injection of dsRNAs because systemic RNAi works efficiently in this model insect.

6.4. Efficiency of RNAi

The RNAi effect varies greatly among species. Successful gene silencing via RNAi has been reported in the nematode *C. elegans* (Fire et al., 1998; Montgomery et al., 1998; Timmons et al., 2001), the fly *D. melanogaster* (Dzitoyeva et al., 2001; Goto et al., 2003; Miller et al., 2008), the beetle *T. castaneum* (Arakane et al., 2005; Bucher et al., 2002; Tomoyasu and Denell, 2004) and the honeybee *A. mellifera* (Antonio et al., 2008; Maleszka et al., 2007; Patel et al., 2007) etc. However, some species seem to be refractory to RNAi. For example, RNAi has many times proven to be difficult to achieve in Lepidoptera (moths and butterflies). Therefore, several possible factors influencing the efficiency of RNAi in Lepidoptera were discussed (Terenius et al., 2011), such as the methods of dsRNA delivery, systemic RNAi, environmental RNAi, tissue susceptibility, length and stability of dsRNA etc. In addition to lepidopterans, pea aphid *Acyrthosiphon pisum* is often reported with less effective RNAi response (Christiaens et al., 2014), although several successful experiments were published (Jaubert-Possamai et al., 2007; Mao and Zeng, 2012; Mutti et al., 2006; Pitino et al., 2011).

For the efficiency of dsRNA-mediated RNAi in *T. castaneum*, a recent study determines comprehensively that the length and concentration of dsRNAs have profound effects on the efficacy of the RNAi response in regard to both the initial efficiency and the duration of the silencing effect in *T. castaneum* (Miller et al., 2012). In that study, long dsRNAs and dsRNAs at a higher concentration are proven to be more effective on triggering RNAi effect with a

longer duration. DsRNAs are ranged approximately 120-520 bp in most *T. castaneum* studies, which proves to be effective (Arakane et al., 2005, 2008; Tomoyasu and Denell, 2004).

7. *Tribolium castaneum*

7.1. Taxonomy and introduction

Phylum: Arthropoda
Class: Insecta
Order: Coleoptera
Family: Tenebrionidae
Genus: *Tribolium*

The red flour beetle *T. castaneum* (Herbst, 1797) is a representative species from the holometabolous order Coleoptera (beetles). Many beetles are pests in agricultural systems and *T. castaneum* is a global pest of stored agricultural products. It has been reported in grain, flour, beans, cacao, cottonseed, shelled nuts, dried fruit, dried vegetables, spices, chocolate, dried milk and animal hides. Approaches for controlling the beetles have been tried and assessed given the severe damage that they cause (Arthur, 2010; Arthur and Puterka, 2002; Soderstrom et al., 1992).

T. castaneum has been used as a model organism in studies on population ecology and pest control since the 1930s (Sokoloff, 1974). Additionally, it is a model organism that offers considerable advantages for fundamental biological study, including ease of rearing in large numbers, a sequenced genome (Richards et al., 2008) and a strong systemic RNAi response (Tomoyasu et al., 2008). *Tribolium*-related projects have been described, such as the *Tribolium* ontology (TrOn) (Dönitz et al., 2013) and iBeetle RNAi screening. Moreover, a *Tribolium*-derived cell line TcA (BCIRL-TcA-CLG1) is developed recently (Goodman et al., 2012).

7.2. Life cycle and growth conditions

As a typical holometabolous insect, *T. castaneum* has four developmental stages: egg, larva, pupa and adult (Figure I-9). Female adults are very fecund and can lay 300-400 eggs during a 5- to 8-month period. Eggs are microscopic, cylindrical and white, laid in the grain product. They begin to hatch into larvae within 5 to 12 days. A larva is brownish-white, up to 12 mm

long, with six almost invisible legs. Larvae live inside grains for 22-100 days until they enter the pupa stage. There are 5-9 larval instars, depending on the growth conditions. The pupa stage lasts for about 8 days before a full-grown beetle emerges. The adult beetle is very active and prefers living away from light. The complete life cycle takes 40-90 days, and adults can live up to three years.

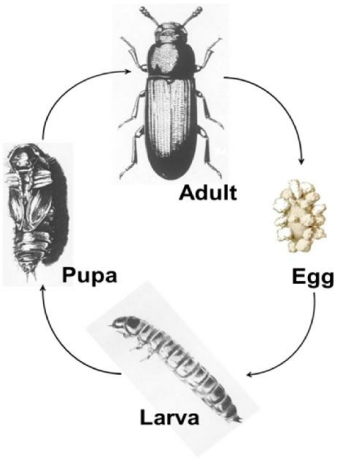


Figure I-9 Life cycle of *Tribolium castaneum*. The beetle develops through egg, larva, pupa and adult (image sources: APHLIS Africa and PestWeb).

The development parameters depend very much on the living conditions, particularly the temperature and humidity. The standard conditions for keeping the beetle stock is 25 °C, relative humidity (R.H.) 60%, although the beetles are tolerant to a wide range of temperature (20-40 °C) and R.H. (40-80%). Beetles show fast embryonic development and high RNAi effect at 30 °C (*Tribolium* beetle book).

The *T. castaneum* colony (strain GA-1; Haliscak and Beeman, 1983) in our lab is fed with whole wheat flour with 5% yeast and maintained in a climate chamber of 30 °C, R.H. 70% and constant darkness. Under these conditions, most larvae have six instars.

Chapter II. Characterization of sulfakinin and sulfakinin receptors and their roles in feeding in the red flour beetle *Tribolium castaneum*

Parts of this chapter are published in:

Yu, N., Nachman, R.J., Smagghe, G. (2013) Characterization of sulfakinin and sulfakinin receptor and their roles in food intake in the red flour beetle *Tribolium castaneum*. General and Comparative Endocrinology, 188: 196-203.

Yu, N., Smagghe, G. (2014) Characterization of sulfakinin receptor 2 and its role in food intake in the red flour beetle, *Tribolium castaneum*. Peptides 53, 232-237.

Yu, N., Smagghe, G. (2014) CCK (-like) and receptors: structure and phylogeny in a comparative perspective. General and Comparative Endocrinology, in press.

1. Introduction

Sulfakinin (SK) peptides were first isolated from the extract of cockroach head based on their myotropic activity (Nachman et al., 1986b, 1986c). Since then, they have been identified via either peptide isolation (Duve et al., 1995; Maestro et al., 2001) or gene cloning (Meyering-Vos and Müller, 2007b; Nichols, 1992). With the increasing genome sequencing projects, sequence information of SK genes is readily accessible in more species, such as the wasp *Nasonia vitripennis*, the blood-sucking bug *Rhodnius prolixus* and the red flour beetle *Tribolium castaneum*. (Amare and Sweedler, 2007; Hauser et al., 2010; Ons et al., 2011; Tanaka et al., 2013). SKs reduce insect food intake in assays with either direct peptide injection (Maestro et al., 2001; Wei et al., 2000) or SK gene silencing (Meyering-Vos and Müller, 2007a).

In *T. castaneum*, one SK precursor gene (*sk*) and two SK receptor (SKR) genes (*skr1* and *skr2*) are predicted, and the Trica-SK-I and Trica-SK-II peptides are confirmed by mass spectrometry (Li et al., 2008). However, their physiological functions remain unclear.

The present study aims at figuring out the characterization and function of SK signaling in *T. castaneum*. First, the coding sequence of the genes *sk*, *skr1* and *skr2* were cloned and their transcript profiles were determined in regard to developmental stages and tissues, as well as the nutritional status. Second, the three genes and their deduced proteins were characterized based on their sequences. Also, the evolution of SK signaling was discussed after the phylogenetic analysis. Third, the three genes were individually silenced by dsRNA-mediated gene silecing in *T. castaneum* larvae and the consequential effect on feeding was examined by measuring the mass of food consumption. Finally, a synthetic sulfated peptide *Trica*-SK-II$^{[1-9]}$ (FDDY(SO_3H)GHMRF-NH_2) was injected in larvae to enhance the SK signaling where food intake was monitored. An opposite observation on food intake was expected between *Trica*-SK-II$^{[1-9]}$ peptide injection and *sk*-silencing assay. Through these experiments, we wanted to investigate the involvement and the role of the SK signaling in the regulation of feeding in *T. castaneum*.

2. Materials and methods

2.1. Insects

The wild type GA-1 strain of *T. castaneum* (Haliscak and Beeman, 1983) was reared under standard conditions (30 °C, R.H. 70% and constant darkness) in the lab. Larvae of the penultimate and last instars (with body mass of 12.3 ± 0.28 mg) were used in RNAi experiments and the peptide assay.

2.2. Sequence information

Sequence of *T. castaneum sulfakinin* (*sk*) (BeetleBase: TC030085), *sulfakinin receptor 1* (*skr1*) (BeetleBase: TC007536) and *sulfakinin receptor 2* (*skr2*) (BeetleBase: TC008438) was retrieved from the annotated *T. castaneum* BeetleBase (Kim et al., 2010; Wang et al., 2007) by tblastn with protein queries: sulfakinin (GenBank: EFA04708.1), similar to perisulfakinin receptor (GenBank: XP_975226) and similar to CCK-like receptor at 17D3 (GenBank: XP_972750.1). Two reference genes *ribosomal protein L32e* (*rpl32*; GenBank: XM_964471.2) and *alpha-tubulin 1* (*α-tubulin*; GenBank: XM_961399.1) were included. Primers were designed based on sequences from annotation and/or cloning and are displayed in Table II-1.

2.3. Total RNA extraction and cDNA synthesis

Insects were dissected in phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, $NaHPO_4$ 10 mM, KH_2PO_4 1.76 mM; pH 7.2) and different body parts were stored in *RNAlater*® reagent (Sigma-Aldrich) at -70 °C prior to total RNA extraction. Samples included 10 entire larvae, 10 entire pupae and 10 entire adults as well as head, gut and the decapitated body that were collected from 20 larvae and 20 adults. In the nutrition-SK assay, whole larval and adult individuals were collected after 6 days of starvation. In the RNAi experiment, three larvae were collected and pooled daily and stored for transcript analysis of the target gene.

Total RNA was extracted with the RNeasy® Mini Kit (Qiagen) following the manufacturer's instruction. A DNase I treatment with the DNA-*free*™ DNA Removal Kit (Ambion) was included to eliminate the potential genomic DNA contamination. One microgram of total RNA was used in cDNA synthesis with SuperScript™ II Reverse Transcriptase (Invitrogen)

and oligo(dT)$_{12}$ (Invitrogen) after quantification and verification by spectrophotometer (Nanodrop ND-1000, Thermo Scientific) and gel electrophoresis with 1.5% agarose gel. Finally, the cDNA solution was ten times diluted with nuclease-free water and stored at -20 °C for subsequent experiments.

2.4. Cloning and phylogenetic analysis

Genes *sk*, *skr1* and *skr2* were amplified with Platinum® *Pfx* DNA polymerase (Invitrogen) and primer pairs sk, skr1 and skr2 (Table II-1), respectively. The following thermocycling profile was used: 4 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 56 °C and then 40 s (*sk*) or 100 s (*skr1* and *skr2*) at 68 °C. The single band of the target fragment was purified from the agarose gel, cloned into pGEM®-T vector (Promega) and then confirmed by sequencing.

The obtained nucleic acid sequences and deduced amino acid sequences were compared with the existing sequence of the corresponding gene. Multiple sequence alignments of DNA and protein and phylogenetic analysis were performed with MEGA 5 (Tamura et al., 2011). The properties of deduced proteins were analyzed using online tools: SignalP 4.0 (Petersen et al., 2011) for signal peptide prediction, TMHMM Server v.2.0 and Protter v1.0 for transmembrane helices prediction (Krogh et al., 2001; Omasits et al., 2013; Sonnhammer et al., 1998) and Phyre^2 for three-dimensional (3D) structure prediction (Kelley and Sternberg, 2009).

Phylogenetic analysis of receptors was conducted with available sequences of SK(-like) receptors from invertebrates to vertebrates (Table II-2) based on two distance analysis methods: the neighbor-joining (NJ) and the maximum-likelihood (ML). After removing gaps, the amino acid sequences encoding the seven transmembrane domains, the intracellular loop I and II, and the extracellular loop I and III of the receptors were used to construct phylogenetic trees in MEGA 5 with default setup (Tamura et al., 2011). *Canis lupus familiaris* bradykinin receptor (BDKR), *Carassius auratus* vasoactive intestinal polypeptide receptor (VIPR) and *Homo sapiens* secretin receptor (SCTR) were used as an outgroup.

2.5. Transcript profiles

The temporal transcript was determined by quantitative real-time PCR (qPCR) with cDNA from larval, pupal and adult stages and the spatial transcript with cDNA from tissues as head, gut and the decapitated body. cDNA from starved beetles was sampled to investigate the gene expression in relation to the nutritional status.

Primers sk_q, skr1_q and skr2_q (Table II-1) were validated with a standard curve based on a serial dilution of cDNA to determine efficiency and a melt curve analysis with temperature range from 60 °C to 95 °C to ensure specificity. Each reaction was performed in duplicate and contained 10 µl SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.4 µl of 10 µM forward primer (Invitrogen), 0.4 µl of 10 µM reverse primer (Invitrogen), 8.2 µl water and 1 µl cDNA prepared in section 2.3. The qPCR reaction was performed and analyzed using a CFX96™ Real-Time System and the CFX Manager (both from Bio-Rad). Two reference genes, *rpl32* and *α-tubulin* were chosen, based on previous reports (Lord et al., 2010; Morris et al., 2009; Parthasarathy et al., 2008) and our optimization with qbase+ software (Biogazelle). "No template control" and "minus reverse transcriptase control" reactions were included in qPCR to ensure that there is no foreign DNA or genomic DNA. The relative transcript level of a target gene was normalized to the amount of the two reference genes.

2.6. DsRNA synthesis

A single fragment of *sk*, *skr1* and *skr2* was amplified with primer pairs sk, skr1_s and skr2_s (Table II-1), respectively. The fragments were cloned into pGEM®-T vector (Promega) and then confirmed by sequencing. The linearized plasmids were used to generate dsRNA templates with primer pairs dssk, dsskr1 and dsskr2 (Table II-1; Figure II-S1). The thermocycling profile started with 5 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, and then continued with 28 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s and extension at 72 °C for 30 s.

One microgram of purified dsRNA templates was used in the dsRNA synthesis with the MEGAscript® RNAi Kit (Ambion) following the instruction. DsRNAs were eluted from the column with nuclease-free water. The yielded dsSK (382 bp), dsSKR1 (459 bp) and dsSKR2 (409 bp) (Table II-1) were quantified and verified by a spectrophotometer and 1.5% agarose

gel. Following the same protocol, dsRNA for green fluorescent protein (dsGFP; 495 bp) was synthesized with plasmid containing *gfp* fragment and the primer pair dsgfp (Table II-1).

2.7. RNAi experiment

For the RNAi experiment, the penultimate and last instar larvae were collected from the laboratory colony. Approximate 200 ng of dsRNA was injected into the larval haemocoel from the dorsal side of their 3rd and 4th abdominal segments (Posnien et al., 2009; Tomoyasu and Denell, 2004). Injection of dsGFP or nuclease-free water did not cause difference compared to the no-injection insects in previous trials; therefore, 200 ng of dsGFP was injected as a negative control to confirm the sequence-specific effect of dsRNA.

The transcript level of a target gene was determined by qPCR using total RNA extracted from the three-pooled injected larvae. These larvae were collected at designated time points after dsRNA-injection. Primer pairs sk_q, skr1_q and skr2_q were located in different regions from the dsRNA-region (Table II-1; Figure II-S1). The same qPCR profile was used as in section 2.5.

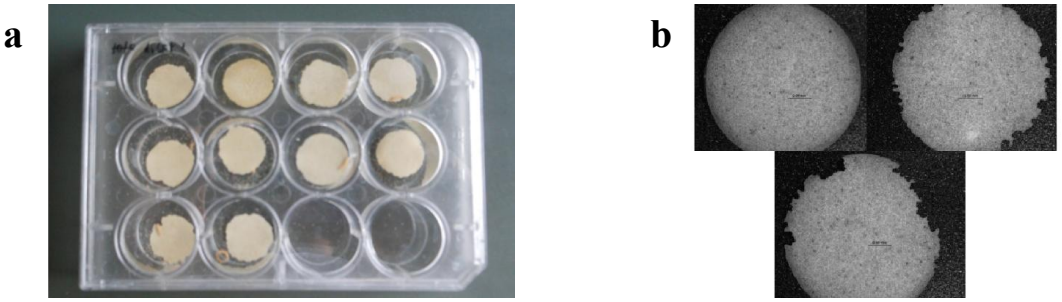


Figure II-1 Flour disk feeding assay. *T. castaneum* larvae were kept individually with a flour disk per larva (a) and the consumption of flour disk represents the food consumption (b).

Gene silencing effect was evaluated phenotypically with the larval food intake. Thirty larvae were divided into six replicates with five larvae per replicate. Each dsRNA-injected larva was supplied with a flour disk (Figure II-1a) in a 12-well tissue culture plate (TPP, Switzerland) under standard conditions. Before fed to larvae, the flour disks were kept in the insect culture incubator for three days to saturate with the standard humidity. A saturated flour disk weighed on average 0.29 ± 0.02 mg. Then, the flour disk was weighed on a daily basis. All larvae

were scored for cumulative food intake for eight days, when most turned into pupa. The decrease in the mass of a flour disk represented the food intake of one larva (Figure II-1b).

2.8. Injection of sulfated Trica-SK-II$^{[1-9]}$ peptide in larvae

Synthetic peptide sulfated Trica-SK-II$^{[1-9]}$ (sSK-II for short; with sequence FDDY(SO_3H)GHMRF-NH_2) was injected in larvae to investigate its effect on feeding. The sSK-II was synthesized via FMOC methodology under previously described conditions (Nachman et al., 1986c). The sSK-II solution was freshly prepared with a buffered saline (10 mM Hepes, pH 7.4, 1% BSA, 0.85% NaCl; Bloch Qazi et al., 1998) before the experiment.

Before injection, larvae were kept individually for 12 h without food in small plastic boxes. The sSK-II was injected to a final concentration of 80 μM, which is calculated to be higher than the effective concentration in desert locust (Wei et al., 2000). Larvae of the control series were injected with the same volume of saline solution. A no-injection control was also included in the experiment. Both the treatment and control series contained each six replicates with five larvae per replicate. Each larva was kept individually with a flour disk. All larvae were then kept under the standard conditions and scored daily for food intake.

3. Results

3.1. Cloning and characterization of SK and SKR genes and proteins

The cDNA of the SK prepropeptide gene was cloned (GenBank: KC161574) as *sk*. The SK gene contained two exons and encoded 113 amino acids. The deduced protein had the same sequence as the predicted SK prepropeptide EFA040708.1. The SK prepropeptide (Figure II-2a) contained a hydrophobic signal peptide with a cleavage site after the residue A29 (Dyrløv Bendtsen et al., 2004; Nielsen et al., 1997) and two peptides, designated as Trica-SK-I and Trica-SK-II. Both peptides were flanked by putative mono- and di-basic endoproteolytic cleavage sites: -KR- (K79, R80 and K93, R94) and -R- (R109) (Nakayama et al., 1992; Sossin et al., 1989; Von Heijne, 1985). Both peptides had a characteristic Y (Y86 and Y102) and G (G92 and G108), which is a potential sulfation and amidation site, respectively (Eipper and Mains, 1988; Eipper et al., 1992; Merkler, 1994; Weinshilboum et al., 1997). The residue Y (Y86 and Y102) is reported to have both sulfated and nonsulfated forms (Predel et al., 1999) and the putative amidation on the C-terminal G (G92 and G108) is considered to protect the peptides (Gregory et al., 1964).

Table II-1 Oligonucleotide primers used in PCR for cloning and quantitative real-time PCR

Primer*	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Location**
Cloning PCR				
sk	CACTTGCAGTCAGTAATGGGTATGAAGAGTT	ATTTATTGGCTTAATCGCTGCCGCTTC	342	1-342
dssk	T7-CTTGCAGTCAGTAATGGGTATGAAGAG	T7-TTAATCGCTGCCGCTTCTTCC	382	1-342
skr1	GGCGGAAAATGTCAGAAGTGGAAATGAACT	CGCTGCTAAACACGATCTTCGGC	1665	1-1665
skr1_s	GCCAGTTACGCCACTTCTTC	ATGCCGTTCTTCTGAGCTGT	413	1134-1547
dsskr1	T7-GCCAGTTACGCCACTTCTTC	T7-ATGCCGTTCTTCTGAGCTGT	459	1134-1547
skr2	GTAACAATGGACTGGGCTGAAAAC	CACAAATTATCTACAAAAGTCGGCA	1263	1-1264
skr2_s	CAAGGGAATGAAGACGGAAA	CTCGAACAGTACGCCAACAA	399	693-1091
dsskr2	T7- CAAGGGAATGAAGACGGAAA	T7-CTCGAACAGTACGCCAACAA	409	693-1091
dsgfp	T7-TACGGCGTGCAGTGCT	T7-TGATCGCGCTTCTCG	495	
T7	TAATACGACTCACTATAGGGAGA			
Quantitative real-time PCR				
rpl32	TGACCGTTATGGCAAACTCA	TAGCATGTGCTTCGTTTTGG	136	144-279
α-tubulin	CGCCTGTTGGGAATTGTACT	ACGACAGTGGGTTCCAAGTC	171	167-339
sk_q	GGCAGCGATTAAGCCAATAA	TGCAAAATTTATTACCAGCCATT	80	331-410
skr1_q	AAACAACTCCGACGTCTCGT	TCGGCTTCCAAAACCACTAC	90	1563-1652
skr2_q	AAGTGTGCCACAATGGTACG	TTCCGATGACTGCCAACATA	80	90-169

***, Abbreviation used: sk, sulfakinin; skr, sulfakinin receptor; gfp, green fluorescence protein; rpl32, ribosomal protein L32e; T7, T7 polymerase promoter sequence.**

****, position of a neucleotide is counted from the first neuclotide in the open reading frame of a gene.**

When the amino acid sequence of the *T. castaneum* SK prepropeptide was compared to the prepropeptides of SK homologs in Hymenoptera, Orthoptera, Diptera and Ixodida, a relatively high identity was found in the region corresponding to the two peptides (Figure II-2b), sharing at least seven identical C-terminal amino acid residues.

For the SKRs, two genes were annotated (BeetleBase: TC007536 and TC008438) in *T. castaneum* (Amare and Sweedler, 2007; Hauser et al., 2008; Li et al., 2008). To make things easier, we designated them as *skr1* and *skr2* corresponding to the sequences cloned with primer pairs from TC007536 and TC008438, respectively.

skr1 (GenBank: KC161573) contained eight exons and encoded 554 amino acids with a calculated molecular weight of 62.95 kDa. The SKR1 amino acid sequence differed with two insertions between residues 261 to 266 and residues 499 to 501 (Figure II-3), as compared to the amino acid sequence from TC007536. We suspect that these differences are due to two splicing differences in the 4^{th} and 6^{th} introns. The open reading frame of *skr1* matched the canonical GT/AG splice. In addition, *skr1* showed four nucleotides different from the genomic sequence in the BeetleBase, which were all present in the 3^{rd} position of codons without changing the amino acids. Therefore, KC161573 showed the correct sequence of one sulfakinin receptor and could replace the predicted TC007536.

skr2 was organized into five exons and encoded a protein of 420 amino acids with a calculated molecular weight of 47.91 kDa. It had four nucleotides different from TC008438 but both deduced the same amino acid sequence.

a

MG**M**KSFFTGVFLISSVYLLFIHQFQNVSA↓APGNANNV

DSHRLRARPFARLTPRTQYSRIKAEPFNEFIVDDDDLFEL

SKR**QTSDDYGHLRF**GKR**GEEPFDDYGHMRF**GRSGSD-

Trica-SK-I Trica-SK-II

b

```
Trica   ----------------MGMKSFFTGVFLISSVYLLFIHQFQNVS---------------- 28
Psahi   ------------------MKSFFTGMFLLISLYLLLTYQFGFSS--------------- 26
Grybi   ----------------MGC-SMLTAAFFVVSVYLLVHHQHHAVSGHA-----LPLPPSE 37
Rhopr   ----------------MGS-SFLITLLLAIGVYMFIENSHFMCL--------------- 27
Culqu   --------MARLSVTILATLILYLAYQTLASEAVPTGSGNSKLLSSSVDTSENLLSDENL 52
Derva   ------------MQLPARFLLFLLVAIMAAASSALGYSAASPVSS-------------- 33
Apime   ------------------------------YNVENLLLEEDD----------------- 12
Drome   MGPRSCTHFATLFMPLWALAFCFLVVLPIPAQTTSLQNAKDDRRLQ-----------EL 48
Anoga   ---------------MARVTLALAVSLAAYAAYYYVHIAEAQPIGSL------VLAAPH 38
Bommo   ----------------MRIAAVMLLAVSVAVTFC------------------------- 18

Trica   -AAPGNANN--VDSHRLRARPFARLT----PRTQYSRIKAEP----------------- 63
Psahi   -AATGGPTN--QEVHRPRTRILGRFAR---PVTQYARLKPEP----------------- 62
Grybi   GGAGGGAGGGAGGALRGRALVLGRRAPPVAPQLLRARLAVADDATV------------- 83
Rhopr   -AEPAERRS------------------------LIRIRPEP----------------- 43
Culqu   NKLQTAWFKSAYNRRSSPVGGSDARAAVATYPVLRRTAPAVAAPNP------------- 98
Derva   QQQQQQRHRISVGRWLKSVLPAAAAAAASAGDSDSRNTADLDAAD------------- 77
Apime   ----------FTNLN---------KRQQ---------------------------- 21
Drome   ESKIGGEIDQPIANLVGPSFSLFGDRRNQKTMSFGRRVPLISRPI------------- 93
Anoga   SQQQQPGSSTSDEATINHLQQQHQRLKDTNVYRARSKMRPHDRKYPGVIGAYEAYRRTVQ 98
Bommo   -------VCCCDGANLRRVQPDDD-----------EDFRPHP--------LYRDY----- 47

Trica   ------------------------------------FNEFIVDDDDLFELSKR----- 80
Psahi   ------------------------------------YLNDFIDDDDLIEFSKR----- 79
Grybi   ----------------------------------QGLLGDFVVDDEELGEMSKR----- 103
Rhopr   ---------------------------------------ALFAAEDDPLDIVDKR----- 59
Culqu   ----------------------------------MGYFADISLLDDEDIQK-------- 115
Derva   ---------------------------------------MIDPVLLASGFAKR-------- 91
Apime   ------------------------------------------------------------
Drome   ----------------------------------IPIELDLLMDNDDERTKA----- 111
Anoga   GPQLMQRNPATADRFADDPGVDEQDQMRFSLEGFLTGARTPTLLNDDEEEEEDEDHEQGG 158
Bommo   ------------------------------GLIR----SRVIRGDD------------ 59

Trica   ----QTSDDYGHLRFGKRG--EEPFDDYGHMRFGRSGSD-------------------- 113
Psahi   ----QTSDDYGHLRFGKRG--EESFDDYGHMRFGRSGTEE------------------- 113
Grybi   ----Q-SDDYGHMRFGKR----EPFDDYGHMRFGRSAE---------------------- 132
Rhopr   ----Q-FNEYGHMRFGKRGGSDEKFDDYGYMRFGRSRPLANSLPN-------------- 99
Culqu   -----RFDDYGHMRFGKRGGEGDQFDDYGHMRFGRRQCAEPESPQSVESKEMSCKGAFGS 170
Derva   -----QEDDYGHMRFGRS-------DDYGHMRFGRK----------------------- 115
Apime   ------FDDYGHLRFGKR----EQFEDYGHMRFGRNHHK------------------- 50
Drome   ----KRFDDYGHMRFGKRGGD-DQFDDYGHMRFGR------------------------ 141
Anoga   DGLVKRFDDYGHMRFGKRGGEGDQFDDYGHMRFGR------------------------ 193
Bommo   -----TFDDYGHLRFGRS-------DD--------------------------------- 74
              ::***:***:        :*
```

Figure II-2 Sequence of deduced *T. castaneum* SK prepropeptide (a) and alignment of insect SK prepropeptides (b). In the *T. castaneum* SK prepropeptide (a), the possible start amino acid Ms are shown in bold letters in the position 1 and 3 from the N-terminus. The putative cleavage site of the signal peptide is indicated by an arrow. The

potential mono- or di-basic endoproteolytic cleavage sites are underlined and the preceding G residues required for α-amidation are marked by "+". The two deduced sulfakinin peptides (Trica-SK-I and Trica-SK-II) are bold. The dash indicates the position of stop codon. Alignment (b) was performed with SK prepropeptides (deduced) from *Tribolium castaneum* (Trica; Coleoptera; GenBank: KC161574), *Psacothea hilaris hilaris* (Psahi; Coleoptera; UniProtKB: B7XH66), *Gryllus bimaculatus* (Grybi; Orthoptera; UniProtKB: A4H1X5), *Rhodius prolixus* (Rhopr; Ixodida; UniProtKB: C6K793), *Culex quinquefaciatus* (Culqu; Diptera; UniProtKB: B0WBY7), *Dermacentor variabilis* (Derva; Ixodida; UniProtKB: B2ZA31), *Apis mellifera* (Apime; Hymenoptera; UniProtKB: H9KFT3), *Drosophila melanogaster* (Drome; Diptera; UniProtKB: P09040), *Anopheles gambiae* (Anoga; Diptera; UniProtKB: Q49U19) and *Bombyx mori* (Bommo; Lepidoptera; UniProtKB: B3IWA7). *, an amino acid position which is completely conserved among all aligned proteins; :, an amino acid position with lower degrees of conservation among all aligned proteins. The regions corresponding to two Trica-SKs are underlined.

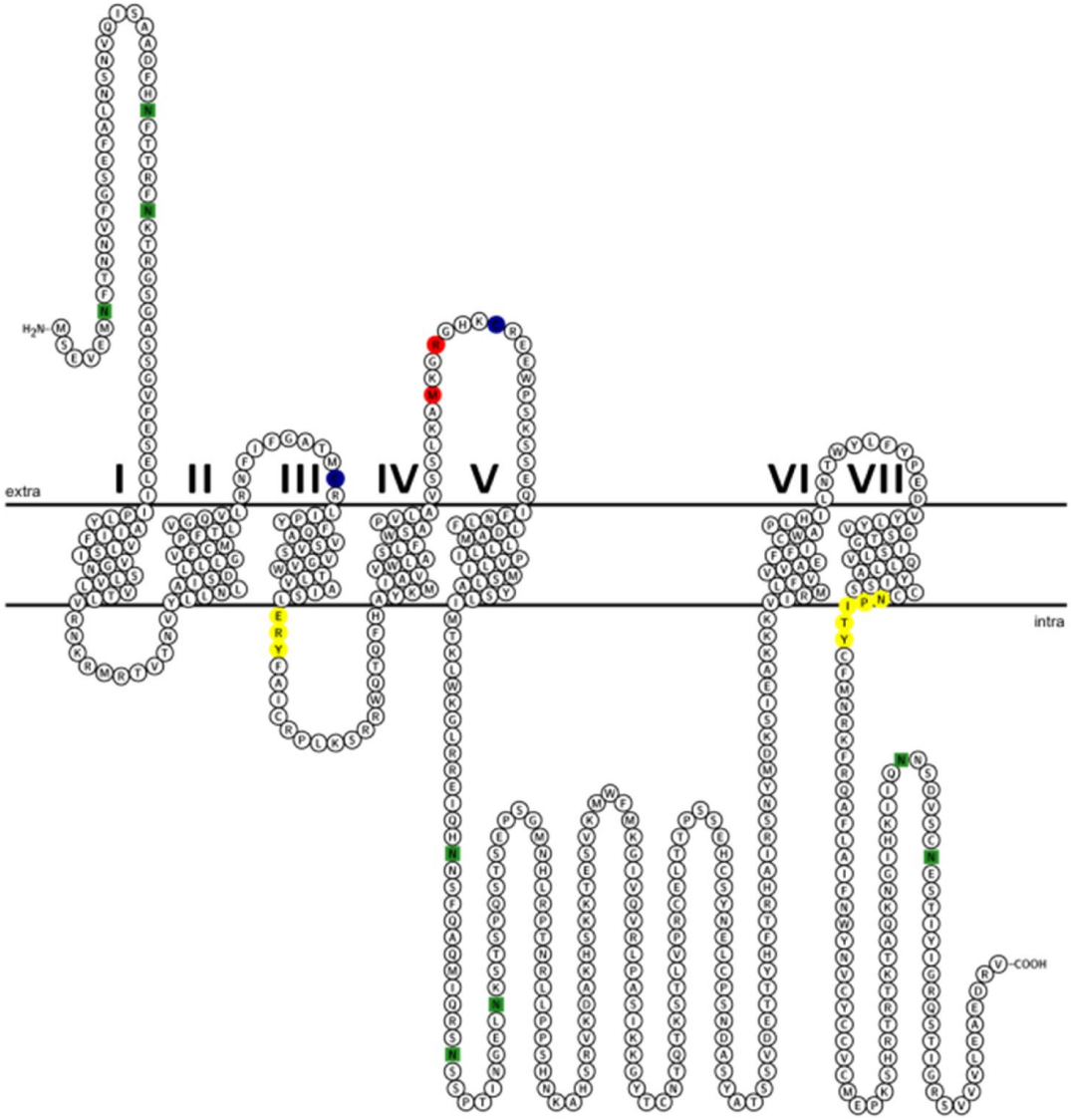
```
XP_975226       MSEVEMNFTNNVFGSEFALNSNVQISAADFHNFTTRFNKTRGSGASSGVFESELIIPLYA
KC161573_P      MSEVEMNFTNNVFGSEFALNSNVQISAADFHNFTTRFNKTRGSGASSGVFESELIIPLYA
                ************************************************************

XP_975226       IIFVLSIVGNSLVLVTLVRNKRMRTVTNVYLLNLAISDLLLGVFCMPFTLVGQVLRNFIF
KC161573_P      IIFVLSIVGNSLVLVTLVRNKRMRTVTNVYLLNLAISDLLLGVFCMPFTLVGQVLRNFIF
                ************************************************************

XP_975226       GATMCRLIPYFQAVSVSVGVWTLVAISLERYFAICRPLKSRRWQTQFHAYKMIAVVWLAS
KC161573_P      GATMCRLIPYFQAVSVSVGVWTLVAISLERYFAICRPLKSRRWQTQFHAYKMIAVVWLAS
                ************************************************************

XP_975226       LFWSAPVLAVSSLKAMKGRGHKCREEWPSKSSEQIFNLFLDAMLLLIPVLIMSLAYSLIM
KC161573_P      LFWSAPVLAVSSLKAMKGRGHKCREEWPSKSSEQIFNLFLDAMLLLIPVLIMSLAYSLIM
                ************************************************************

XP_975226       TKLWKGLRREIQHNNSFQAQSERTNAQRSNSSPTINGELNKSTSPQSTSEPSGMNHLRPT
KC161573_P      TKLWKGLRREIQHNNSFQAQM----IQRSNSSPTINGELNKSTSPQSTSEPSGMNHLRPT
                ********************    ************************************

XP_975226       NRLLPPSHNKAHSRVKDAKHSKKTESVKMWFMKGIVQVRLPASIKKGYTCNTQTKSTLVP
KC161573_P      NRLLPPSHNKAHSRVKDAKHSKKTESVKMWFMKGIVQVRLPASIKKGYTCNTQTKSTLVP
                ************************************************************

XP_975226       RCELTTPSSEHCSYNELCPSNDASYATSSVDETTYHFTRHAIRSNYMDKSIEAKKKVIRM
KC161573_P      RCELTTPSSEHCSYNELCPSNDASYATSSVDETTYHFTRHAIRSNYMDKSIEAKKKVIRM
                ************************************************************

XP_975226       LFVVVAEFFICWAPLHILNTWYLFYPEDVYLYVGSTGISLVQLLAYISSCCNPITYCFMN
KC161573_P      LFVVVAEFFICWAPLHILNTWYLFYPEDVYLYVGSTGISLVQLLAYISSCCNPITYCFMN
                ************************************************************

XP_975226       RKFRQAFLAIFNWYK--------------TRTKTAQKNGIHKIIQNNSDVSCNESTIYIG
KC161573_P      RKFRQAFLAIFNWYNVCYCCVCMEPKSHRTRTKTAQKNGIHKIIQNNSDVSCNESTIYIG
                **************:              ********************************

XP_975226       RQSTIGRSEVVVLEAEDRV
KC161573_P      RQSTIGRSVV-VLEAEDRV
                ******** * ********
```

Figure II-3 Comparison of *T. castaneum* sulfakinin receptor 1 from annotation (XP_975226) and cloning (KC161573_P). Alignment was performed by using the online Clustal Omega. The consensus is presented under each amino acid by "*" representing

complete conservation and ":" lower degree of conservation. The two full boxes indicate the differences between the two proteins.

Both TcSKR proteins showed the characteristics of GPCRs, including the seven transmembrane domains (TMs), an extracellular N-terminal region and a intracellular C-terminus (Figure II-4; Figure II-5). In addition, a large intracellular linker between TM V and TM VI was confirmed, which is known as a noticeable feature of SKRs (Staljanssens et al., 2011). A disulfide bond is predicted between two C residues in the extracellular loop (EL) I and II in both SKR1 (C125 and C203) and SKR2 (C112 and C190) (Figure II-4). The two vertebrate CCKRs contain such disulfide bonds as well, which helps in stabilizing the extracellular ligand binding pocket (Miller and Gao, 2008). Also, the significant sequences for the activation of GPCR (ERY and NPXXY) were found in both TcSKRs (Figure II-4; Dufresne et al., 2006). In CCKRs, an M in the EL II interacts with the aromatic ring of the Y in CCK and a R interacts with the negatively charged sulfate moiety (SO_3H) in CCK (Gigoux et al., 1998, 1999). An M and R were found in the EL II in the TcSKR1 while only a R was found in the TcSKR2 (Figure II-4).

a



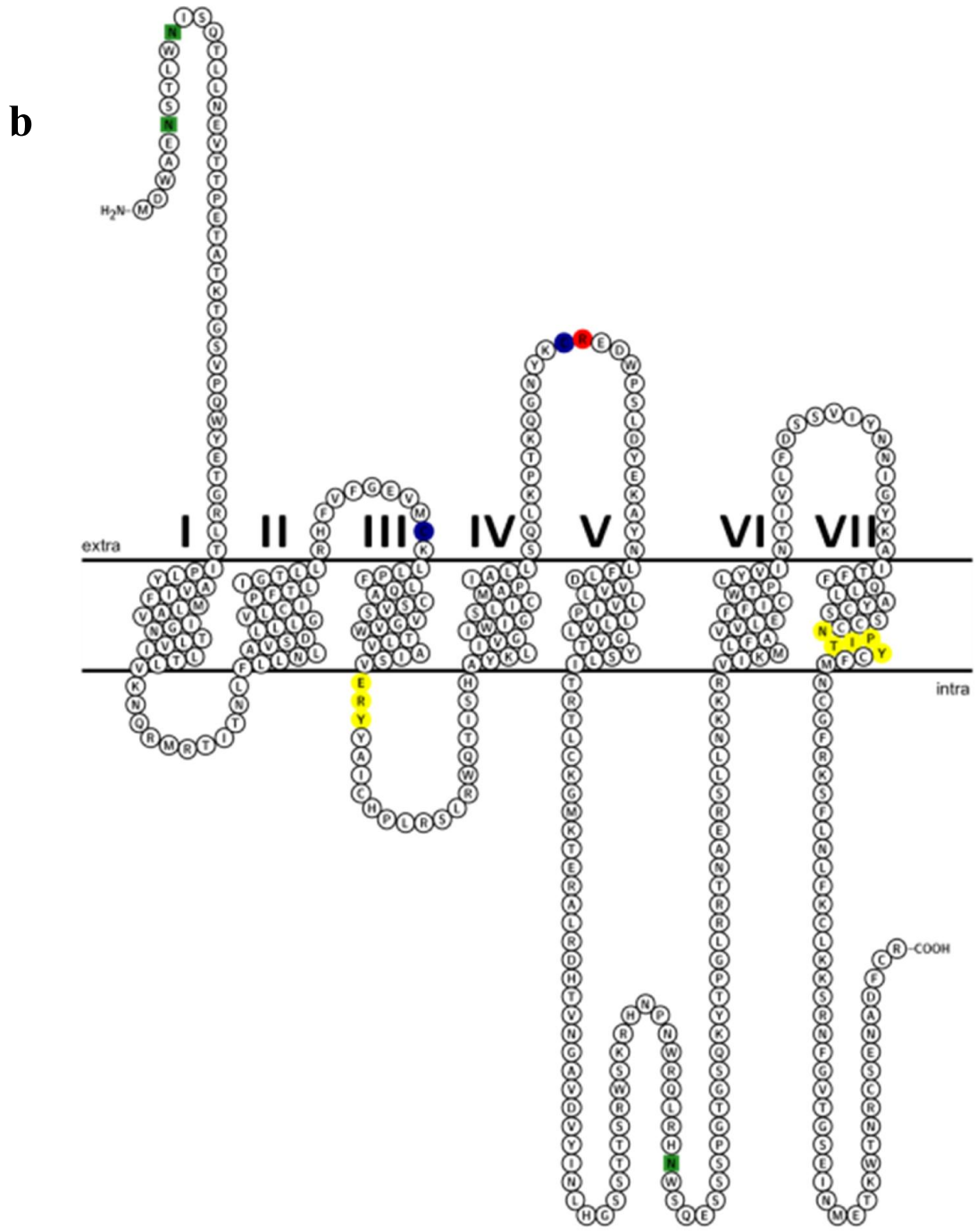


Figure II-4 Schematic two-dimensional representation of the seven transmembrane regions of protein TcSKR1 (a) and TcSKR2 (b) from *Tcskr1* and *Tcskr2* built in Protter v1.0. The protein starts with the first M in the open reading frame. The potential N-glycosylation residues are in green; potential disulfide bond residues are in dark blue; residues important for efficient binding of the sulfated Y of SK are in red; motifs essential for receptor activation (ERY and NPITY) are in yellow.

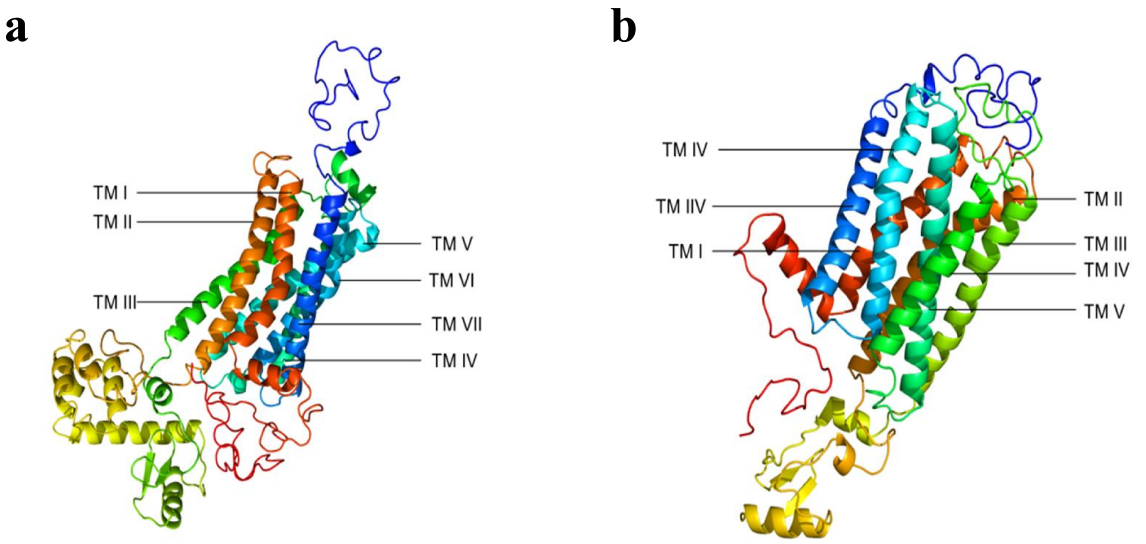
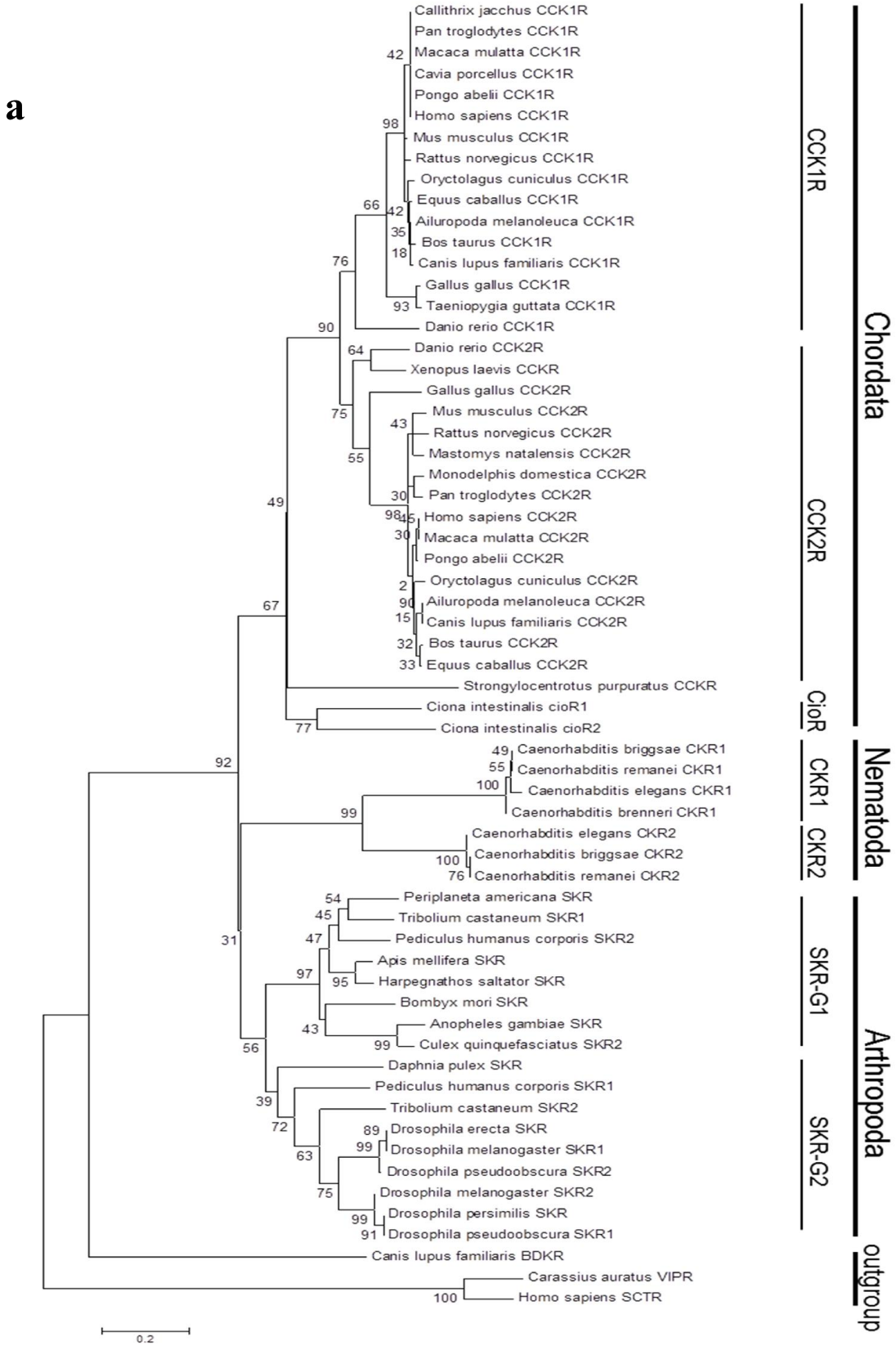


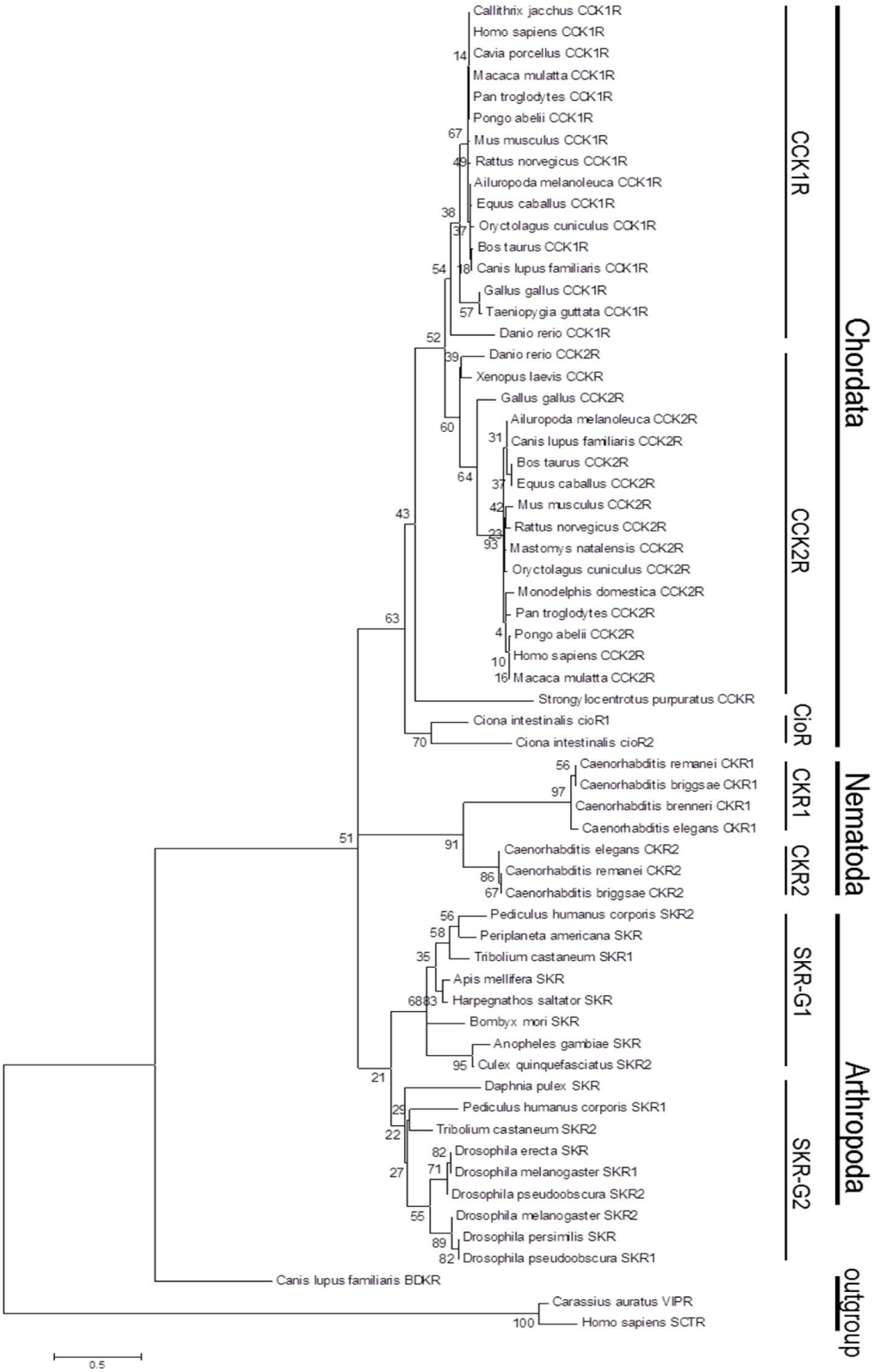
Figure II-5 Three-dimensional structure of proteins TcSKR1 (a) and TcSKR2 (b). The structures were constructed with Phyre2. The protein starts with the first M in the open reading frame. The seven transmembrane domains of the receptor protein are marked as TM I-VII. Images are colored by rainbow N → C terminus. In image (a), 84% of residues are modelled at >90% confidence. In image (b), 89% of residues are modelled at >90% confidence.

3.2. Evolution of the metazoan SK(-like) signaling system

In general, insect SKR, vertebrate CCKR/CioR and nematode CKR share relatively conserved sequences in their seven-transmembrane domains (Figure I-4). In order to discuss the evolution of the metazoan SK(-like) receptors, two phylogenetic trees (Figure II-6) were generated based on the NJ and ML methods. Both the NJ tree (Figure II-6a) and the ML tree (Figure II-6b) showed that the SK(-like) receptor family in each phylum consisted of two distinct branch clusters: receptor 1 and receptor 2. In chordates, a clear separation of the CCK1R and CCK2R groups was obtained and this was also observed within nematodes where sequences clustered within the CKR1 and CKR2 groups. However, this contrasted with the arthropods scenario and the two *Drosophila* SKRs grouped separately from the other insect representatives. In Arthropoda, SKRs were clustered in two groups where no clear distinction between SKR1 and SKR2 could be made; thus, we designated them as SKR-group 1 (SKR-G1) and SKR-group 2 (SKR-G2). The analysis demonstrated that all SK(-like) receptors share a very high degree of sequence similarity, not only within clusters, but also between clusters.


a
Callithrix jacchus CCK1R
Pan troglodytes CCK1R
Macaca mulatta CCK1R
Cavia porcellus CCK1R
Pongo abelii CCK1R
Homo sapiens CCK1R
Mus musculus CCK1R
Rattus norvegicus CCK1R
Oryctolagus cuniculus CCK1R
Equus caballus CCK1R
Ailuropoda melanoleuca CCK1R
Bos taurus CCK1R
Canis lupus familiaris CCK1R
Gallus gallus CCK1R
Taeniopygia guttata CCK1R
Danio rerio CCK1R
Danio rerio CCK2R
Xenopus laevis CCKR
Gallus gallus CCK2R
Mus musculus CCK2R
Rattus norvegicus CCK2R
Mastomys natalensis CCK2R
Monodelphis domestica CCK2R
Pan troglodytes CCK2R
Homo sapiens CCK2R
Macaca mulatta CCK2R
Pongo abelii CCK2R
Oryctolagus cuniculus CCK2R
Ailuropoda melanoleuca CCK2R
Canis lupus familiaris CCK2R
Bos taurus CCK2R
Equus caballus CCK2R
Strongylocentrotus purpuratus CCKR
Ciona intestinalis cioR1
Ciona intestinalis cioR2
Caenorhabditis briggsae CKR1
Caenorhabditis remanei CKR1
Caenorhabditis elegans CKR1
Caenorhabditis brenneri CKR1
Caenorhabditis elegans CKR2
Caenorhabditis briggsae CKR2
Caenorhabditis remanei CKR2
Periplaneta americana SKR
Tribolium castaneum SKR1
Pediculus humanus corporis SKR2
Apis mellifera SKR
Harpegnathos saltator SKR
Bombyx mori SKR
Anopheles gambiae SKR
Culex quinquefasciatus SKR2
Daphnia pulex SKR
Pediculus humanus corporis SKR1
Tribolium castaneum SKR2
Drosophila erecta SKR
Drosophila melanogaster SKR1
Drosophila pseudoobscura SKR2
Drosophila melanogaster SKR2
Drosophila persimilis SKR
Drosophila pseudoobscura SKR1
Canis lupus familiaris BDKR
Carassius auratus VIPR
Homo sapiens SCTR
0.2
CCK1R
CCK2R
CioR
CKR1
CKR2
SKR-G1
SKR-G2
Chordata
Nematoda
Arthropoda
outgroup

b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Equus caballus CCK1R
Oryctolagus cuniculus CCK1R
Bos taurus CCK1R
Canis lupus familiaris CCK1R
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Danio rerio CCK1R
Danio rerio CCK2R
Xenopus laevis CCKR
Gallus gallus CCK2R
Ailuropoda melanoleuca CCK2R
Canis lupus familiaris CCK2R
Bos taurus CCK2R
Equus caballus CCK2R
Mus musculus CCK2R
Rattus norvegicus CCK2R
Mastomys natalensis CCK2R
Oryctolagus cuniculus CCK2R
Monodelphis domestica CCK2R
Pan troglodytes CCK2R
Pongo abelii CCK2R
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Macaca mulatta CCK2R
Strongylocentrotus purpuratus CCKR
Ciona intestinalis cioR1
Ciona intestinalis cioR2
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Caenorhabditis briggsae CKR1
Caenorhabditis brenneri CKR1
Caenorhabditis elegans CKR1
Caenorhabditis elegans CKR2
Caenorhabditis remanei CKR2
Caenorhabditis briggsae CKR2
Pediculus humanus corporis SKR2
Periplaneta americana SKR
Tribolium castaneum SKR1
Apis mellifera SKR
Harpegnathos saltator SKR
Bombyx mori SKR
Anopheles gambiae SKR
Culex quinquefasciatus SKR2
Daphnia pulex SKR
Pediculus humanus corporis SKR1
Tribolium castaneum SKR2
Drosophila erecta SKR
Drosophila melanogaster SKR1
Drosophila pseudoobscura SKR2
Drosophila melanogaster SKR2
Drosophila persimilis SKR
Drosophila pseudoobscura SKR1
Canis lupus familiaris BDKR
Carassius auratus VIPR
Homo sapiens SCTR
0.5
CCK1R
CCK2R
CioR
CKR1
CKR2
SKR-G1
SKR-G2
Chordata
Nematoda
Arthropoda
outgroup

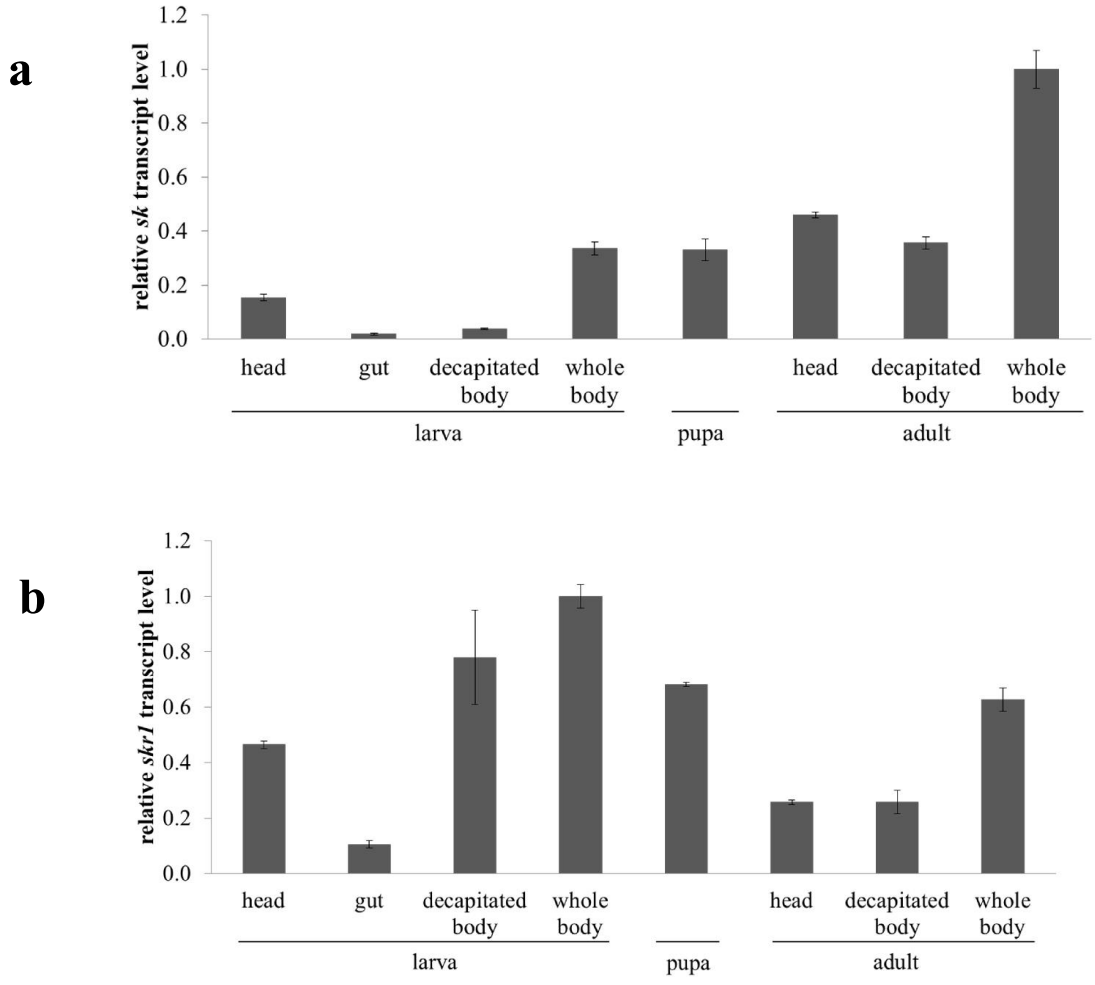
Figure II-6 Phylogenetic tree of SK (-like) receptors constructed using the neighbor-joining (NJ) method (a) and maximum-likelihood (ML) method (b). The amino acid sequences encoding the seven transmembrane domains, the intracellular loop I and II, and the extracellular loop I and III of the receptors were used to conduct the phylogenetic anayses. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. In the NJ tree (a), the evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The evolutionary history in the ML tree (b) was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The scale bar indicates an evolutionary distance of 0.2 (A) or 0.5 (B) amino acid substitutions per protein. *Canis lupus familiaris* bradykinin receptor (BDKR), *Carassius auratus* vasoactive intestinal polypeptide receptor (VIPR) and *Homo sapiens* secretin receptor (SCTR) were used as an outgroup. The sequence accession numbers are shown in Table II-2.

The SKR is divergent in quantity among Arthropoda. For instance, two SKRs are identified in both *D. melanogaster* and *T. castaneum* (Hauser et al., 2006b, 2008), whereas only one copy is found in *Anopheles gambiae* (VectorBase) and *Apis mellifera* (Hauser et al., 2006a). In the silkworm *Bombyx mori*, only one protein A9 receptor (namely SKR in Figure II-6) is found and predicted as a SK-like receptor (Fan et al., 2010). In the arachnids *Ixodes scapularis* and *Mesobuthus martensii*, and the insect *R. prolixus*, *Culex quinquefasciatus* and *Aedes aegypti*, partial SKR sequences were found via sequence similarity searches (VectorBase; Cao et al., 2013). However, due to the lack of certain conserved transmembrane domains in these protein sequences, we did not include them in the phylogenetic analysis. Moreover, the SKR gene in Crustacea has only been found in the water flea *Daphnia pulex*, although some studies reported the presence of SK peptides or SK precursor genes in Crustaceans (Dickinson et al., 2007; Johnsen et al., 2000; Torfs et al., 2002).

3.3. Transcript profiles

The temporal and spatial expression of genes *sk*, *skr1* and *skr2* were determined by qPCR in the three developmental stages as larva, pupa and adult, and different tissues as head, gut and decapitated body. The *rpl32* and *α-tubulin* were used as reference genes. No foreign DNA or genomic DNA was found in the "no template control" and "minus reverse transcriptase control" reactions.

As shown in Figure II-7, the *sk* and two *skr*s were expressed throughout all tested developmental stages (larva, pupa and adult) and the examined tissues. *sk* and *skr2* were highly expressed in the head of both larva and adult, and this phenomenon was most prominent for the larval stage. *skr1* was expressed in head to a similar (adult) or less (larva) extent compared to the rest part of body.



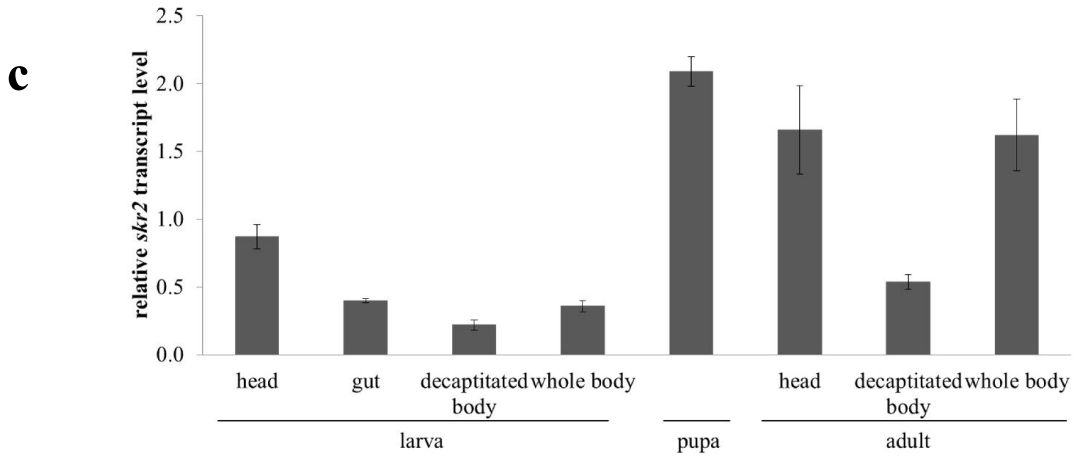
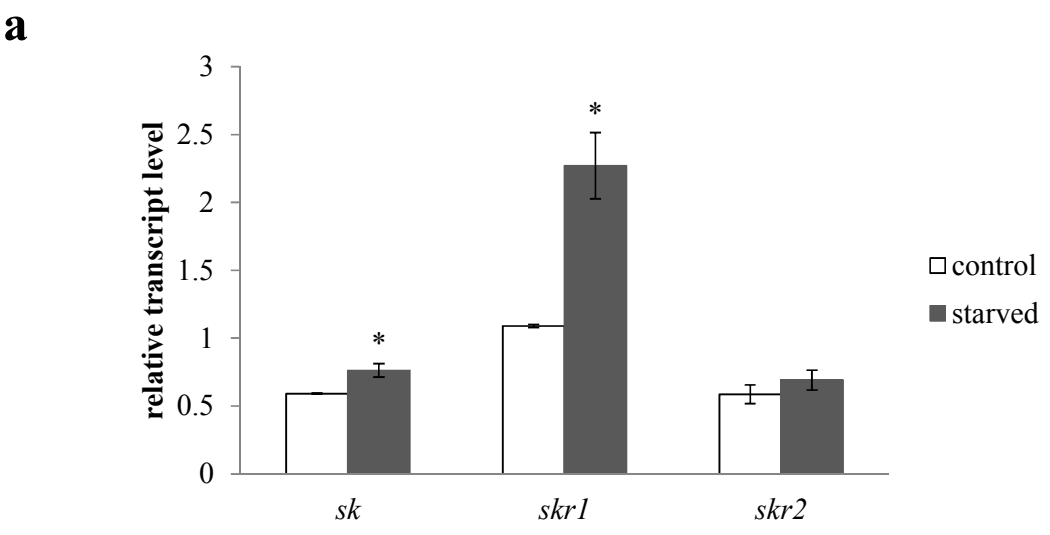


Figure II-7 Expression profiles of *sk* (a), *skr1* (b) and *skr2* (c). qPCR was performed with the total RNA extracted from whole bodies of larvae, pupae and adults, and also of separated tissues as head, decapitated bodies and gut. Data are based on three biological replicates and expressed as mean ± SEM relative to the reference genes *rpL32* and *α-tubulin.*

With respect to the nutritional status, the expression of *sk* and *skr1* were detected to be higher in both larvae and adults that were starved for six days, while the expression of *skr2* remained similar as in the normal larvae (Figure II-8). The beetles were likely to respond to the poor nutritional status by enhancing the SK signaling to induce satiety and reduce food intake.



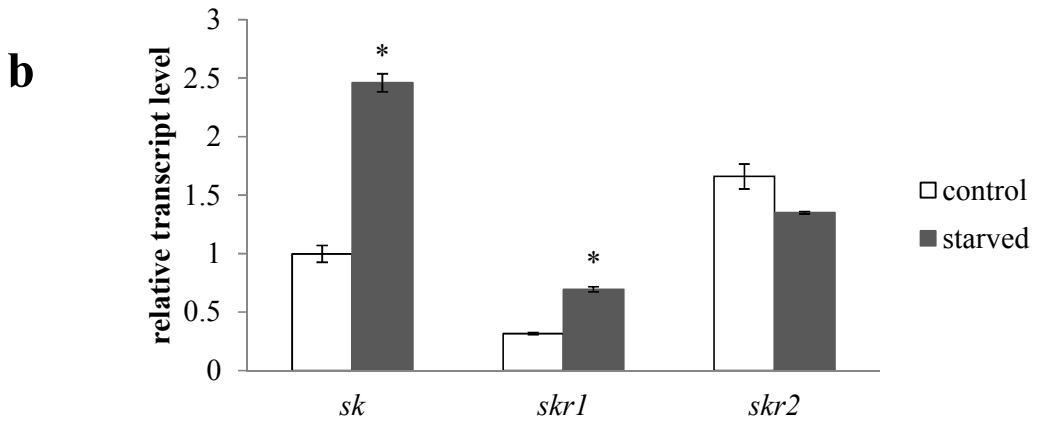
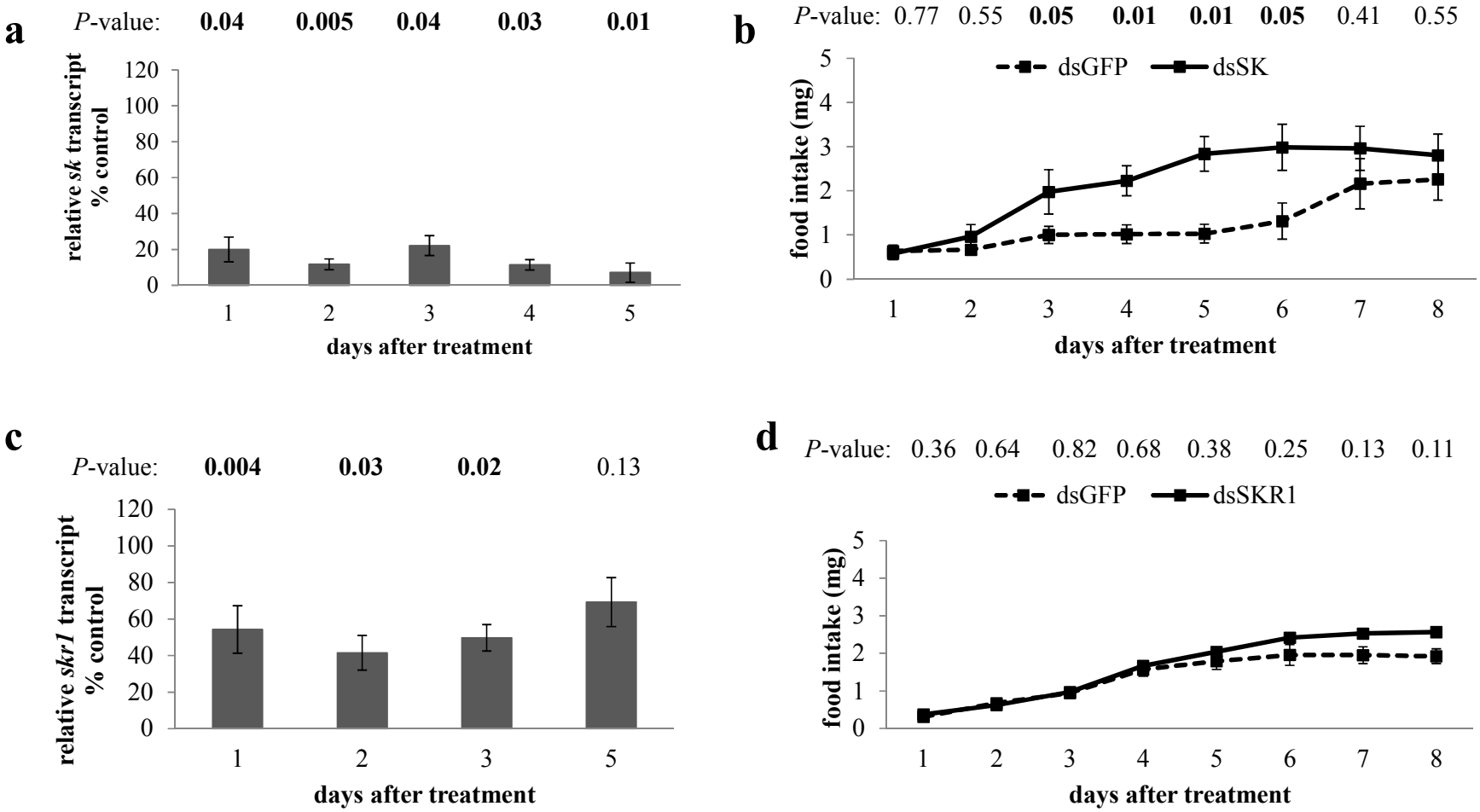


Figure II-8 Expression of *sk*, *skr1* and *skr2* in larva (a) and adult (b) after six days of starvation. qPCR was performed with the total RNA extracted from whole bodies of three larvae and adults. Data represent the mean expression ± SEM (n=3) relative to the reference genes *rpL32* and *α-tubulin.* *, statistically significant difference between "control" and "starved" samples with the student's *t*-test conducted in GraphPad Prism version 5.00.

3.4. Effects of dsRNA-mediated RNAi on gene expression and food intake

3.4.1. Effect of dsRNA-mediated RNAi on gene expression

Ds*sk* strongly suppressed the expression level of *sk* by 80-90% in the first five days after injection, compared to ds*gfp* (Figure II-9a). The expression of *skr1* was reduced by about 50% in the first three days but only 31% on day five after ds*skr*-injection (Figure II-9c). Ds*skr2* significantly suppressed the expression level of *skr2* by 30-70 % in the first four days, with the silencing effect dropped to only 16% on day five (Figure II-9e).


a
P-value: 0.04 0.005 0.04 0.03 0.01
relative sk transcript % control
120
100
80
60
40
20
0
1 2 3 4 5
days after treatment
b
P-value: 0.77 0.55 0.05 0.01 0.01 0.05 0.41 0.55
dsGFP
dsSK
food intake (mg)
5
4
3
2
1
0
1 2 3 4 5 6 7 8
days after treatment
c
P-value: 0.004 0.03 0.02 0.13
relative skr1 transcript % control
120
100
80
60
40
20
0
1 2 3 5
days after treatment
d
P-value: 0.36 0.64 0.82 0.68 0.38 0.25 0.13 0.11
dsGFP
dsSKR1
food intake (mg)
5
4
3
2
1
0
1 2 3 4 5 6 7 8
days after treatment

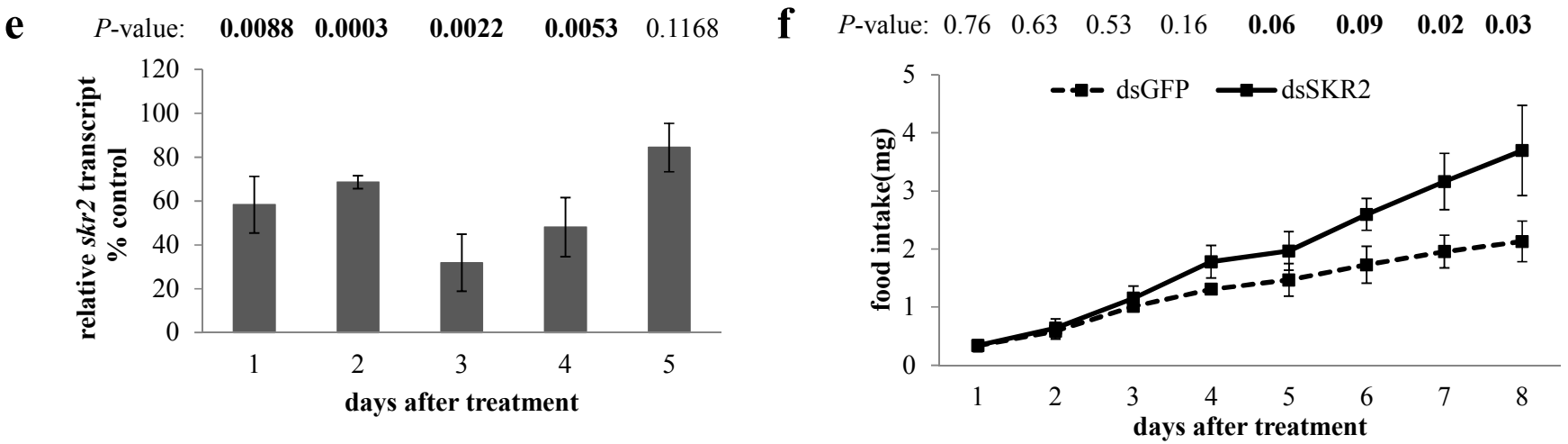


Figure II-9 Effects of dsRNA-mediated RNAi on the transcript level of the target genes (*sk*: a, *skr1*: c and *skr2:* e) and larval food intake (*sk*: b, *skr1*: d and *skr2:* f). Transcript level was determined by qPCR with total RNA from three injected larvae at indicated time points after injection. ds*gfp* was injected as a negative control. Data are normalized to the reference genes *rpl32* and *α-tubulin* and then shown as a percentage of the expression of the control, mean ± SEM (n=3). Data of food intake are expressed as mean ± SEM (n=6). The *P*-values of the significance between control and treatment at the indicated time points, as calculated by the student's *t*-test, are shown in the figure. The *t*-test was conducted in GraphPad Prism version 5.00.

3.4.2. Effect of dsRNA-mediated RNAi on larval food intake

The phenotypic effect of the silencing of genes *sk* and *skr*s was followed by measuring the consumption of the flour disk during a period of eight days after injection. The *sk*-silenced larvae consumed significantly more food than the ds*gfp*-injected control larvae (Figure II-9b). Silencing of *skr1* also led to an increased larval food intake from day five to day eight; however the effect was not as strong as in the *sk*-silenced larvae (Figure II-9d). In contrast, silencing of *skr2* resulted in a significant increase in larval food intake from day five to day eight (Figure II-9f).

3.5. Effect of sulfated Trica-SK-II$^{[1-9]}$ peptide on larval food intake

The effect of sSK-II on food intake was examined by injecting a synthetic sulfated Trica-SK-II$^{[1-9]}$ (sSK-II; FDDY(SO_3H)GHMRF-NH_2) in larvae and then measuring the consumption of flour disk. Figure II-10 displays that the injection of sSK-II strongly reduced larval food intake by about 50% after four days with a strong effect observed on after one day. The injection of an equal amount of saline had no impact on feeding as compared to the no-injection control larvae, ruling out the effect that the injection manipulation could have played.

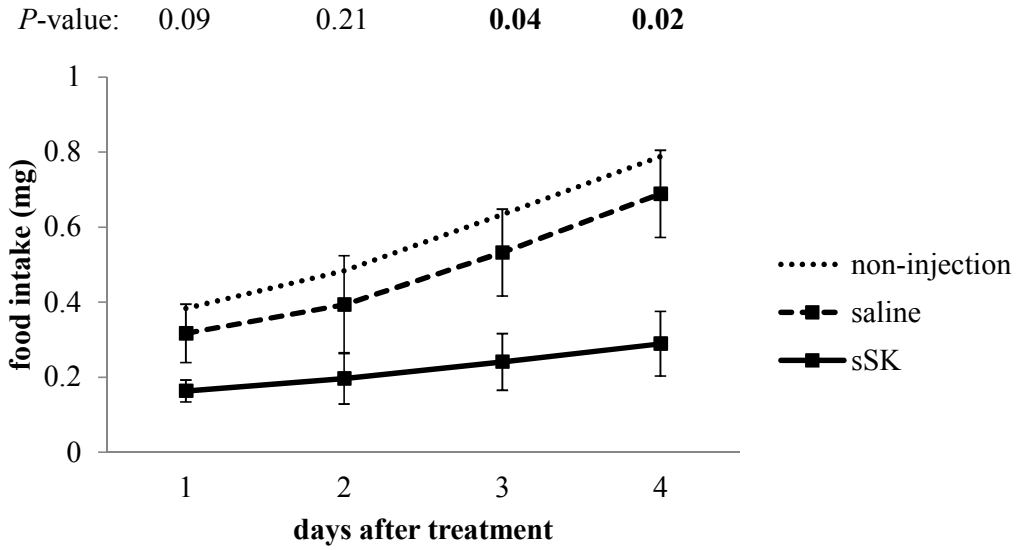


Figure II-10 Food intake by larvae injected with the sSK-II peptide and saline. Data are presented as mean ± SEM (n=6). The *P*-values of the significance between saline-injected and sSK-II-injected larvae at the different time points, as calculated by the student's *t*-test in GraphPad Prism version 5.00, are shown in the figure. The *P*-values between the

saline-injected and non-injection larvae were 0.58, 0.63, 0.63 and 0.64 on day 1, 2, 3 and 4 after injection, respectively, demonstrating that injection itself had no impact on larval food intake.

4. Discussion

The sequences of *sk* and two *skr*s were determined in the coleopteran insect *T. castaneum*. The deduced proteins TcSK and TcSKR2 are the same as the ones from existing genomic gene annotation, whereas TcSKR1 is different due to an insertion, which probably results from a dysfunctional annotation. Two *Tcskr*s with similar deduced proteins were also cloned by Zels et al. (2014).

When the TcSK prepropeptide was compared to other known insect SK prepropeptides (Figure II-2b), the regions corresponding to the two Trica-SK peptides are highly conserved, together with the amidation signal and the C-terminal dibasic cleavage sites, while the remainder is quite divergent. This is consistent to the data obtained in previous studies in *Calliphora vomitoria* and *Gryllus bimaculatus* (Duve et al., 1995; Meyering-Vos and Müller, 2007b).

The strong similarity in structure and biological activities between insect SKs and vertebrate CCK/gastrin has led to the hypothesis that the neuropeptide signaling systems and mechanisms underlying the regulation of ingestive behavior between arthropods and vertebrates have been conserved throughout evolution (Janssen et al., 2008, 2010; Nachman et al., 1986b, 1986c). SK(-like) peptides in animals are highly homologous in both amino acid sequence and function as discussed in Chapter I. Because CCK and gastrin genes are identified as separate genes in the dogfish, a duplication of the ancestral gene most likely occurred during the evolution of cartilaginous fishes or earlier, giving rise to two distinct hormones, CCK and gastrin (Johnsen, 1998). A recent synteny analysis provides strong evidence that the CCK/gastrin family in vertebrates has been mainly shaped by whole genome duplications (Dupré and Tostivint, 2014).

Insects are reported to have higher evolutionary rate compared to vertebrates (Wyder et al., 2007). In the analysis of a genome-wide acceleration of protein evolution, Savard et al. (2006) concluded that the *Tribolium* genes show the lowest rates of divergence compared to Diptera.

In both phylogenetic trees (Figure II-6), the two *Drosophila* SKRs clustered together in SKR-G2, while the two *T. castaneum* SKRs were separated in two groups. This indicates that species-specific events may influence the receptor evolution.

Mirabeau and Joly (2013) conducted a comprehensive analysis of the peptidergic signaling systems in bilaterians by interrogating genomic sequence databases and using phylogenetic reconstruction tools, which paves the way for comparative studies. In their study, the analysis of the intronic structure of genes suggests that receptors share orthologous introns and are likely to have evolved from a common ancestral bilaterian receptor gene. Generally, there is co-evolution of peptides with their receptors and SK(-like) peptides that have been recognized in lineages to human, nematodes and arthropods (Mirabeau and Joly, 2013; Taghert and Nitabach, 2012). The phylogenetic analysis here (Figure II-6) demonstrates the early origin of the CCKRs in metazoans. The identification of representatives in protostomes and deuterostomes suggests that a putative SK(-like) receptor gene was already present in the ancestral Bilateria genome.

Christie et al. (Christie, 2008a, b; Christie et al., 2008, 2011a, 2011b) carried out a series of identification of neuropeptides using bioinformatics of genome and publicly accessible expressed sequence tags (ESTs) among *D. pulex* and species from Chelicerata, Ecdysozoa, Aphidoidea and Ixodoidea. SK genes are only found in *I. scapularis* and *D. pulex*, in consistent with our searching result. The spider mite *Tetranychus urticae* is a representative of Chelicerata that misses the SK signaling. Of interest is the fact that SK signaling could not be found in the hemimetabolous pea aphid *Acyrthosiphon pisum* (The International Aphid Genomics Consortium, 2010), the parasitic wasp *Nasonia vitripennis* (Hauser et al., 2010; NasoniaBase) and the plant parasitic nematode *Meloidogyne incognita* (*Meloidogyne incognita* resources). These results showed that SK signaling was missing in some species during evolution. Pea aphids take up a big volume of food to get enough essential nutrients. Li et al. (2013) proposed that the loss of the SK signaling is likely due to increases in food intake. Hauser et al. (2010) compared the neuropeptides in two hymenopteran *A. mellifera* and *N. vitripennis*, with SK being present in *A. mellifera* but missing in *N. vitripennis*. The authors suggest that differences in neuropeptides might reflect differences in behavior (social vs. parasitic), feeding (herbivores vs. carnivores) and/or habitats of these two related insect species, which is also discussed by Nygaard et al. (2011).

Moreover, in arthropods, rhodopsin GPCR evolution is characterized by species-specific gene duplications and deletions and in nematodes by gene expansions in species with a free-living stage and gene deletions in representatives of obligate parasitic taxa. Based upon variation in GPCR gene number and potentially divergent functions within phyla, it is hypothesized that the life style and feeding diversity practiced by nematodes and arthropods is one factor that contributes to rhodopsin GPCR gene evolution (Cardoso et al., 2012). Accordingly, we hypothesize that the SK signaling is likely to evolve in relation to the feeding behavior. An example is that the larvae of the endoparasitic wasp *N. vitripennis* are living inside their host allowing feeding continuously. At the moment, a large number of species are being sequenced, for instance, the i5k genome project (ArthropodBase) which will benefit the study of SK signaling in various species with different feeding behavior and nutrition requirements.

SK(-like) receptors share a common ancestor among metazoans. The two *ckr*s that are present in nematodes resulted from a lineage-specific duplication and homologs of the two *C. elegans* genes were identified in many nematode genomes (Cardoso et al., 2012). In arthropods, two separate SKR genes are found in *D. melanogaster* and *T. castaneum*. However, the two *D. melanogaster skr*s seem to be a result of a species-specific evolutionary event, which is distinct from the scenario from *T. castaneum* (Figure II-6). Moreover, in the vertebrate lineage, the two receptor genes share a high similarity, leading to the assumption that both CCK1R and CCK2R evolved from the same duplicated ancestral genes (Huppi et al., 1995). A sophisticated tree (gene tree: ENSGT00730000110635) is available in the Ensembl website (http://www.ensembl.org/Multi/GeneTree/Image?gt=ENSGT00730000110635), in which, remarkably, the *cck2r* is duplicated in many teleost fishes, whereas *cck1r* is present in one copy in these teleost fishes. It suggests that the duplication took place in the basal teleost tetraploidization (3R). It is also speculated that the vertebrate *cck1r* and *cck2r* arised through the vertebrate basal tetraploidizations (1R and 2R) which has been studied for *cck/gastrin* (Dupré and Tostivint, 2014). Investigations on the neighboring genes and rearrangements of chromosomal regions in vertebrates will extend our knowledge on the evolution of SKR-like proteins.

In terms of the transcript profile, *sk* and *skr*s are expressed in all the examined developmental stages and tissues, although the tissue sampling is fairly poor. Recently, a very detailed transcript analysis of the two *Tcskr*s (Zels et al., 2014) revealed that both receptors are expressed highest in the central brain, followed by the optic lobes, but appears less abundant

or detectable in the other examined tissues, such as salivary glands, gut, fat body, testes and ovaria. Specifically, the expression of *Tcskr*s appears to be most prominent in the fat body apart from the nervous system, which indicates the possible role of SK signaling in the energy storing and processing in the fat body. The transcript levels of *Tcsk* and *Tcskr1* appears to be dependent on the nutritional state of the beetles. Deprivation of food resulted in higher expression of *Tcsk* and *Tcskr1* (Figure II-8). It seems the beetle responds to the insufficient nutrition via actively modulating the mechanism in regulation of feeding.

In respect to the structure, the two TcSKRs are both highly similar in the seven transmembrane domains to other insect SKRs. The TcSKR1 shows significant homology to the two *Drosophila* (55% identity to AAF48875; 52% identity to AAF48879), one *Periplaneta* (58% identity to AY865608) and one *Anopheles* (61% identity to AAR28375) SKR proteins. The TcSKR2 shares 53% similarity with TcSKR1. The differences mainly exist in the N-terminal and C-terminal regions.

The regulation of feeding is a complex behavioral system (Geiselman, 1996). In insects, several neuropeptides are considered to be pivotal in regulating feeding (Audsley and Weaver, 2009; Downer et al., 2007; Schoofs et al., 1997; Spit et al., 2012). Among these neuropeptides, SKs and their receptors are expected to form one part of the signaling network in the regulation of feeding and digestion, because its counterpart in vertebrate, gastrin/CCK signaling is a well-known satiety factor.

In this chapter, a drastic increase in larval food intake was observed in *sk*-silenced *T. castaneum* larvae, which supports the fact that SK signaling inhibits feeding. Similarly, silencing of *sk* leads to a stimulation of food intake in cricket *G. bimaculatus* (Meyering-Vos and Müller, 2007a). Moreover, the injection of sSK-II peptide dramatically reduced larval food intake. The reduction in food intake was also observed in other (pest) insects such as the desert locust (Wei et al., 2000) after the injection of SK homologs. Taken together, the SK signaling acts in an inhibitory way to regulate feeding in insects. Given the importance of the regulation of feeding in insect development, SK signaling is a promising route to exploit in the development of novel pest control strategies.

The sSK peptide reduced larval food intake after injected to the beetles (Figure II-10). A notable drop of food intake by the sSK-injected beetles was observed on day one and the effect lasted for the rest three days. The effect of sSK peptide accumulated to a significant

reduction of food intake from day three on. Wei et al. (2000) reported an almost immediate effect of injected sSK on reducing feeding in the desert locust. The feeding assay here and Wei et al. may hint towards factors such as the species-specificity and the viability of sSK in the haemolymph after administration. Moreover, the present experiment was only followed for four days, when the inhibitory effect of sSK was still present. Therefore, the information of the stability of sSK in haemolymph and duration of the inhibitory effect remains unknown.

The efficiency and duration of dsRNA-mediated gene silencing effects are influenced by many factors such as the concentration, length and sequence specificity of dsRNAs as well as the tissue-dependency of the RNAi effect (Miller et al., 2012). DsRNA-mediated RNAi has been performed successfully for a variety of target genes at different developmental stages and tissues in *T. castaneum*, which makes this beetle species a useful model for functional genomics (Miller et al., 2012; Tomoyasu and Denell, 2004). In the present study, similar doses of dsRNAs (about 200 ng) were used in the RNAi experiments targeting three different genes. They all resulted in a significant down-regulation of the specific target mRNA. However, the extent to which the target was silenced differed. This was not unexpected, because the expression levels of these target genes differ in the larval stage (Figure II-7). In addition, the dsRNA fragments used in this experiment were carefully chosen in the least similar region (Figure II-S1), which are regions of less than five consecutive identical nucleotides between the two *Tcskr*s in the two dsRNA fragments, in order to avoid the off-target RNAi effect (Jackson et al., 2003; Kulkarni et al., 2006). Higher efficiency and longer duration of gene silencing could be achieved by optimizing the dose of dsRNAs, the target region within the gene as well as the administration of multiple injections.

The silencing of *Tcskr2* increased food intake significantly while silencing of *Tcskr1* was not so fast and strong. We postulate here the following two hypotheses. First, there might be enough TcSKR1 left in the larva after *Tcskr1* was down-regulated by 50%. Second, TcSKR2 also responded to TcSKs for its activity when TcSKR1 was missing. In the *Tcskr2*-silencing experiment, a dramatic increase of food intake was observed from day five onwards after *Tcskr2* gene was silenced up to 70% on day three. The stronger phenotypic effect of silencing of *Tcskr2* over *Tcskr1* suggests that TcSKR2 responds more actively than TcSKR1 in the SK signaling in the regulation of feeding. However, in the case of poor food supply, the expression of *Tcskr1* was upregulated while *Tcskr2* was not influenced in the tested period. Therefore, these two hypotheses require further investigation.

SKs and SKRs constitute a multifunctional signaling system in insects. In addition to inhibiting food intake, SKs have been reported to influence gut muscle contraction (Maestro et al., 2001; Nachman et al., 1986b, 1986c; Predel et al., 2001) and to stimulate the release of digestive enzymes (Harshini et al., 2002; Nachman et al., 1997). In our study with *T. castaneum*, the *Tcsk* and *Tcskr*s were found to be expressed throughout the three developmental stages, including the pupa stage where insects do not feed. We therefore believe that the considerable expression of *Tcsk* and *Tcskr*s in pupa supports that the SK signaling is also involved in multiple activities in addition to food intake.

In summary, SK signaling is involved in inhibiting feeding in *T. castaneum*. Therefore, study on the mechanism of SK signaling is promising to understand the regulation of feeding in insects and to find a novel approach of pest control.

Table II-2 SKR homologs used in alignment and phylogenetic analysis

Species	Protein	Accession Number
Ailuropoda melanoleuca	CCK1R	XP_002924347.1
	CCK2R	XP_002925026.1
Anopheles gambiae	SKR	AAR28375.1
Apis mellifera	SKR	XP_006562432.1
Bombyx mori	SKR	NP_001127744.1
Bos taurus	CCK1R	NP_001095335.1
	CCK2R	NP_776687.2
Caenorhabditis brenneri	CKR1	CBN10364
Caenorhabditis briggsae	CKR1	XP_002640196.1
	CKR2	XP_002642853.1
Caenorhabditis elegans	CKR1	NP_491918.3
	CKR2	ACA81683.1
Caenorhabditis remanei	CKR1	XP_003114892.1
	CKR2	XP_003103966.1
Callithrix jacchus	CCK1R	XP_002745977.1
Canis lupus familiaris	CCK1R	AAX12114.1
	CCK2R	NP_001013868.1
	BDKR	NP_001014306.1
Carassius auratus	VIPR	AAB05459.1
Cavia porcellus	CCK1R	Q63931.1
Ciona intestinalis	CioR1	NP_001027945.1
	CioR2	BAL70271.1
Culex quinquefasciatus	SKR2	EDS26978.1
Danio rerio	CCK1R	XP_697493.2
	CCK2R	CAQ14219.1
Daphnia pulex	SKR	EFX77608.1
Drosophila erecta	SKR	XP_001977700.1
Drosophila melanogaster	SKR1	NP_001097021.1
	SKR2	NP_001097023.1

(Table II-2 continued)

Species	Protein	Accession Number
Drosophila persimilis	SKR	XP_002026777.1
Drosophila pseudoobscura	SKR1	XP_002134520.1
	SKR2	XP_002134525.1
Equus caballus	CCK1R	XP_001499250.2
	CCK2R	XP_001504633.2
Gallus gallus	CCK1R	NP_001074970.1
	CCK2R	NP_001001742.1
Harpegnathos saltator	SKR	EFN85362.1
Homo sapiens	CCK1R	NP_000721.1
	CCK2R	NP_795344.1
Homo sapiens	SCTR	AAA64949.1
Macaca mulatta	CCK1R	XP_001084186.1
	CCK2R	XP_001102094.1
Mastomys natalensis	CCK2R	AAB41677.1
Monodelphis domestica	CCK2R	XP_001380242.1
Mus musculus	CCK1R	NP_033957.1
	CCK2R	NP_031653.1
Oryctolagus cuniculus	CCK1R	NP_001075852.1
	CCK2R	NP_001164594.1
Pan troglodytes	CCK1R	XP_526545.1
	CCK2R	XP_521813.1
Pediculus humanus corporis	SKR1	EEB20399.1
	SKR2	EEB18252.1
Periplaneta americana	SKR	AAX56942.1
Pongo abelii	CCK1R	XP_002814697.1
	CCK2R	NP_001127690.1
Rattus norvegicus	CCK1R	NP_036820.1
	CCK2R	NP_037297.1

(Table I-2 continued)

Species	**Protein**	**Accession Number**
Strongylocentrotus purpuratus	CCKR	XP_782630.3
Taeniopygia guttata	CCK1R	XP_002191034.1
Tribolium castaneum	SKR1	XP_975226.2
	SKR2	XP_972750.1
Xenopus laevis	CCKR	AAB09052.1

Supplementary data

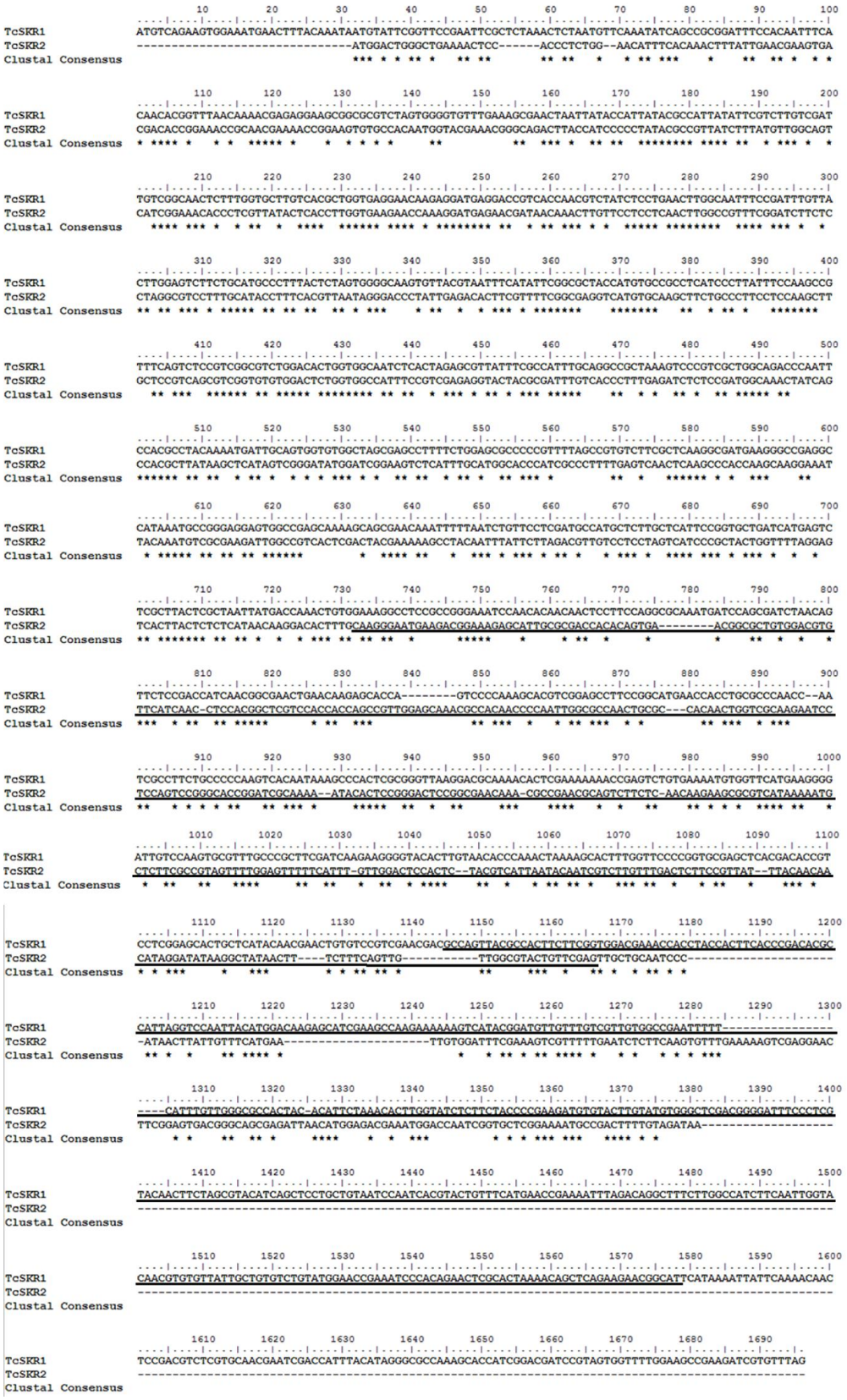


Figure II-S1 Position of two ds*skr* fragments in the two Tc*skr*s. Alignment of two Tc*skr*s was conducted with the default setting using online server Clustal Omega. The asterisk represents indentical nucleotides between two genes. Nucleotides corresponding to the two ds*skr* fragments are underlined.

Chapter III. Effect of sulfakinin(-related) peptides on feeding in *Tribolium castaneum*

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1. Introduction

Insect SKs share a conserved C-terminus (DYGHM/LRF-NH_2) among various species (Table I-1). The structure-activity relationship (SAR) of SKs has been investigated in a series of studies with an emphasis on the myotropic activity (Table III-1). In Leuma-SK-I, substitutions such as $G^5 \rightarrow A^5$ or $M^3 \rightarrow A^3$ retained partial activity, while A substitution in position H^4, R^2 or F^1 lead to inactive compounds (Nachman et al., 1989). However, partial retention of activity was observed when the R^2 residue was replaced with a basic K^2. Leuma-SK-I lost activity completely when the C-terminal amide was replaced with an acid moiety. Replacement of $Y^6(SO_3H)$ by $S^6(SO_3H)$ resulted in the loss of activity. In contrast, aliphatic amino diacid α-aminosuberic acid (Asu) functioned as an effective mimic of $Y^6(SO_3H)$ in Leumi-SK for both myotropic and food intake-inhibition activity, whereas neither α-aminoadipic acid (Adi) nor E substitution was active (Nachman et al., 2005). It was proposed that the degree of the activity of SK analogs is correlated with the carboxyl/α-carbon distance in the cockroach hindgut contractile assay. The oxidant-susceptible M^3 could be replaced by Norleucine (Nle^3) without losing activity. However, replacement of $M^3 \rightarrow L^3$ and $G^5 \rightarrow V^5$ led to compounds with decreased myostimulatory activity (Nachman et al., 1993). The Leuma-SK-I demonstrated the tolerance to modification in N-terminus, e.g., introduction of pE^8 to E^8 had negligible impact on activity (Nachman et al., 1993).

The sulfate moiety on Y^6 ($Y^6(SO_3H)$) of SKs has been demonstrated to be necessary to not only the contraction of hindgut and heart but also the inhibition of food intake (Maestro et al., 2001; Nachman et al., 1986b, 1986c, 1989; Predel et al., 1999; Wei et al., 2000). Nevertheless, nonsulfated SKs (nsSKs) are demonstrated functional in certain biological processes. Nichols (2007) reported that nonsulfated *Drosophila* SKs (nsDSK-I and nsDSK-II) decreased the frequency of the spontaneous contraction of the larval anterior midgut, to which the sulfated *Drosphila* SKs (sDSKs) were not statistically effective; nsDSKs were more effective than sDSKs to decrease the frequency of spontaneous contractions of the adult crop. NsDSKs proved to increase the frequency of heart contraction in larva and/or adult, but not in pupa (Nichols et al., 2008b). Later, the same group demonstrated that both forms of DSK-I influence larval locomotion and both forms of DSK-II influence larval odor preference in *D. melanogaster* (Nichols et al., 2008a). Recently, nsSK-I from *Zophobas atratus* (Zopat-SK-I) showed a myostimulatory action on the isolated hindgut of the adult beetles, but a

myoinhibitory action on the adult and pupal heart contraction. Besides, Zopat-SK-I increased free sugar levels in the larval haemolymph (Marciniak et al., 2011).

Table III-1 Structure-activity relationship study of Leuma-SK-I on its myostimulatory activity

Change [a b]	Effect on myostimulatory activity [c]	Reference
$E^8 \rightarrow pE^8$	no impact	Nachman et al., 1993
$\cancel{D}^7$	3-fold increase	Nachman et al., 1989
$\underline{Y}^6 \rightarrow \underline{S}^6$	trace activity	Nachman et al., 1989
$\underline{Y}^6 \rightarrow Asu^6$	effective as parent peptide (also on feeding)	Nachman et al., 2005
$\underline{Y}^6 \rightarrow Adi^6$	40% activity of parent peptide	Nachman et al., 2005
$\underline{Y}^6 \rightarrow E^6$	loss of activity	Nachman et al., 2005
$G^5 \rightarrow A^5$	retention of activity	Nachman et al., 1989
$G^5 \rightarrow V^5$	loss of activity	Nachman et al., 1993
$\cancel{G}^5$	loss of activity	Nachman et al., 1993
$H^4 \rightarrow A^4$	loss of activity	Nachman et al., 1989
$M^3 \rightarrow A^3$	retention of activity	Nachman et al., 1989
$M^3 \rightarrow Nle^3$	effective as parent Leuma-SK-I	Nachman et al., 1993
$M^3 \rightarrow L^3$	2-order of magnitude decreased activity	Nachman et al., 1993
$R^2 \rightarrow A^2$	loss of activity	Nachman et al., 1989
$R^2 \rightarrow K^2$	partial retention of activity	Nachman et al., 1989
$F^1 \rightarrow A^1$	loss of activity	Nachman et al., 1989
$-NH_2 \rightarrow -COOH$	loss of activity	Nachman et al., 1989

[a] Leuma-SK-I: EQFEDYGHMRF-NH_2

[b] $\underline{Y}$, sulfated Y residue; →, substituted to; $\cancel{X}$, removed residue.

[c] activity relative to parent peptide Leuma-SK-I

So far, most functional studies on SKs have been conducted for their contractile activity, although SKs are also reported to inhibit feeding in many insects (Downer et al., 2007; Maestro et al., 2001; Meyering-Vos and Müller, 2007a; Wei et al., 2000). Thus, we are especially interested in the SAR of SK peptides on feeding in *T. castaneum*.

In this chapter, the SAR of SK on feeding in *T. castaneum* was examined by assays with a series of SK(-related) peptides. These peptides were evaluated for their activity on feeding of *T. castaneum*. Truncation and substitution (by A, Asu, S and Nle) were introduced in these peptides to investigate the importance of the sulfate moiety and the conserved hexapeptide YGHMRF-NH_2. The peptides were injected in *T. castaneum* adults, where the food intake

was scored for a period of three days. In addition, the effect of nematode NLP-12 and NLP-13 peptides wasalso studied.

2. Materials and Method

2.1. Insects

T. castaneum wild-type strain GA-1 was reared in whole wheat flour with 5% yeast under standard conditions in the lab. Adults were used in the subsequent experiments.

2.2. Peptide synthesis

Peptide sequences were chosen from known insect and nematode SK-related neuropeptides. These peptides (Table III-2) were synthesized via FMOC methodology under previously described conditions (Nachman et al., 1986c). The identity of the peptides was confirmed via matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) and quantified via amino acid analysis. The synthesis and verification were carried out in the lab of Dr. Nachman in USDA.

2.3. Solution preparation and injection

Peptides were prepared in a stock solution of 80% acetonitrile (in water). Dilutions with buffered saline (10 mM Hepes, pH 7.4, 1% BSA, 0.85% NaCl) were freshly prepared before each experiment. The saline solution was used as a negative control.

Adult beetles weighing 2.0 ± 0.2 mg were kept separately to standardize their hunger state by depriving them of food for 12 h before the assay. Beetles were anaesthetized with ether, placed with their abdomens down on double-sided tape and injected with peptide solution or saline into their dorsal abdomens under the elytra (Posnien et al., 2009) to a final concentration of 16 μM beetle, approximate to the effective concentration of SK in the desert locust (Wei et al., 2000). Ten randomly picked adults were injected for each peptide or saline. Each beetle was kept individually with a flour disk (Figure II-1). Prior to the assay, the flour disks were saturated in a culture incubator with R. H. 70% for 72 h and weighed before fed to beetles. All injected beetles were kept under the standard conditions (temperature 30 °C, R. H. 70%) for food intake observation. The peptide assay was repeated three times.

2.4. Measurement of food intake and data analysis

The flour disks were weighed before and 72 h after fed to the *T. castaneum* adults. The decrease of a disk weight represented the food intake by a beetle over the three-day period. The relative activity of a peptide on inhibiting food intake was calculated following the formula: activity of peptide (% nsSK-II) = (food $intake_{control}$ − food $intake_{peptide}$) / (food $intake_{control}$ − food $intake_{nsSK-II}$) ×100. The food intake by beetles of different treatment was compared to saline control and nsSK-II, separately, via Student's *t*-test in GraphPad Prism version 5.00.

3. Results

Six groups of peptides were tested in *T. castaneum* adults. Table III-2 displays their activities to inhibit feeding in *T. castaneum* relative to nsSK-II, with a final concentration of 16 μM.

Table III-2 Relative acitivity of SK(-related) peptides (16 μM) to inhibit food intake in *T. castaneum* adults.

Group	Peptide	Sequence [a b]	Relative activity (% nsSK-II) [c]	Statistical significance [e]	
				to negative control [d]	to nsSK-II [d]
SK	1010 (nsSK-II)	FDDYGHMRF-NH_2	100 ± 5	s	ns
	1521 (sSK-II)	FDD<u>Y</u>GHMRF-NH_2	93 ± 6	s	ns
SK-Ala substitution	2003	FDDYGHMR**A**-NH_2	94 ± 24	s	ns
	2004	FDDYGHM**A**F-NH_2	76 ± 21	s	ns
	2005	FDDYGH**A**RF-NH_2	93 ± 25	s	ns
	2006	FDDYG**A**MRF-NH_2	56 ± 19	s	ns
	2007	FDDY**A**HMRF-NH_2	12 ± 19	ns	s
	2008	FDD**A**GHMRF-NH_2	59 ± 10	s	ns
SK-truncation	2009	DDYGHMRF-NH_2	74 ± 30	s	ns
	2010	DYGHMRF-NH_2	47 ± 33	ns	ns
	2011	YGHMRF-NH_2	95 ± 32	s	ns

(Table III-2 continued)

Group	Peptide	Sequence [a] [b]	Relative activity (% nsSK-II) [c]	Statistical significance [e]	
				to negative control [d]	to nsSK-II [d]
SK-truncation	2053	GHMRF-NH_2	124 ± 28	s	ns
	2052	HMRF-NH_2	108 ± 15	s	ns
	2051	MRF-NH_2	84 ± 16	s	ns
	2076	FDDYGHMR-NH_2	144 ± 19	s	ns
SK-related	1835	**S**DDYGHMRF-NH_2	85 ± 10	s	ns
	1070	FDD(**Asu**)GHMRF-NH_2	96 ± 12	s	ns
	1107	DD(**Asu**)GHMRF-NH2	113 ± 5	s	ns
	1658	DDYGH(**Nle**)RF-NH_2	50 ± 12	ns	ns
	1591-1	EAYGH(**Nle**)**K**F-NH_2	50 ± 13	ns	ns
	1598-2	EYGH(**Nle**)RF-NH_2	-15 ± 9	ns	s
	1586	***cyclo***(EAYGH(**Nle**)**K)**F-NH_2	74 ± 16	s	ns
	1592	***cyclo***(EYGH(**Nle**)**K)**F-NH_2	52 ± 17	s	ns
C. elegans NLP-13	1679 (NLP-13a)	pQPSYDRDIM**S**F-NH_2	93 ± 7	s	ns
	1432-2 (NLP-13b)	SPVDYDRPIMAF-NH_2	102 ± 10	s	ns
	1569	PVDYDRPIMAF-NH_2	87 ± 9	s	ns
	1567	SPVDYDRPIMF-NH_2	83 ± 9	s	ns
	2018	***cyclo***(YDRPIMAF)	-34 ± 12	ns	s
	2020	***cyclo***(RPIMAF)	38 ± 39	ns	s
C. elegans NLP-12	1678a (NLP-12a)	DYRPLQF-NH_2	-61 ± 30	ns	s
	1678b (NLP-12b)	DGYRPLQF-NH_2	-65 ± 11	s	s
Combination	nsSK-II + NLP-12b	FDDYGHMRF-NH_2 + DGYRPLQF-NH_2	-48 ± 20	ns	s

[a] **Y, Y(SO_3H).**

[b] Bold letters highlight the positions with modification of the parent peptide. Asu: α-aminosuberic acid; Nle: Norleucine; *cyclo*: cyclic structure.
[c] Activity of a peptide is presented as percentage of the activity of nsSK-II (% nsSK-II) at a final concentration of 16 µM. Data are presented as mean ± SEM from three biological replicates.
[d] Saline was used as a negative control. The final concentration of a peptide was 16 µM. The food intake of *T. castaneum* (mean ± SD, n=30) for three days after treatment was: saline-treated adult, 0.70 ± 0.13 mg; sSK-II-treated adult, 0.27 ± 0.06 mg; nsSK-II-treated adult, 0.14 ± 0.05 mg. The food intake of adults treated with sSK-II and nsSK-II showed no significant difference.
[e] s, statistically significant difference of food intake; ns, no statistically significant difference of food intake.

3.1. Effect of SK-II (FDDYGHMRF-NH_2) on feeding

Both sSK-II and nsSK-II inhibited food intake dramatically by 60% and 80%, with food intake 0.27 ± 0.06 mg and 0.14 ± 0.05 mg, respectively, compared to 0.70 ± 0.13 mg of the saline-injected control beetle. Thus, nsSK-II and sSK-II exhibited similar activity on inhibiting feeding, regardless of the sulfate moiety.

3.2. Effect of nsSK-II analogs (Ala-substitution and truncation of FDDYGHMRF-NH_2) on feeding

3.2.1. G^5 of nsSK-II was critical for inhibiting feeding

Peptides 2003-2008 are a series of nsSK-II analogs with Ala-substitution (A-nsSK) from the position 1 to 6. Most of these A-nsSKs inhibited feeding to diverse extents. However, the peptide 2007 ($G^5 \rightarrow A^5$) was inactive with only 12% of the nsSK-II activity, indicating that the G^5 is essential for nsSK-II. Moreover, the peptides 2006 ($H^4 \rightarrow A^4$) and 2008 ($Y^6 \rightarrow A^6$) also exerted less inhibitory effect compared to the other A-nsSKs, suggesting that H^4 and Y^6 were relatively important to nsSK-II.

3.2.2. Removal of N-terminus did not affect the activity of nsSK-II on inhibiting feeding

Truncated nsSK-II analogs 2009, 2010, 2011, 2051, 2052, 2053 and 2076 are peptides with amino acids removed from the N-terminus of nsSK-II. Six of these peptides (2009, 2011,

2051, 2052, 2053 and 2076) were tolerated to the residual deletion, retaining similar activity as their parent peptide nsSK-II. Peptide 2010 retained only 47% activity with F^9 and D^8 removed, for which the reason is not clear yet.

3.3. Effect of SK-II analogs (substitution, deletion or cyclic structure) on feeding

Peptide 1835 ($F^9 \rightarrow S^9$) exerted a similar effect as nsSK-II, suggesting that the aromatic structure of F^9 is not an important characteristic for the activity at this N-terminal position. Peptides 1070 and 1107 are with the substitution $Y^6 \rightarrow Asu^6$ of nsSK-II. Both peptides were strongly active on inhibiting food intake (with relative activity as 96% and 113%, respectively). Therefore, the substitution of Y^6 with Asu^6 did not affect the activity of nsSK-II, further emphasizing that aromaticity is not a critical feature at this position.

Peptides 1658, 1591-1, 1598-2 are peptides with the replacement $M^3 \rightarrow Nle^3$. Peptide 1658 and 1591-1 showed no statistical significance from saline although they retained 50% activity of nsSK-II. 1598-2 was completely inactive, which suggested that either the residue A^7 or the length of peptide is critical for the activity of SK. Peptides 1586 and 1592 are cyclic peptides on the basis of peptide 1658 and 1598-2, respectively. The cyclic structure in 1586 and 1592 did not affect or even elevated their activity as they both exhibited higher activity than its parent peptides 1658 and 1598-2. The two cyclic structures may therefore prove useful in the determination of the active conformation of the SKs in food intake inhibition in *T. castaneum* beetles. In addition, peptides 1591-1, 1586 and 1592 contain the substitution $R^2 \rightarrow K^2$ of SK and they retained most activity.

3.4. Effect of NLP-13 (YDRPIMAF-NH_2) analogs on feeding

The *C.elegans* NLP-13 (neuropeptide-like protein-13; YDRPIMAF-NH_2) peptides, which exhibit some sequence similarity with insect SKs, elicited similar inhibitory effects on food intake as SK. Peptide 1679 is sulfated and blocked in N-terminus, with the same sequence as NLP-13a. Peptide 1432-2 has the amino acid sequence of the NLP-13b. Therefore, both NLP-13 peptides displayed similar activity to nsSK-II. The other two peptides 1569 and 1567 were also active on inhibiting feeding.

Peptide 2018 and 2020 are both cyclic peptides of the conserved motif of NLP-13 and showed different activities from NLP-13. They were both inactive to inhibit food intake although they

displayed quite different activities from each other. Peptide 2020 had 38% activity in inhibiting feeding relative to nsSK-II, whereas 2018 had the opposite activity to nsSK-II by increasing feeding.

3.5. Effect of NLP-12 (YRPLQF-NH_2) on feeding

NLP-12 peptides are recognized as SK homologs in *C. elegans* (Janssen et al., 2008). Here, both NLP-12a and NLP-12b increased food intake in *T. castaneum* and especially NLP-12b exerted a dramatic increase in food intake, indicating a negative activity to Trica-SK-II on inhibiting feeding.

3.6. Effect of the combination of nsSK-II and NLP-12b on food intake

The nsSK-II and NLP-12b were mixed at a molar ratio of 1:1 and then injected in beetles at a final concentration of 16 μM for each peptide. The peptide-treated beetles ate similar amount of food as the saline-treated control, suggesting NLP-12b may be an antagonist of nsSK-II.

4. Discussion

In the present experiment, both sSK-II and nsSK-II led to significantly low levels of food intake in *T. castaneum* adults, suggesting that the acidic sulfate group (SO_3H) on Y^6 may be not necessary for this activity. The sulfate moiety was shown to be critical to feeding in previous studies. In the desert locust *Schistocerca gregaria*, nsLeumi-SK had no effect on food intake (Wei et al., 2000). In *Blattella germanica*, the sLeuma-SK-II and sPeram-SK-I inhibited food intake at low concentrations, but the nonsulfated peptides were inactive even at higher concentrations (Maestro et al., 2001). In addition, the sDSK is much more potent than the nsDSK to activate DSK receptors in *D. melanogaster* (Chen et al., 2012; Kubiak et al., 2002). Nevertheless, nsSKs have also been present and physiologically active. In the American cockroach *Periplaneta americana*, both sSK and nsSK are detected in the corpora cardiaca/corpora allata complexes, providing an evidence that the two forms of SK naturally co-exist in insects (Predel et al., 1999). In *D. melanogaster* and *Z. atratus*, nsSKs function on myoactivity, odor preference and locomotion (Marciniak et al., 2011; Nichols, 2007; Nichols et al., 2008a). Moreover, NLP-12 peptides, the SK homologs in *C. elegans*, were also suggested to be likely unsulfated *in vivo* (Janssen et al., 2008).

In vertebrates, both sulfated and nonsulfated CCKs are active and they play their biological roles via two receptors with CCK1R preferring sulfated CCK and CCK2R no preference (Staljanssens et al., 2011). Two SKRs have also been identified in *Drosophila* and *Tribolium*, which suggests that potentially distinct mechanisms underlay the SK signaling (Nichols et al., 2008a).

Peptides with Ala-substitution produced similar effect as the parent peptide except the peptide 2007 with $G^5 \rightarrow A^5$. G^5 is known as one of the most conserved residues in not only insect SKs but also human gastrin II and CCK (Nachman et al., 1986b; Figure I-3). In addition, G^5 is one of the sequence differences between SKs and leucomyosuppressin (LMS). SKs and LMS have contrasting biological activity, despite the fact that they share sequence similarity at several positions (Nachman et al., 1993). Therefore, we conclude that G^5 is conserved and important to the activity of SKs on inhibiting feeding.

Analogs with deletion of one to eight residues from N-terminus (peptides 2009-2076 in Table III-2) retained most of the activity on inhibiting food intake. Thereby, the N-terminus shows more tolerance to residue change than the C-terminus. It is consistent to a previous report that truncated leucopyrokinin analogs retained the myotropic activity (Nachman et al., 1986a).

Two analogs (1070 and 1107) of nsSK-II contained an unnatural residue α-aminosuberic acid (Asu) that mimics the $Y(SO_3H)$. Asu features an acidic carboxyl group that projects the same distance from the peptide backbone as does the acidic SO_3H group on Y^6 by virtue of attachment to a chain of methylene groups (Nachman et al., 2005). The strong inhibitory activity of these analogs observed in *T. castaneum* feeding assay indicates that the aromatic phenyl ring of Y is not a critical characteristic for this position. The M^3 is in the active core for the feeding-inhibitory activity of SK in *T. castaneum* because the replacement $M^3 \rightarrow Nle^3$ resulted in no statistically significant difference (Table III-1). Similarly, [Nle^3]Leuma-SK-I retains only 26% activity of the synthetic Leuma-SK-I when the oxidation-sensitive M^3 is replaced with isosteric Nle^3 (Nachman et al., 1989). The sequence of peptide 1835 is present in several insect SKs such as Leuma-SK-II, Trica-SK-I and Ixosc-SK-I (Table I-1). Peptide 1835 has similar feeding-inhibitory activity as sSK-II (Table III-2), indicating that aromaticity is not a critical characteristic in position 9. Peptides 1569 and 1432-2 feature a replacement of $R^2 \rightarrow A^2$ and 1679 features a $R^2 \rightarrow S^2$. R is positively-charged and strongly basic, but A and S have no charged character. The strong inhibitory activity observed for all these three

peptides indicates that basicity is not a critical characteristic in the position 2, in contrast with the activity of the SKs in the cockroach hindgut myotropic assay (Nachman et al., 1989).

The *C. elegans* NLP-12 was reported to stimulate nematode CKR2 *in vitro* (Janssen et al., 2008) and to elicitate the contraction of nematode body wall muscle preparations (McVeigh et al., 2006). In the feeding assay, NLP-12 peptides demonstrated an opposite effect on food intake to SK-II in *T. castaneum*, although they share sequence similarity to some extent. This observation suggests NLP-12 as a putative antagonist to nsSK-II. The presence of -**Q**F-NH_2 rather than -RF-NH_2, -AF-NH_2 or -SF-NH_2 in the C-terminus of the nematode sNLP-12 peptides might account for the putative antagonist response in the *T. castaneum* feeding assay. However, the mechanism by which NLP-12 exerts the opposite effect to canonical SKs is not clear yet.

The *C. elegans* NLP-13 peptide gene is reported to be expressed in pharyngeal neurons that modulate pharyngeal pumping of food (Nathoo et al., 2001), although exact effect of NLP-13 peptides on feeding has not been investigated. NLP-13 peptides exerted the similar effect on feeding as the *T. castaneum* sSK in beetles regardless of the low homology of their amino acid sequences. It is not likely that NLP-13 peptides act on the *T. castaneum* SKRs, but other neuropeptide receptor associated in the regulation of feeding.

SKs inhibit feeding in insects, which provides a possibility to apply SK peptide analogs as a pesticide in pest control. NsSK-II exhibits similar activity as sSK-II in the present study, although the exact role and mechanism of the two forms of SK in SK signaling is not clear yet. NsSK could be a practical candidate of potential pesticide, because nsSK is a short peptide and that it does not have the sulfate group, making the synthesis and modification of the peptide easier and more cost effective than sSK. In addition, the SK signaling is relatively conserved in insects and SKs possess a conserved C-terminus and a diverse N-terminus, which can be implemented to define the spectrum of the nsSK pesticide, for instance, to be generally targeted or to be species-specific. Nevertheless, more details on the mechanism of SK signaling need to be revealed before the application.

In conclusion, the C-terminal hexapeptide (YGHMRF-NH_2) is predominant in the feeding-inhibitory activity of SK while the N-terminus is relatively tolerant to manipulation. Certain properties are important to specific positions such as the aromaticity and basicity. In addition,

nsSK is a potential pesticide given its simple structure and strong activity on inhibiting feeding. However, the diversity of tested peptides is not sufficient to come to a reliable conclusion on the SAR of SK on feeding, although it provides some clues which can direct further investigations.

Chapter IV. Development and application of a cell-based sulfakinin receptor bioassay

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Yu, N., Swevers, L., Nachman, R.J., Smagghe, G. (2014) Development of cell-based bioassay with Sf9 cells expressing TcSKR1 and TcSKR2 and differential activation by sSK and nsSK peptides. Peptides 53, 238-242.

1. Introduction

The silencing of *Tcskr2* dramatically altered food intake while the silencing of *Tcskr1* did not exert as strong the effect on food intake as the silencing of *Tcskr2* (Chapter II). However, food intake is a final outcome of a complex network of events triggered in an organism with many different layers of regulation. It is not known whether this complex event occurs as an intrinsic property of a single class of receptors or as the result of ligand binding to multiple populations of receptors present in the cells (Yule et al., 1993). It is of our interest to ascertain if the TcSKRs are capable of stimulating similar effects to that are seen in the whole insect and specifically to determine if a particular receptor is capable of differentially activating signal transduction systems on stimulation by different SK analogs.

SKRs are G-protein coupled receptors (GPCRs). GPCRs activate diverse signaling pathways via different types of Gα-protein. The two homologs of SKRs in mammals, namely CCK1R and CCK2R, are both reported to activate the PLC pathway by coupling to $G\alpha_q$-protein, and CCK1R is also coupled to $G\alpha_s$-protein in some cases (Dufresne et al., 2006; Yule et al., 1993). In contrast to mammals, there are few studies on signaling pathways related to SKs and SKRs in invertebrates. Given the high similarity in both structure and function, we expect that SKRs transduct signaling in a manner similar to the CCKRs.

SKs contain a consensus C-terminal hexapeptide YGHM/LRF-NH_2 (Table I-1). They exist in sulfated and nonsulfated forms depending on the sulfated group (SO_3H) on Y^6. Regarding the activation of SKR, sulfated *Drosophila* SK-I (sDSK-I) is about 3000-fold more potent than its nonsulfated counterpart to activate exogenously expressed CCKLR-17D3 (Kubiak et al., 2002) and only the sulfated form of DSK-I and DSK-II are functional ligands of CCKLR-17D1 (Chen et al., 2012). In *Ciona intestinalis*, sulfate moiety is present on the Y residues in the position 6 and 7 of cionin, [$Y^6(SO_3H)$]cionin and [$Y^7(SO_3H)$]cionin. To activate either CioR1 or CioR2, the potency of di-sulfated cionin is higher than mono-sulfated cionin; of the two mono-sulfated cionin, [$Y^7(SO_3H)$]cionin is more potent than [$Y^6(SO_3H)$]cionin; nonsulfated cionin is inactive (Sekiguchi et al., 2012). In mammals, sulfated CCK-8 activates CCK1R with a potency of 1000-fold more than nonsulfated CCK-8, while both forms of CCK-8 activate CCK2R with similar efficacy (Dufresne et al., 2006; Smeets et al., 1998).

In *T. castaneum*, a series of synthetic SK(-related) peptides was tested in adults for their effects on feeding, providing information on the structure-activity relationship (SAR) of SK *in*

vivo (Chapter III). Noticeably, the sulfated moiety of SK seemed to be not critical to the feeding-regulatory activity of SK in *T. castaneum*, whereas it is required in species such as *Schistocerca gregaria* (Wei et al., 2000). Therefore, it is interesting to evaluate the SK(-related) peptides for their activity with individual TcSKR, which will gain us more information on the SAR of SK.

In this chapter, a cell-based TcSKR bioassay was established with the two *T. castaneum* SKRs (TcSKR1 and TcSKR2, Chapter II) stably expressed in insect Sf9 cells. With this bioassay, 1) both sSK-II and nsSK-II were evaluated by their ability to activate the two individual TcSKRs, specifically aiming at the role of the sulfate moiety; 2) the type of Gα-protein involved in the SK signaling was determined. Here we focused on two potential signaling pathways, AC pathway and PLC pathway, in which $G\alpha_s$- and $G\alpha_q$-protein is involved, respectively. The transcription of reporter genes triggered by cAMP and/or calcium was monitored as indicators of the extent that the signaling pathway was activated; 3) a dose-response curve for sSK-II was calculated in terms of activating TcSKRs; 4) the structure-activity relationship of SK was investigated by evaluating the activity of SK-related peptides on TcSKRs.

2. Materials and methods

2.1. Peptide synthesis and preparation

Peptides listed in Table IV-1 were synthesized via FMOC methodology under previously described conditions (Nachman et al., 1986c). The identity of the peptides was confirmed via matrix-assisted laser desorption ionization (MALDI) time-of flight (TOF) mass spectrometry (MS) and quantified via amino acid analysis.

The peptides were prepared in stock solution of 80% acetonitrile (in water). Working solutions were freshly prepared with cell culture medium SF-900™ II SFM (Life Technologies) prior to each experiment.

2.2. Construction of TcSKR expression vectors

The open reading frames (ORFs) of the two *skr*s were cloned from *T. castaneum* cDNA with primers 5'-TTA*GGATCC*CACC**ATG**GGTATGAAGAGTTTTTTTACTG-3' (forward) and 5'-TTA*GGATCC*ATCGCTGCCGCTTCT -3' (reverse) for *Tcskr1* and

5'-TAT*GGATCC*CACC**ATG**GACTGGGCTGAAAAC-3' (forward) and 5'-CCG*GGATCC*TCTACAAAAGTCGGCATT-3' (reverse) for *Tcskr2*. The BamHI cloning site sequence (italicized) was introduced in the 5' end of both forward and reverse primers. In the forward primer, before the initiation codon ATG (bold), a Kozac initial sequence (underlined) was included. In the reverse primer, the BamHI cloning site immediately preceded the reverse complement sequence of the last codon of the ORFs and allowed in-frame cloning with the C-terminal Myc-His tag in the pEA-MycHis expression vector (Douris et al., 2006). PCRs were performed with the proofreading Platinum® *Pfx* DNA polymerase (Invitrogen) and products were verified by sequencing. PCR products were digested with FastDigest BamHI (Thermo Scientific) and then cloned to the pEA-MycHis vector to generate the pEA-TcSKR1-MycHis and pEA-TcSKR2-MycHis expression vectors.

Vector pEA-PAC was included in the experiment, providing the resistance to puromycin by the gene *pac* to select stably transfected cells. The reporter vectors pGL4.29-CRE and pGL4.30-NFAT-RE (containing cAMP- and calcium-activated response elements, respectively; Figure I-6b; Promega) were chosen for the signaling pathway detection.

2.3. Establishment of stable Sf9-TcSKR cell lines

Sf9 cells were routinely cultured in SF-900™ II SFM medium at 27 °C in darkness. For transfection, Sf9 cells were grown to 50-70% confluence in T75 flasks and then transferred to a 6-well tissue culture plate at a density of 1×10^5 cells/ml culture. The cells in 2 ml culture were then transfected with 2 µg of TcSKR expression construction and 200 ng of vector pEA-PAC using ESCORT™ IV transfection reagent (Sigma-Aldrich) for 6 h in serum-free medium. At 48 h post-transfection, cells were placed in medium containing 50 µg/ml of gentamycin and 20 µg/ml of puromycin. The puromycin-containing medium was replaced every 5 days until a stable colony was formed and proliferated.

To verify the expression of TcSKRs in transfected Sf9 cells, Western blotting was carried out with crude protein samples. The protein samples were collected as follows: cells were harvested 48 h post-transfection by centrifugation and supernatant was collected for detection of the secreted proteins; cell pellets were suspended with 100 µl phosphate buffered saline (PBS) and lysed by freezing at -70 °C for 15 min. Subsequently, the cell suspension was centrifuged at 12000 g for 15 min and both supernatant (soluble protein fraction) and pellet (insoluble protein fraction) were collected. The pellet was suspended in 100 µl PBS and

treated with sonication to avoid protein aggregation. Protein samples were separated by 4-12% NuPAGE® gel (Life Technologies) and bands were transferred to nitrocellulose membranes using an electrophoretic transfer system (Life Technologies). The antibodies were mouse anti-Myc and horseradish peroxidase (HRP)-conjugated anti-mouse used at 1:1000. DAB (3,3'-diaminobenzidine tetrahydrochloride) solution was used for detection.

2.4. TcSKR signaling pathway assay

Sf9, Sf9-TcSKR1 and Sf9-TcSKR2 cells were transiently transfected with 2 ug of reporter vectors pGL4.29-CRE or pGL4.30-NFAT-RE following the transfection procedure described in section 2.3. Hygromycin were added to the concentration of 200 μg/ml in order to select transfected cells. Cells were then harvested for further assay at 24 h post-transfection.

The transient cells (Sf9-CRE, Sf9-NFAT, Sf9-TcSKR1-CRE, Sf9-TcSKR1-NFAT, Sf9-TcSKR2-CRE and Sf9-TcSKR2-NFAT) were seeded at 2×10^4 cells per well in a white 96-well plate (Greiner Bio-One). After cells had attached to the well bottom overnight, the medium was replaced by assay medium containing sSK-II or nsSK-II. sSK-II was tested at concentrations from 0.01 nM to 10 μM and nsSK from 1 nM to 10 μM. Each concentration was tested in three replicates. Solvent acetonitrile at the same final concentration in the cell culture medium did not affect cell response. Two kinds of negative control were included. First, cell medium was used as a negative control reagent to give the background luminescence. Second, Sf9-CRE and Sf9-NFAT cells were used as a negative control to eliminate potential internal factors that were activated by SKs. Cells were incubated with SKs-containing medium or pure medium for 5 h at 27 °C and the Bright-Glo™ luciferase assay (Promega) was carried out according to the manufacturer's instruction. Per well, the light emission resulting from the luciferase activity was measured for 10 s using an Infinite® 200 PRO microplate reader (Tecan).

2.5. Screening of SK-related peptides on TcSKRs

Based on the work in section 2.4, activation of the TcSKRs required 10 μM of nsSK-II. Therefore, SK-related peptides were tested at a final concentration of 10 μM. All assays were run with three biological replicates, each contains three technical replicates. Forskolin (40 μM, Sigma-Aldrich) was included as a positive control. The assay was performed as mentioned in section 2.4 with the same two negative controls.

2.6. Data analysis

The mean luminescence value was calculated from three biological replicates. Data from Sf9-TcSKR cells were first compared to those from Sf9 cells by Student's *t*-test, resulting in the confirmation that response of Sf9-TcSKR cells were mainly from the expressed TcSKRs.

In the TcSKR signaling assay, the activation of TcSKRs was represented by the degree of luminescence increase, calculated with the following formula: % increase in luminescence = ($luminescence_{SK}$ - $luminescence_{control}$) / $luminescence_{control}$ × 100. Data from different concentrations of SKs were compared to that from control via Student's *t*-test in GraphPad Prism version 5.00.

Dose-response curves of sSK-II on TcSKRs were generated by the following procedures. The maximal activation of TcSKRs obtained from the highest concentration of sSK was assigned as 100%. The activation of TcSKRs from negative control was 0%. The percent of activation of receptor was plotted on the Y-axis and logarithm (concentration) on the X-axis in GraphPad Prism version 5.00. The median effect concentration (EC_{50}) together with the 95% confidence interval was calculated; the R^2 was used to evaluate the goodness of the sigmoid curve fitting.

In the screening of SK-related peptides, the activity of a peptide was represented as percentage of the nsSK-II activity calculated with the following formula: relative activity of peptide (% nsSK-II) = $activity_{peptide}$ / $activity_{nsSK\text{-}II}$ × 100. The peptide activity was evaluated by one-way analysis of variance with Dunnett's posttest in GraphPad Prism version 5.00.

3. Results

3.1. Establishment of stable Sf9-TcSKR cell lines

Western blotting was performed to verify the expression of TcSKRs in Sf9 cells. As shown in Figure IV-1(a and b), bands corresponding to the molecular weight of about 63 and 48 KDa were detected predominantly in the membrane (insoluble) sample from Sf9-TcSKR1 and Sf9-TcSKR2 cells, respectively, in accordance with the transmembrane property of the two TcSKRs. Sf9-TcSKR1 and Sf9-TcSKR2 cells were cultured stably every week in SF-900™ II SFM medium supplemented with 20 μg/ml of puromycin. After three passages, the two bands corresponding to the molecular weight of TcSKRs were still clearly detectable, indicating a

stable presence of the expressed proteins in the two respective transformed Sf9 cell lines. Finally, Figure IV-1c confirmed that the transformed Sf9 cells exhibited morphology similar to the native Sf9 cells.

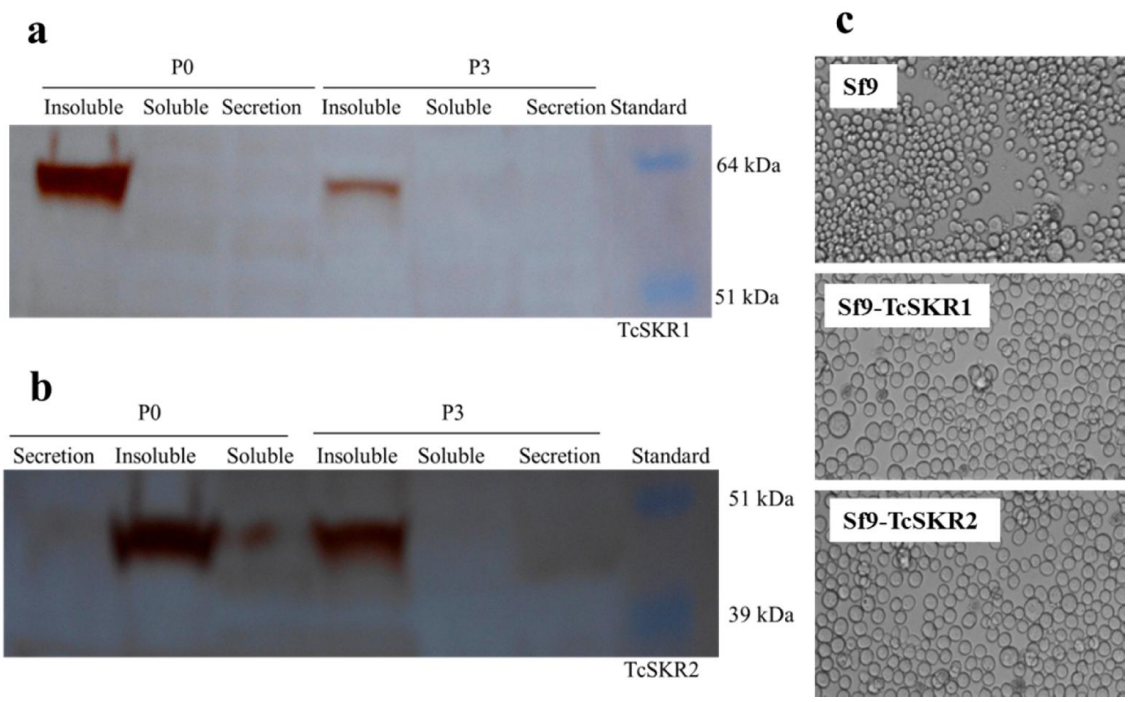


Figure IV-1 Western blotting analysis of the expression of TcSKR1 (a) and TcSKR2 (b) in stably transfected Sf9 cells, and the morphology of Sf9, Sf9-TcSKR1 and Sf9-TcSKR2 cells (c). Crude protein samples were collected from native Sf9 cells and transfected Sf9 cells at 48 h post-transfection (P0) and after three passages of culture (P3). Samples were separated as a secretion, a soluble protein fraction and an insoluble protein fraction. A pre-stained standard was used as a mass marker. Cell images were obtained three days after seeding with magnification of 200.

3.2. TcSKR1 and TcSKR2 were coupled to $G\alpha_s$-protein upon activation by SKs in Sf9 cells

In the TcSKR signaling assay, Sf9-TcSKR1 and Sf9-TcSKR2 cells were transfected with the reporter vectors containing CRE and NFAT-RE and then challenged with both sSK-II and nsSK-II. Only cells with CRE showed considerably high luciferase activity (Table IV-1) when challenged with sSK-II and nsSK-II. In these cells, luciferase transcription was triggered by response of CRE to the intracellular cAMP accumulation. On the other hand, cells with

NFAT-RE reporter did not react to either sSK-II or nsSK-II at tested concentrations of up to 10 μM, suggesting that the intracellular calcium was not the second messenger of the activated TcSKRs in Sf9 cells (data not shown). Taken together, the data suggested that, upon activation by SK-II, the two TcSKRs were coupled to $G\alpha_s$-protein and in turn this increased the intracellular cAMP level, which transmitted the signaling down inside the Sf9 cells.

3.3. sSK was more potent than nsSK to activate TcSKRs

sSK-II and nsSK-II were examined for their activity on activating the two TcSKRs at different concentrations ranging from the lowest (1 nM) to the highest concentration (10 μM) in the cell culture medium. As shown in Table IV-1, sSK-II significantly activated the two TcSKRs at a concentration of 1-10 nM, while the activation of TcSKRs required 1 μM or up to 10 μM of nsSK-II. The efficacy (% increase in luminescence) was also much lower for nsSK-II than for sSK-II (Table IV-1). While sSK-II treatment resulted in the detection of 400-2500 of luminescence units in the transformed cells, much lower values were observed for nsSK-II. Therefore, sSK-II was approximately 1000 to 10000 times more potent than nsSK-II to activate the two TcSKRs.

3.4. sSK activated TcSKRs in a dose-dependent manner

Dose-response curves were generated for sSK-II to activate TcSKR1 and TcSKR2 in the two transfected Sf9 cell lines (Figure IV-2). The EC_{50} was 1.6 nM (1.0-2.7 nM) for TcSKR1 and 5.4 nM (1.8-16.5 nM) for TcSKR2, with 95% confidence interval. The R^2 was 0.9691 and 0.8529 for curves in Figure IV-2a and Figure IV-2b, respectively.

Table IV-1 Activity of sulfated sulfakinin (sSK-II, a) and nonsulfated sulfakinin (nsSK-II, b) on the two expressed TcSKRs in Sf9 cells.

a

		% increase in luminescence*+			
	concentration, nM	**TcSKR1**	***P*-value**	**TcSKR2**	***P*-value**
sSK-II	1,000	2333 ± 89	< 0.0001	403 ± 35	0.0003
	100	2404 ± 63	< 0.0001	358 ± 12	< 0.0001
	10	1754 ± 108	< 0.0001	126 ± 14	0.0013
	1	822 ± 47	< 0.0001	5 ± 8	0.6218
control		0 ± 6		0 ± 6	

b

		% increase in luminescence*+			
	concentration, nM	**TcSKR1**	***P*-value**	**TcSKR2**	***P*-value**
nsSK-II	10,000	28 ± 4	0.0343	19 ± 2	0.0049
	1,000	6 ± 2	1.0000	15 ± 5	0.0541
	100	5 ± 1	0.5462	1 ± 4	0.8698
control		0 ± 7		0 ± 3	

***, % increase in luminescence = (luminescence$_{SK}$ - luminescence$_{control}$) / luminescence$_{control}$ × 100**

+, The effect of SKs on activating SKRs is reported as mean ± SEM (n=3) calculated as percent increase in luminescence with *P* values relative to control from the Student's *t*-test in GraphPad Prism version 5.00.

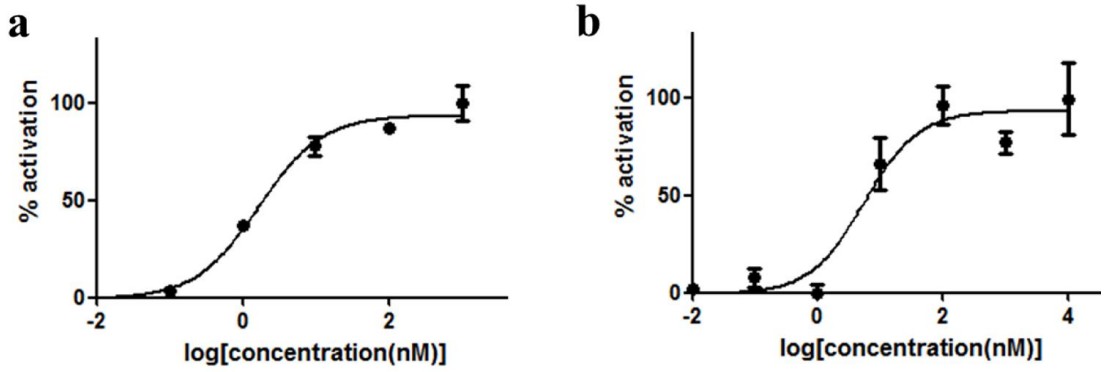


Figure IV-2 Dose-response curve of the activation of TcSKR1 (a) and TcSKR2 (b) when challenged with sSK-II in transformed Sf9 cells. The maximal activation of respective TcSKR was set as 100%. Cells in untreated cell culture medium were used to define the basal level of 0%. Data are presented as mean ± SEM (n=3).

3.5. Activity of SK-related peptides on TcSKRs

Sf9-TcSKR-CRE cells responded to cell medium to the same level as Sf9-CRE cells did, indicating that TcSKRs were not activated by the medium. Sf9-CRE cells responded to all tested peptides to the same level as the medium did, indicating that no significant internal element was activated by these peptides. Forskolin of 40 μM led to similar levels of cAMP production in Sf9-TcSKR1-CRE and Sf9-TcSKR2-CRE cells.

The relative activities of SK-related peptides at the concentration of 10 μM are shown in Table IV-2. The SAR of SK on TcSKRs was analyzed based on the data. A peptide is evaluated in two aspects: the amino acid content and the length.

3.5.1. TcSKR1 required stringent amino acid content

Single substitution of nsSK-II led to inactive peptides to SKR1 such as peptide 2003 ($F^1 \rightarrow A^1$), 2004 ($R^2 \rightarrow A^2$), 2005 ($M^3 \rightarrow A^3$), 2006 ($H^4 \rightarrow A^4$) and 1070 ($Y^6 \rightarrow Asu^6$). The four A-substituted nsSK-IIs lost their activity could be due to the simple structure of A, as amino acids F, R M and H all have a bigger side chain. The inactive peptide 1070 was likely to be a result of the loss of the aromatic ring of Y. Other substitutions resulted in peptides with retention of activity. These were peptides 2007 ($G^5 \rightarrow A^5$), 2008 ($Y^6 \rightarrow A^6$), 1835 ($F^9 \rightarrow S^9$), 1658 ($M^3 \rightarrow Nle^3$) and 1591-1 ($R^2 \rightarrow K^2$). These substitutions were mostly with amino acids of similar properties to certain extent. Therefore, the right property of amino acid content was important to TcSKR1.

The removal of amino acids from the N-teminus of nsSK-II led to a drastic loss of activity when more than two residues were missed from nsSK-II (Table IV-2). This suggested that the minimal length of nsSK-II for activating TcSKR1 was DYGHMRF-NH_2.

3.5.2. TcSKR2 required certain length of a peptide

nsSK-II lost its activity for TcSKR2 when certain substitution was introduced in peptides 2004 ($R^2 \rightarrow A^2$), 2006 ($H^4 \rightarrow A^4$), 2008 ($Y^6 \rightarrow A^6$), 1070 ($Y^6 \rightarrow Asu^6$) and 1591-1 ($R^2 \rightarrow K^2$). In contrast, nsSK-II kept its activity in peptides such as 2003 ($F^1 \rightarrow A^1$), 2005 ($M^3 \rightarrow A^3$), 2007 ($G^5 \rightarrow A^5$), 1835 ($F^9 \rightarrow S^9$) and 1658 ($M^3 \rightarrow Nle^3$). However, removing any amino acid from nsSK-II was fatal for the peptide, resulting in the drastic loss of activity. Therefore, for TcSKR2, the length of nsSK-II seemed to be more important than the exact amino acid content.

Table IV-2 Relative activity of SK(-related) peptide (10 µM) on activating TcSKR1 and TcSKR2 in Sf9 cells.

Group	Peptide	Sequence [a b]	TcSKR1			TcSKR2		
			relative activity [c]	difference from negative control [d]	difference from nsSK-II [d]	relative activity [c]	difference from negative control [d]	difference from nsSK-II [d]
SK	1010 (nsSK-II)	FDDYGHMRF-NH_2	100 ± 3	s		100 ± 26	s	
SK-Ala substitution	2003	FDDYGHMR**A**-NH_2	6 ± 1	ns	s	69 ± 18	s	ns
	2004	FDDYGHM**A**F-NH_2	4 ± 1	ns	s	48 ± 13	ns	ns
	2005	FDDYGH**A**RF-NH_2	4 ± 0	ns	s	71 ± 6	s	ns
	2006	FDDYG**A**MRF-NH_2	2 ± 2	ns	s	30 ± 25	ns	s
	2007	FDDY**A**HMRF-NH_2	80 ± 11	s	s	57 ± 14	s	ns
	2008	FDD**A**GHMRF-NH_2	42 ± 11	s	s	30 ± 9	ns	s
SK-truncation	2009	DDYGHMRF-NH_2	71 ± 1	s	s	-7 ± 12	ns	s
	2010	DYGHMRF-NH_2	21 ± 2	s	s	5 ± 2	ns	s
	2011	YGHMRF-NH_2	2 ± 0	ns	s	11 ± 15	ns	s
	2053	GHMRF-NH_2	2 ± 1	ns	s	17 ± 4	ns	s
	2052	HMRF-NH_2	2 ± 1	ns	s	25 ± 16	ns	s
	2051	MRF-NH_2	2 ± 2	ns	s	16 ± 14	ns	s
	2076	FDDYGHMR-NH_2	1 ± 2	ns	s	-4 ± 12	ns	s

(Table IV-2 continued)

Group	Peptide	Sequence [a b]	TcSKR1			TcSKR2		
			relative activity [c]	difference from negative control [d]	difference from nsSK-II [d]	relative activity [c]	difference from negative control [d]	difference from nsSK-II [d]
SK-related	1835	SDDYGHMRF-NH_2	61 ± 7	s	s	143 ± 17	s	s
	1070	FDD(**Asu**)GHMRF-NH_2	-2 ± 1	ns	s	-9 ± 2	ns	s
	1658	DDYGH(**Nle**)RF-NH_2	53 ± 1	s	s	109 ± 3	s	ns
	1591-1	EAYGH(**Nle**)**K**F-NH_2	57 ± 7	s	s	1 ± 7	ns	s
	1586	***cyclo***(EAYGH(**Nle**)**K**)F-NH_2	-4 ± 1	ns	s	-12 ± 1	ns	s
	1592	***cyclo***(EYGH(**Nle**)**K**)F-NH_2	-7 ± 1	ns	s	-16 ± 1	ns	s
C. elegans NLP-13	1679 (NLP-13a)	pQPSYDRDIMSF-NH_2	31 ± 5	s	s	5 ± 5	ns	s
	1432-2 (NLP-13b)	SPVDYDRPIMAF-NH_2	2 ± 2	ns	s	11 ± 9	ns	s
	1569	PVDYDRPIMAF-NH_2	-11 ± 5	ns	s	-17 ± 5	ns	s
	1567	SPVDYDRPIMF-NH_2	3 ± 6	ns	s	-4 ± 1	ns	s
	2018	***cyclo***(YDRPIMAF)	-6 ± 1	ns	s	-3 ± 1	ns	s
	2020	***cyclo***(RPIMAF)	-6 ± 2	ns	s	-6 ± 3	ns	s
C. elegans NLP-12	1678a (NLP-12a)	DYRPLQF-NH_2	40 ± 10	s	s	36 ± 8	ns	s
	1678b (NLP-12b)	DGYRPLQF-NH_2	18 ± 6	ns	s	-9 ± 6	ns	s

[a] Y , Y(SO_3H).

[b] Bold letters highlight the position of modification of the parent peptide. Asu: α-aminosuberic acid; Nle: Norleucine; cyclo: cyclic structure.

[c] Activity of a peptide is presented as percentage of the activity of nsSK-II (% nsSK-II) at the final concentration of 10 µM. Data are presented as mean ± SEM from three independent experiments.

[d] s, statistically significant difference; ns, no statistically significant difference. Statistical analysis was performed by using one-way analysis of variance with Dunnett's posttest in GraphPad Prism version 5.00.

3.5.3. TcSKR1 and TcSKR2 responded differently to certain amino acid substitution

Several peptides had different activities on TcSKR1 and TcSKR2 at the concentration of 10 µM. Peptide 2004 ($R^2 \rightarrow A^2$) was inactive on TcSKR1 but had 48% activity over TcSKR2. Peptide 1835 ($F^9 \rightarrow S^9$) was able to activate both TcSKRs, but with more activity on TcSKR2 (143%) than on TcSKR1 (61%). A similar case is peptide 1658 ($M^3 \rightarrow Nle^3$) showing more activity on TcSKR2 (109%) than TcSKR1 (53%). We speculated that a certain peptide could interact with the two TcSKRs in different ways, most likely due to the structural difference between the two TcSKRs.

3.5.4. The two TcSKRs responded to *C. elegans* NLP-13 and NLP-12 differently

The *C. elegans* NLP-13a and NLP-12a of 10 µM were able to activate TcSKR1 with 30-40% activity of nsSK-II, while they were inactive to TcSKR2. The other peptides derived from NLP-13 and NLP-12 could not elicit any response from both TcSKRs.

4. Discussion

Insect SKs are multifunctional neurohormones modulating various physiological processes such as the regulation of feeding. However, most studies were conducted at the organism level, resulting in an overall observation of the result of likely more than one process. There are only a few systematic studies on SK signaling in insects to our knowledge. Therefore, we established the stable cell lines expressing TcSKR with an eventual aim to investigate the *T. castaneum* SK signaling *in vitro*. Two aspects of the SK signaling were included here as the relevant Gα-protein and the SAR of SK to activate TcSKRs.

The study focused on $G\alpha_s$- and $G\alpha_q$-protein because they are the most likely Gα-proteins that

an activated SKR would be coupled to. The principle of this receptor assay is that the type of involved Gα-protein could be determined by measuring the luciferase activity which is a consequence of either intracellular cAMP accumulation or intracellular calcium mobilization. The luciferase activity is designed to be proportional to the change of cAMP and/or calcium, providing a way to quantify the relative affinity of different TcSKR modulators.

In the present experiment, the data suggest that both TcSKRs are coupled to $G\alpha_s$-protein upon activation because luciferase activity was only measured in the CRE-driven reporter system, where the intracellular cAMP was linked, regardless of the type and concentration of SK peptide. This is different from what is known about mammalian CCK receptors as the CCK1R couples both $G\alpha_s$-protein and $G\alpha_q$-protein, while CCK2R couples only with $G\alpha_q$-protein (Dufresne et al., 2006; Staljanssens et al., 2011). However, we are aware of the false-negative possibility due to the absence of a positive control in inducing intracellular calcium flux in Sf9 cells. The NFAT-RE reporter vector, as optimized for mammalian cells, might be not correctly functional in Sf9 cells. Because of this, the calcium flux induced by the activation of TcSKR could not be detected. In addition, there are two Trica-SK peptides from the *T. castaneum* SK prepropeptide (Chapter II) and only the Trica-SK-II was tested in this experiment. There is also the possibility that different mechanisms underlie the interaction of two Trica-SKs with TcSKRs.

The CRE-driven reporter gene system recruits the CRE binding protein (CREB) signaling to initiate the transcription of the reporter gene. Activated GPCR binds to the Gαs-protein, which activate the adenylate cyclase (AC) and increases the intracellular cAMP level. cAMP activates the protein kinase A (PKA), which then activates CREB by phosphorylating serine-133. Through interaction with its nuclear partner CREB binding protein (CBP), CREB drives the transcription of reporter gene. However, in some studies, forskolin, an activator of AC is not able to induce CRE-driven reporter gene, which leads to the hypothesis that a Ca^{2+}-induced elevation of the expression of the CRE-driven reporter gene in *Drosophila* S2 cells (Poels et al., 2004) or cAMP-dependent PKA signaling pathway is not sufficient to activate CREB in Bm12 cells (Yang et al., 2013). However, forskolin did cause the transcription of luciferase reporter gene in our present CRE-driven reporter system in Sf9 cells (Figure IV-S1). There is no information on the CREB in Sf9 cells by the thesis is being written. There are several possibilities for the cAMP-CREB pathway in Sf9 cells. First, inactive CREB exists in Sf9 cells. Forskolin or activated GPCR cause increased cAMP, which leads to PKA activating

CREB via phosphorylation. The active CREB binds to CRE and initiates the transcription of luciferase gene. Second, inactive CREB is present in Sf9 cells in a state in which certain amino acid residues are phosphorylated by protein kinase C. Forskolin or activated GPCR elevates the intracellular cAMP. The inactive CREB has no phosphorylation site for PKA, which means the inactive CREB does not react to the elevation of cAMP via PKA. cAMP induced a Ca^{2+} mobilization, resulting in the activation of Ca^{2+}-dependent calcineurin. Calcineurin activates CREB via dephosphorylation, leading to the binding of CREB to CRE and eventually athe transcription of luciferase gene (Yang et al., 2013). No matter which possibility, cAMP is the trigger of the CREB signaling pathway and the transcription of luciferase gene is proportional to the change of intracellular cAMP level.

SKs are processed by proteolysis of precursor protein and undergo post-translational modifications (Predel et al., 1999). Sulfation is a modification that introduces a sulfate group on Y^6. Similar to the CCKs in mammals, SKs occur naturally in both sulfated and nonsulfated forms in insects (Nichols, 2007; Nichols et al., 2008b). Many reports indicate that the sulfate group is necessary for activity. In mammals, CCK is 500- to 1000-fold more active than nonsulfated CCK (Dufresne et al., 2006). In *Drosophila*, a 3000-fold higher concentration of nsDSK than sDSK is required to stimulate the receptor (DSK-R1) expressed in mammalian cells (Kubiak et al., 2002). However, sDSK and nsDSK are reported to have different activities in odor preference and locomotion (Nichols, 2007; Nichols et al., 2008a; Nichols et al., 2008b), suggesting that distinct mechanisms may be involved.

In this study, the cell-based TcSKR bioassays showed that sSK-II was 1000 to 10000 times more potent than nsSK-II to activate the two TcSKRs. sSK-II activates both TcSKRs at a concentration of 1-10 nM, but at least 1 μM of nsSK-II is required to activate TcSKRs. In a Chinese Hamster Ovary (CHO) WAT11 cell-based TcSKR system, 1 nM of sSK was enough to reach the maximal activation of TcSKRs and 1 nM of nsSK showed activity, although at a lower level (Zels et al., 2014). The required active concentration of SK peptides on exogenously expressed TcSKRs differs most likely due to the sensitivity of the cell system. The CHO cell line expressing apoaequorin and $G\alpha_{16}$-protein is a mature system for the pharmacological characterization of GPCRs.

In contrast to the different activity on TcSKRs, sSK and nsSK elicited a similar food intake inhibitory activity when they were applied to *T. castaneum* adult *in vivo* (chapter III). The

discrepancy was also observed in two previous studies where the peptide SDNAMRF-NH_2 did not bind the expressed FMRF-NH_2 receptor protein, yet exhibited biological activity in *in vivo* semi-isolated heart rate assay (Maynard et al., 2013; Meeusen et al., 2002). For this disparity, the authors discussed the ligand concentration applied in bioassay and the expression condition of receptor proteins. To explain the discrepancy between the data obtained *in vitro* and *in vivo* in our research, we propose here that 1) the concentration of SK peptides injected in *T. castaneum* adults was about 16 μM, which is efficient for both sSK and nsSK to activate SKRs. Therefore, both sSK and nsSK could exert a similar and lower feeding response. A series of amounts of sSK and nsSK peptides could be tried in the future, which will reveal the threshold amount of peptide as well as the kinetic pattern; 2) two Trica-SK peptides are present in the *T. castaneum* SK prepropeptide (Chapter II) and only the Trica-SK-II was tested in this experiment; 3) moreover, different mechanisms might be recruited by the two Trica-SKs to interact with the TcSKRs.

Only with the one-receptor cell-based system, were we able to investigate the ligand-receptor interactions. The current cell-based TcSKR bioassay can be further used to screen SK-related peptides and mimetics to identify the SAR of SK. Therefore, a series of SK-related peptides with amino acid substitution or deletion was tested for their activities on TcSKRs, where some ideas on the SAR of SK can be obtained.

The minimal active length of nsSK-II is seven residues (DYGHMRF-NH_2) for TcSKR1 but nine residues (FDDYGHMRF-NH_2) for TcSKR2. Within the full length of nsSK-II, substitution of certain amino acid exerted different responses of TcSKR1 and TcSKR2. For example, peptides 2003 ($F^1 \rightarrow A^1$) and 2005 ($M^3 \rightarrow A^3$) were only active for TcSKR2, whereas 2008 ($Y^6 \rightarrow A^6$) and 1591-1 ($R^2 \rightarrow K^2$) were only active for TcSKR1. In contrast, peptides 2007 ($G^5 \rightarrow A^5$), 1835 ($F^9 \rightarrow S^9$) and 1658 ($M^3 \rightarrow Nle^3$) were active for both TcSKR1 and TcSKR2; 1070 ($Y^6 \rightarrow Asu^6$), 2006 ($H^4 \rightarrow A^4$) and 2004 ($R^2 \rightarrow A^2$) were inactive for either TcSKR1 or TcSKR2. These may reflect the different structural requirements of the two TcSKRs for the binding of ligand. Introduction of a cyclic structure led to inactive compounds (peptides 1586 and 1592), which adds values to the study on the structural requirements. However, peptide 1070 showed activity on both TcSKR1 and TcSKR2 at the concentration of 1 μM in Zels et al. (2014). Asu has been proposed a biostable mimic of Y(SO_3H) because the replacement retained activity (Nachman et al, 2005). sSK can exert the maximal activation of SKRs at the concentration of 100 nM. A possibility is that the

receptors were desensitized when they were exposed to 10 μM of 1070. The desensitization of receptor could happen to the peptides containing Y(SO_3H), such as 1569, 1567 and NLP-12s. Therefore, the activity of peptides should be evaluated with lower concentrations. Peptide 1591-1 was also only active on TcSKR1 but not TcSKR2 in Zels's work. In addition, peptide 1598-2 (EY(SO_3H)GH(Nle)KF-NH_2) was active on both TcSKR1 and TcSKR2. Together with peptide 1658, Nle proves to be a good mimic for M, which improves the peptide stability.

C. elegans NLP-12 peptides were recognized as SK homologs in nematodes due to their similarity in function (Janssen et al., 2008). Here, NLP-12a was only able to activate TcSKR1 with 40% activity of nsSK-II but not TcSKR2. NLP-12b had no activity on TcSKR1 and TcSKR2. NLP-12 differs from nsSK-II in amino acid sequence, though the –QF-NH_2 peptide NLP-12b may bind with the TcSKR receptor(s) but fail to activate it. In other words, it may act as an antagonist, an actiivty which has been observed in the feeding experiments in Chapter III. In those experiments a combination of NLP-12b with nsSK blocked the food intake inhibition activity of the latter. In future experiments, it would interesting to test NLP-12b against sSK on the two receptors to see if it might demonstrate an antagonist response. In addition, NLP-13-related peptides were inactive to TcSKRs because NLP-13 peptides contain quite different amino acid sequences from SK.

To compare with their parent peptide nsSK-II, peptides with Ala-substitution and truncation show similar activity as nsSK-II on inhibiting feeding *in vivo* (Chapter III), while some are inactive on neither TcSKR *in vitro*. This result is not to our surprise as we are aware that insect food intake is a complex physiological process employing more than one mechanism. *In vivo*, these nsSK-II analogs could be recognized by other receptors, processed into other active peptides or affect other feeding-regulation mechanism so that similar food intake regulation could be triggered.

One fact should be pointed out here is that the bioavailability of the peptide either in the insect haemolymph (Chapter III) or in the cell culture (this chapter) remains to be determined carefully. One cannot exclude the possibility that part of the peptides were degraded or modified in haemolymph or cell culture, although activities to various extents were observed in both experiments. Therefore, evaluation of the stability of peptides should be included in the follow-up experiments.

In a previous study (Chapter II), the silencing of *Tcskr2* significantly stimulated *T. castaneum* larval food intake, while silencing of *Tcskr1* resulted in less increase in food intake. Thus, we expect that TcSKR2 is more relevant in the SK signaling in food intake regulation, while TcSKR1 is likely to respond to other functions of SKs. Regarding to the response to sSK-II, TcSKR1 was more active than TcSKR2 (Table IV-1) although the EC_{50} values were similar (Figure IV-2). Factors such as the amount of the expressed TcSKR in the cells can influence the overall measured response of TcSKR to SK peptide. The comparison of the expression of the two TcSKRs in *T. castaneum* adult is not yet available at the protein level. Therefore, it is not conclusive to absolutely value the role of TcSKR1 and TcSKR2 in the regulation of feeding at this time.

To conclude this chapter, a cell-based TcSKR bioassay was established and used for the study on the SAR of SK. Assays on individual TcSKR reveals different structural requirements for the interaction of SK and TcSKR. To continue the study, more SK-related peptides and more concentrations can be introduced and a combination of assays *in vivo* and *in vitro* is necessary to complete.

Supplementary data

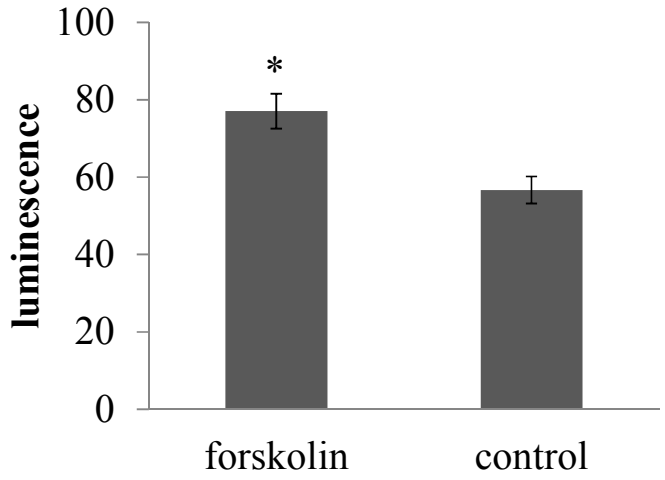


Figure IV-S1 Effect of forskolin (10 µM) on CRE-driven luciferase expression in Sf9 cells. Data are expressed as mean ± SME from three independent assays, each assay with three technical replicates. *, *P*=0.0234 from *t*-test between control sample and forskolin-treated sample in GraphPad Prism version 5.00.

Chapter V. Molecular modeling of sulfakinin receptors and docking of SK analogs to sulfakinin receptors

Manuscript in preparation:

Yu, N., Zotti, M. J., Nachman, R.J., Smagghe, G. (2014) Computational analysis of the structure-activity relationship of sulfakinin with bioactivity on activating sulfakinin receptors.

1. Introduction

The principle of structure-activity relationship (SAR) study is to understand how protein receptors recognize, interact and associate with molecular substrates and inhibitors. One approach to this goal is the *in silico* protein-ligand docking, which aims to predict and rank the structures arising from the association between a given ligand and a target receptor of a known three-dimensional (3D) structure (Sousa et al., 2006). Before a docking can be realized, the 3D structures of the potentially interactive molecules (receptor and ligand) should be known or predicted from existing information.

The most accurate structural characterization of proteins is provided by X-ray crystallography and NMR spectroscopy. However, the number of protein structures solved by experimental methods is so limited, because the technical difficulties and labor intensiveness of these methods (Wu and Zhang, 2009). Therefore, protein structure prediction (PSP, also called protein modeling) is widely employed to achieve the maximal approximation of the 3D structure of a target protein. Two main PSP methods are template-based prediction and free modeling (Figure V-1).

Historically, template-based methods can be categorized into two types, comparative modeling (CM) and threading. CM builds models based on the evolutionary information between target and template sequences, while threading is designed to match target sequences directly onto 3D structures of templates with the goal to detect target-template pairs even without evolutionary relationships. In recent years, as a general trend in the field, the borders between CM and threading are becoming increasingly blurred since both CM and threading methods rely on evolutionary relationships. For a given target sequence, template-based prediction methods build 3D structures based on a set of solved 3D protein structures, termed the template library. When structural analogs do not exist in the protein data bank (PDB) library or could not be detected by threading, the structure prediction has to be generated from scratch, where free modeling is applied.

Threading, also referred to fold recognition, predicts the 3D structure for a target protein by aligning its primary sequence to proteins in PDB in an attempt to find a similar folding (Xu et al., 2008) without regard to their degree of homology. The canonical procedure of template-based modeling consists of four steps (Figure V-1): 1) finding known structures (templates) related to the sequence to be modeled (target); 2) aligning the target sequence on the

template structures; 3) building the structural framework by copying the aligned regions, or by satisfying spatial restraints from templates; 4) constructing the unaligned loop regions and adding side-chain atoms. The first two steps are usually performed as a single procedure because the correct selection of templates relies on their accurate alignment with the target. Similarly, the last two steps are also performed simultaneously since the atoms of the core and loop regions interact closely.

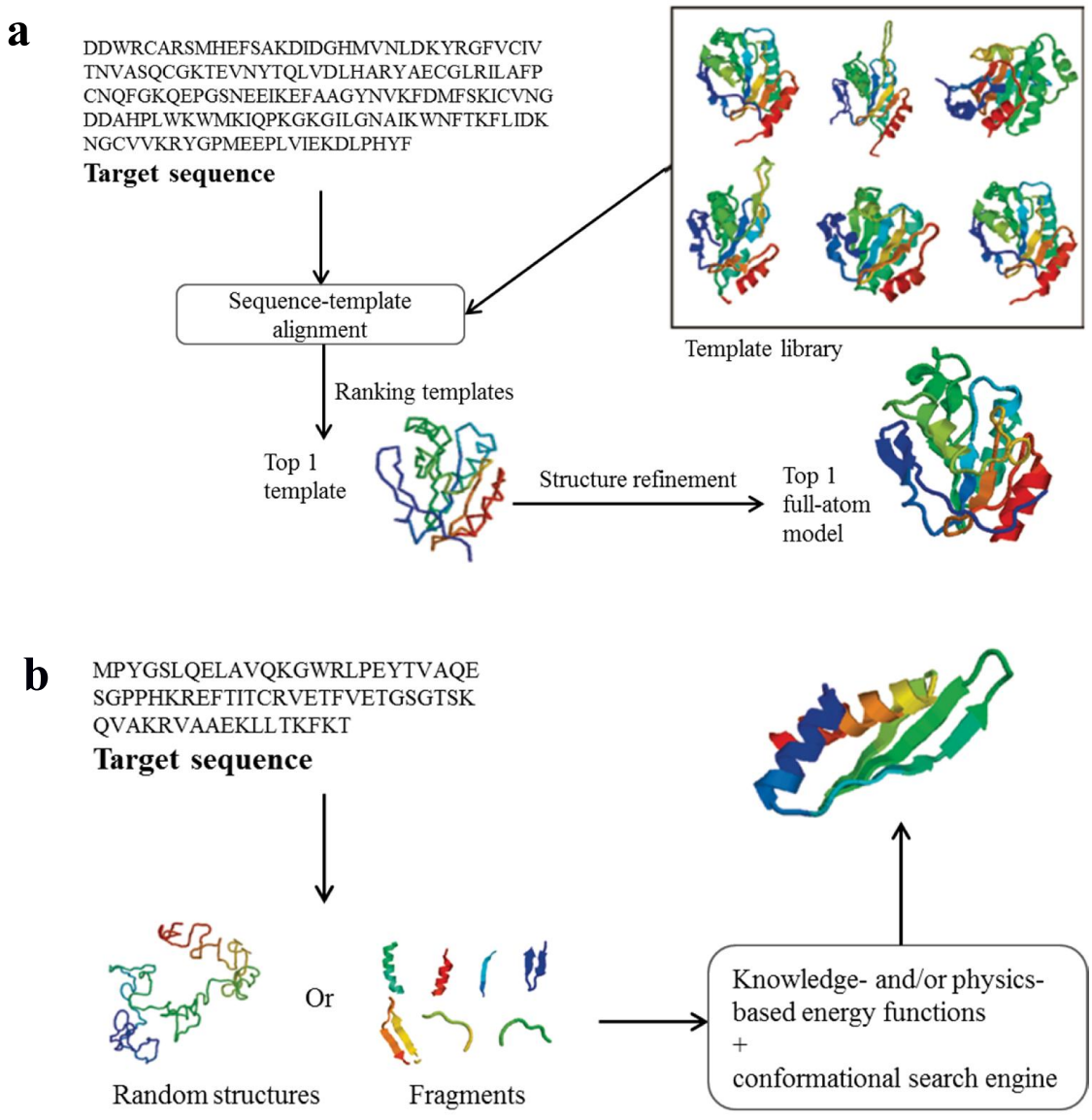


Figure V-1 Schematic overview of the methodologies employed in template-based modeling (a) and free modeling (b). (After Wu and Zhang, 2010)

In this chapter, the SAR of SK signaling was investigated through modeling. First, the 3D structures of the two *Tribolium castaneum* sulfakinin receptors (TcSKRs, TcSKR1 and TcSKR2) were predicted via protein modeling, where the structural similarity and deference between the two SKRs were on focus. Then, the interactive sites of TcSKRs and SK analogs were interpreted via protein-ligand modeling, with emphasis on the sulfate moiety. Lastly, the results from modeling and bioassay in previous chapters were combined and compared to further discuss the SAR of SK signaling.

2. Methods

2.1. Protein modeling

A strategy on protein threading philosophy (Figure V-2) was performed for the modeling of the two TcSKRs. Human CCKRs can not be the template, although they showed high homology to TcSKRs, because the crystal structures of human CCKRs are not available now. Therefore, the simple knowledge-based homology modeling was not applicable. In addition, the degree of identity and homology between TcSKRs and potential templates from protein data bank (PDB) were poor, which did not allow direct use of information from known structures to predict the structure of TcSKRs (Sander and Schneider, 1991).

Modeling of TcSKRs was performed using an approach combined both homology-based modeling and threading modeling. The structurally conserved regions (SCRs), loops, gaps and sidechains in TcSKRs were modeled sequentially and interactively on different templates. 1) Squid rhodopsin (PDB id=2z73) was picked up because it has the crystal structure available and hexhibits 35% and 38% similarity in the transmembrane regions to TcSKR1 and TcSKR2, respectively. However, the similarity between squid rhodopsin and TcSKRs is not ideal to modeling. Hence, *T. castaneum* rhodopsin was first modeled with squid rhodopsin as a template, because the identity and similarity between *T. castaneum* rhodopsin and squid rhodopsin is 33% and 59%, respectively, which is ideal to modeling. In addition, the two rhodopsins are both GPCRs and show functional relationships. 2) The templates for TcSKR modeling was searched using LOMETS (Local Meta Threading Server) (http://zhanglab.ccmb.med.umich.edu/; Wu and Zhang, 2007) with an eye to HOMSTRAD (Homologous Structure alignment database) (Mizuguchi et al., 1998). The *T. castaneum* rhodopsin was also included in the bulk of templates (Figure V-2). 3) The structurally conserved regions (SCRs) were built using ORCHESTRAR interface of SYBYL based on the

templates generated from LOMETS and the ranked structures by Z-score. The best folding for each query sequence was detected with the meta-server technology, instead of simply applying the models as generated by MODELLER in LOMETS. During this procedure several new searches were performed in LOMETS and then the detected structures were retrieved from PDB and implemented into the growing model. 4) Gaps and loops were selected from both FREAD and *ab initio* PETRA loop prediction, both available in ORCHESTRAR. FREAD is a loop database to select loop candidates from a protein structure fragments with environmentally constrained substitution tables. If the candidates are not good, the *ab initio* PETRA was used. 5) The model was optimized by a staged minimization and the stereochemical quality was evaluated utilizing the PROCHECK program (Laskowski et al., 1993). More than 90% and 89% of the residues of the modeled TcSKR1 and TcSKR2, respectively, were correctly assigned on the best allowed regions of the Ramachandran plot, and the remaining residues were located in the marginal regions of the plot (data not shown).

2.2. Protein-ligand docking

Rigid molecular docking of the SK analogs into TcSKRs was performed with OEDocking suite for OSX (version 3.0.1; OpenEye, Santa Fe, NM) and flexible docking using FlexiDock from SYBYL. For the SK analogs (Table V-1), several conformations were generated for each peptide with local minimum energies using OMEGA (version 2.5.1.4; OpenEye, Santa Fe, NM; Hawkins, P.C.D. et al., 2010) force field mmff94 and root-mean-square-deviation (RMSD) set as 1.0. These conformers were docked separately in the TcSKR1 and TcSKR2 extramembrane regions using FRED interface of OEDocking. In this first run of docking, the protein was kept rigid while an exhaustive search was applied for peptides. The exhaustive search systematically searched rotations and translations of each peptide conformer within the selected region.

The generated docking poses were ranked by each of the four individual scores (shape, hydrogen bound, protein desolvation and ligand desolvation). The sum of the normalized scoring functions generated the FRED Chemgauss4 score. The best-ranked pose of each peptide was inspected for steric hindrance. Those protein residues in close contact with the ligand or considered in potential for clashes were assigned as flexible for a new run of docking using FlexiDock. This last procedure was performed individually and continually for each peptide until the absence of clashes (Figure V-2).

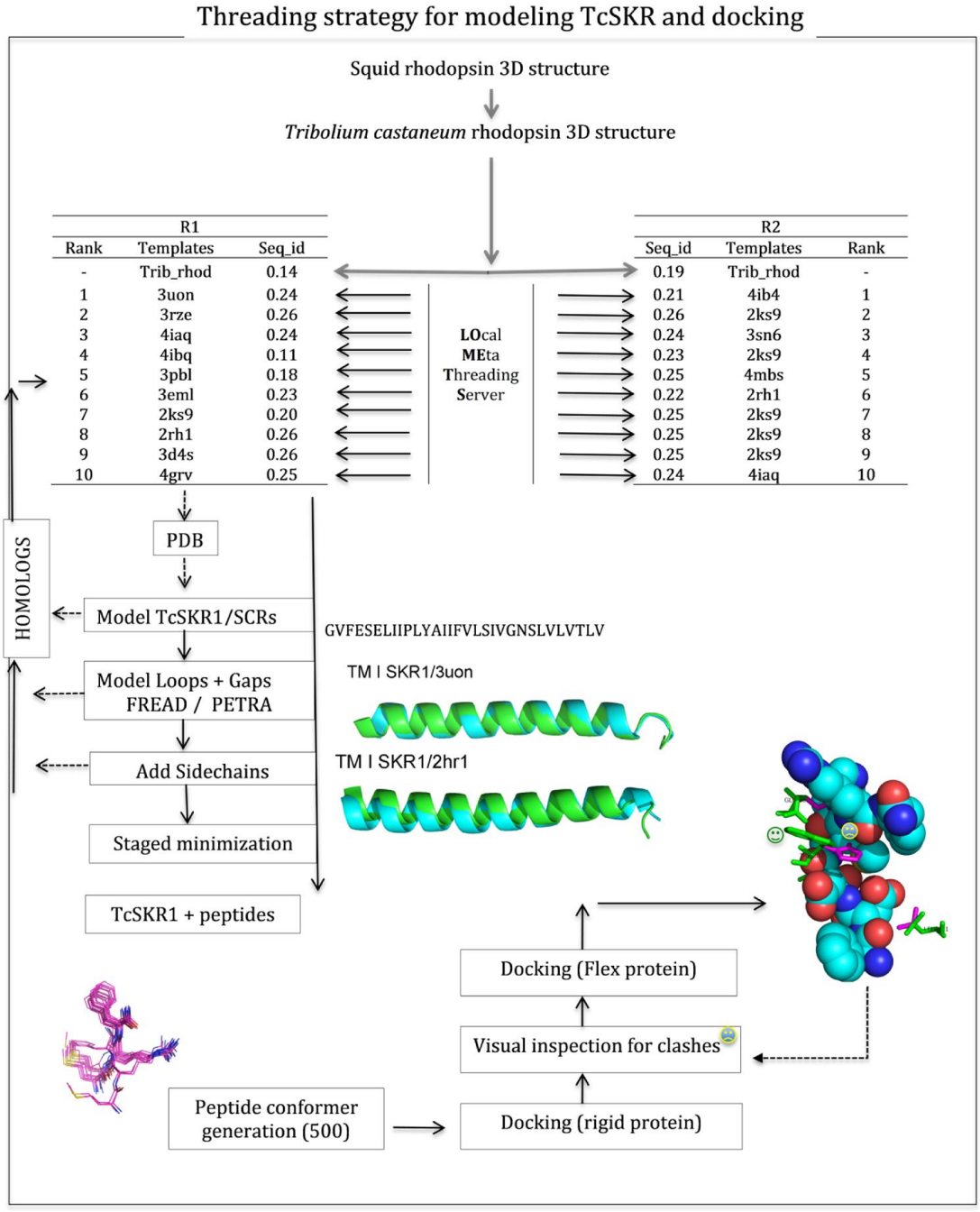


Figure V-2 Threading strategy used in the modeling of TcSKRs and the docking of peptides. For easy view, only the steps applied for TcSKR1 are depicted. The strategy applied for TcSKR2 is similar. The smile face indicates a successful docking while a sad face indicates an unsuccessful docking. The W207 marked in pink caused a clash by a deep intrusion of its indole group into peptide volume. To solve this problem, the residue was made flexible (depicted in green) during the docking. This procedure was performed for all peptides as well as for all residues causing clashes until no visible clash

was found. The α-helices represent the transmembrane I from TcSKR1 superimposed with counterpart region of templates.

3. Results

3.1. TcSKR1 and TcSKR2 contained cavities with different outer opening

The TcSKR1 and TcSKR2 both consisted of the canonical seven α-helices crossing the cell membrane as commonly found in GPCRs (Figure V-3). Both TcSKRs exhibited three extracellular loops (ELs) with similar number of residues except EL 2 with 14 residues in TcSKR1 and 37 residues in TcSKR2. The structures showed a long C-terminal region composed of 84 and 46 residues in TcSKR1 and TcSKR2, respectively. TcSKR1 shows eight intracellular small α-helices and two β-sheets with no counterpart structure in SKR2.

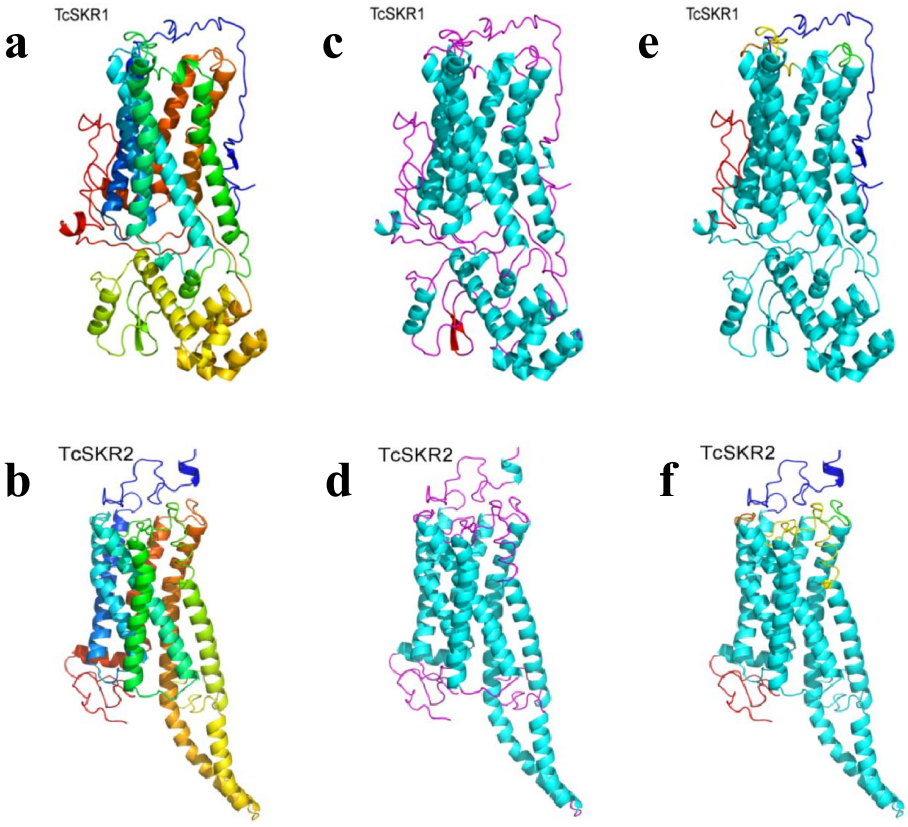


Figure V-3 Cartoon diagram of *Triboilum casteneum* sulfakinin receptors, TcSKR1 and TcSKR2. The seven transmembrane α-helices building the three-dimensional fold of the proteins are differently colored from blue (N-terminus) to red (C-terminus) (a and b) by secondary structure (c and d) and with extracellular loops (EL) colored differently (e

and f). The EL 1 is in orange, EL 2 in yellow and EL 3 in green. The N-terminus is in blue while C-terminus red.

Figure V-4 depicts the largest channel found in each modeled receptor that was calculated by MOLCAD. The cavities of both TcSKRs were similar in deepness. However, TcSKR2 had a narrow outer opening that prevented a deep intrusion of ligands into protein volume. In contrast, TcSKR1 exhibited a similar cavity in depth but with a much larger outer opening than TcSKR2. This larger opening allowed peptides to be docked deeper into the cavity, differently from what occurred in TcSKR2 where peptide could not be docked into protein volume. Therefore, all peptides were laid on the top of receptor in TcSKR2-ligand complexes, while in TcSKR1-ligand complexes, peptides were posed in the receptor cavity. The consequence of these 'lay-docked' poses in TcSKR2-peptide complex was a close contact with the extracellular loops and N-terminus region.

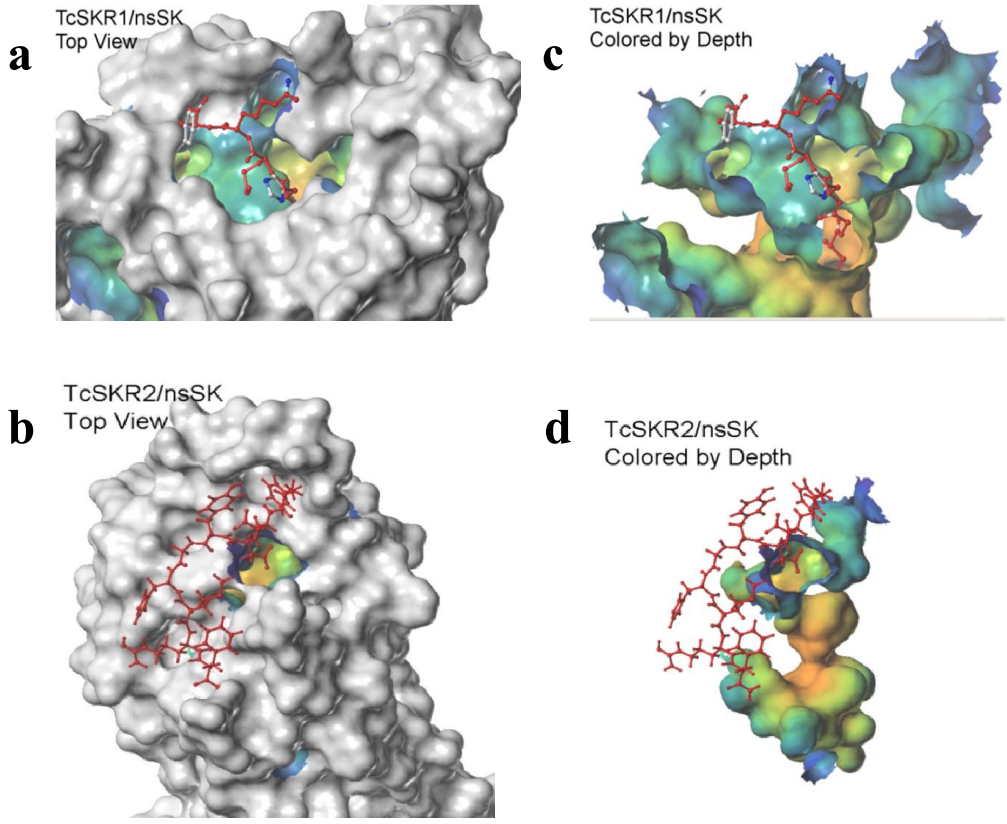


Figure V-4 Surface diagram top view of the 3D structure of TcSKR1 (a) and TcSKR2 (b) with nsSK peptide docked. Cavity colored by depth in TcSKR1 (c) and TcSKR2 (d). The picture demonstrates a deep cavity in both structures. The cavity depth is colored from blue (shallow) to orange (deep).

Table V-1 and *, amino acid is represented with the single-letter code. The following numerical indicates the position from the N-terminus of peptide or protein.

+, relative bioactivity was collected from Table IV-2, relative to the activity of nsSK-II.
&, TM, transmembrane region; EL, extracellular loop.

Table V-2 summarize the amino acid residues involved in the binding of TcSKRs and SK analogs. More information is available in Table V-s1. The interactive residues in receptors were found in both the extracellular region and transmembrane (TM) region in TcSKR1 while they were only located in the extracellular region in TcSKR2 (Table V-1; Table V-2; Figure V-5). For these peptides, TM regions were not involved in the ligand-TcSKR interaction.

Table V-1 Residues in TcSKR1 and TcSKR2 involved in the binding of SKs through polar interactions.

Peptide	Sequence	Amino acid*	TcSKR1			TcSKR2		
			Region &	Amino acid*	Relative bioactivity+	Region &	Amino acid*	Relative bioactivity+
sSK	FDDY(SO_3H) GHMRF-NH_2	F1	N-terminus	R41	>4000	EL 2	S178	>800
		H4	EL 2	E205				
		Y6	TM V	D221		N-terminus	D2	
			TM VI	H432		N-terminus	L9	
		D7	TM V	N217		N-terminus	T8	
		D8	TM V	N217				
nsSK	FDDYGHMR F-NH_2	F1	TM III	Q132	100	EL 2	L177	100
			EL 2	R199				
		H4	TM V	Q214		EL 1	E109	
			EL 3	Y438		EL 2	K181	
		Y6				N-terminus	L9	
		D7				EL 2	S196	

***, amino acid is represented with the single-letter code. The following numerical indicates the position from the N-terminus of peptide or protein.**
+, relative bioactivity was collected from Table IV-2, relative to the activity of nsSK-II.
&, TM, transmembrane region; EL, extracellular loop.

Table V-2 Residues in TcSKR1 and TcSKR2 involved in binding of Ala-nsSK peptides through polar interactions.

Peptide	Sequence	Amino acid*	TcSKR1			TcSKR2		
			Region &	Amino acid*	Relative bioactivity+	Region &	Amino acid*	Relative bioactivity+
2003	FDDYGHMR	A1	N-terminus	K39	6	EL2	L177	69

	$A\text{-}NH_2$		N-terminus	T40			
			TM VII	S451			
		R2	N-terminus	K39			
			EL 1	N117			
			EL 2	R204			
		H4			EL 1	E109	
					EL 2	K181	

(*, amino acid is represented with the single-letter code. The following numerical indicates the position from the N-terminus of peptide or protein.

+, relative bioactivity was collected from Table IV-2, relative to the activity of nsSK-II.

&, TM, transmembrane region; EL, extracellular loop.

Table V-2 continued)

Peptide	Sequence	Amino acid*	TcSKR1			TcSKR2		
			Region &	Amino acid*	Relative bioactivity+	Region &	Amino acid*	Relative bioactivity+
2003	FDDYGHMR $A\text{-}NH_2$	G5	TM VII	Q458				
		Y6				N-terminus	L9	
		D7	TM V	N217		N-terminus	T8	
						EL 2	S196	
		F9				N-terminus	D2	
2004	FDDYGHMA $F\text{-}NH_2$	H4			4	EL 1	E109	48
		G5				N-terminus	W10	
						EL 2	D198	
		D7	TM VII	S455		EL 2	S196	
			TM VII	S458				
		D8	EL 2	E205		N-terminus	T8	
			TM VII	S451				
		F9	EL 1	N117		N-terminus	T8	
			TM IV	S184		N-terminus	T22	
			EL 2	R204				
2005	FDDYGHAR $F\text{-}NH_2$	F1	EL 2	E205	4			71
		R2	EL 2	E205		N-terminus	W10	
						N-terminus	T22	
		H4	EL 2	E206		N-terminus	T8	
		Y6	TM V	N217		EL 3	S342	
		D7				EL 2	S196	
						EL 2	L197	
2006	FDDYGAMR $F\text{-}NH_2$	R2	TM V	E213	2	EL 2	E109	30
						EL 2	L180	
		Y6				N-terminus	L9	
		D7	TM V	N217		N-terminus	T8	
		D8	TM III	P129				
		F9	TM V	N217		EL 2	L177	
2007	FDDYAHMR $F\text{-}NH_2$	R2	TM VII	A461	80			57
		H4	TM VII	Q458		EL 2	K181	

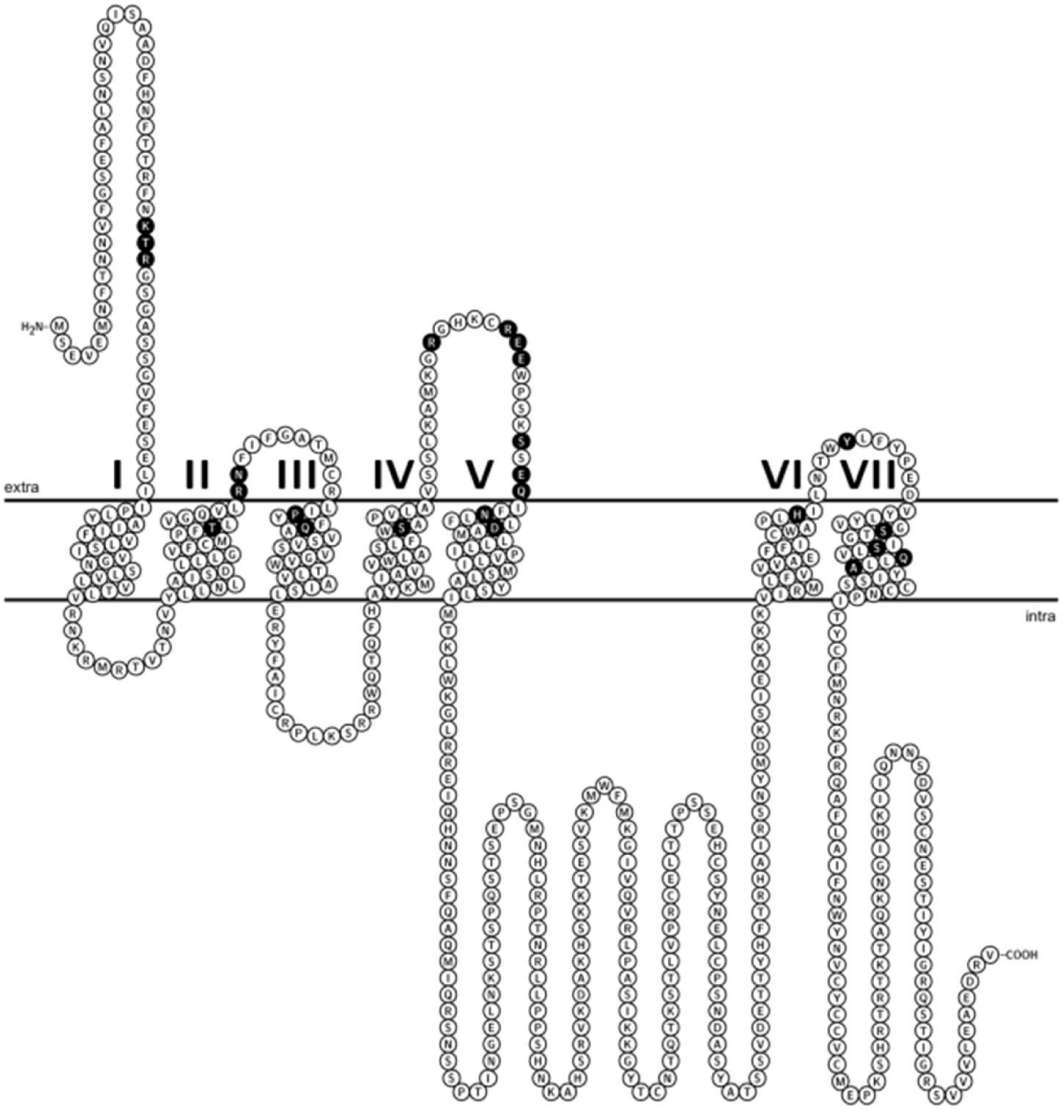
						EL 2	D198	
		D7				N-terminus	N11	
		F9				N-terminus	N18	
2008	FDDAGHMR F-NH_2	F1 H4 G5	EL 2 EL 1 TM II	S211 R116 T109	42			30

***, amino acid is represented with the single-letter code. The following numerical indicates the position from the N-terminus of peptide or protein.**

+, relative bioactivity was collected from Table IV-2, relative to the activity of nsSK-II.

&, TM, transmembrane region; EL, extracellular loop.

a



(Figure V-5)

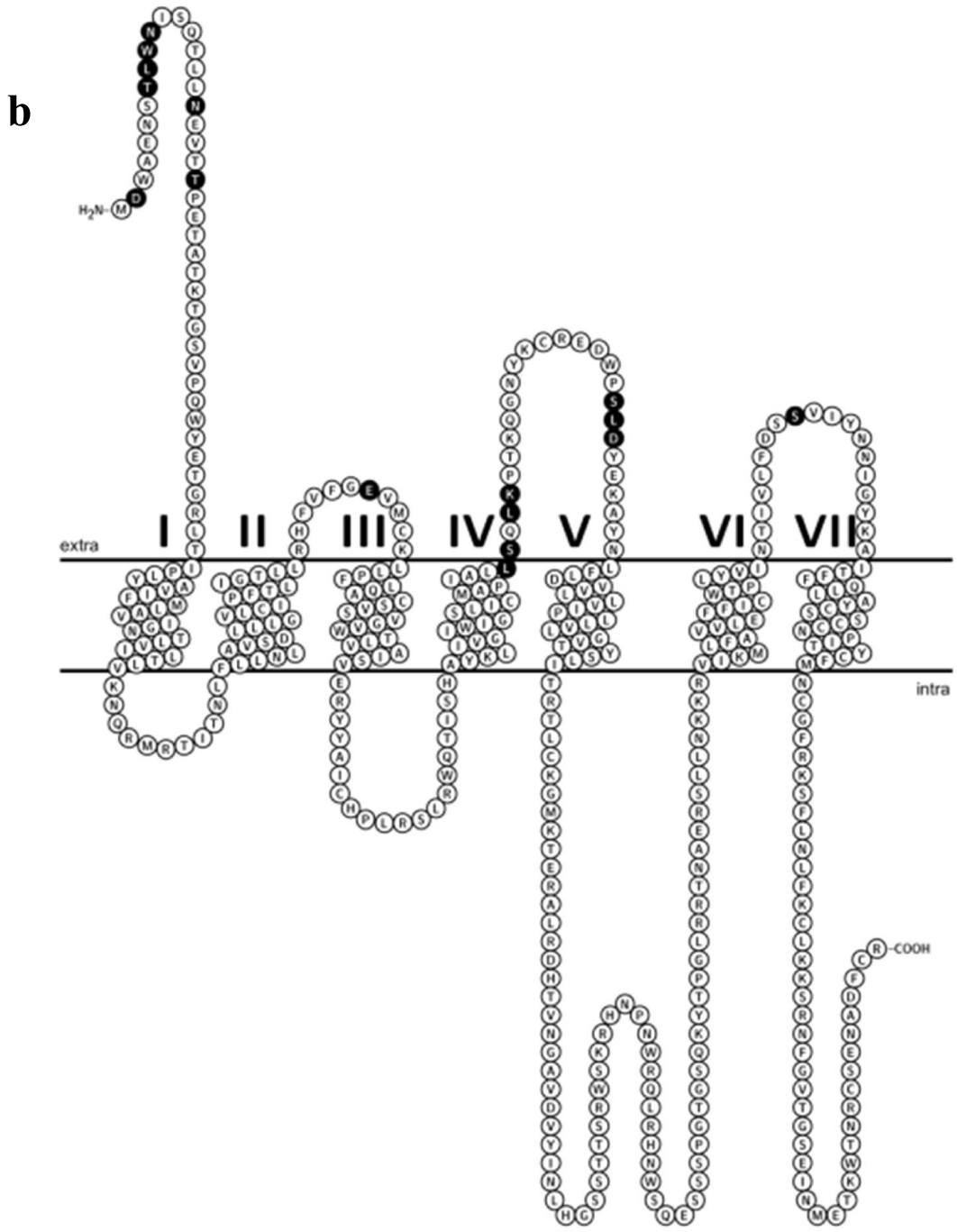


Figure V-5 Residues involved in binding of SK analogs in TcSKR1 (a) and TcSKR2 (b). The residues were collected for the binding of TcSKRs with sSK, nsSK and Ala-substituted SK in Table V-1 and Table V-2. The residues are represented with white single-letter codes in black filled circles. Figures were generated with Protter v.1.0.

3.2. Sulfate moiety affected the binding of SK to SKR

Sulfated SK (sSK) and nonsulfated SK (nsSK) were docked in both TcSKR1 and TcSKR2. The interactive residues from both peptide and receptor are summarized in Table V-1. First, in the TcSKR1-SK complexes, 5 sSK residues interacted with 5 TcSKR1 residues but only 2 nsSK residues interacted with 4 TcSKR1 residues. In the TcSKR2-SK complexes, 3 and 4 residues in sSK and nsSK, respectively, interacted with 4 and 5 TcSKR2 residues. Second, in the TcSKR1-SK complexes, the sulfated Y^6 in sSK was involved but not the nonsulfated Y^6 in nsSK. In contrast, in the TcSKR2-SK complexes, the Y^6 was interactive in the binding to TcSKR2 regardless the presence of sulfate group. Lastly, as a general observation, the interactive residues were found in only the extracellular regions in TcSKR2 but both the extracellular regions and the TM regions in TcSKR1.

3.3. Peptides with Ala-substitution were posed differently to SKR1 and SKR2

The peptides with Ala-substitution of nsSK were docked to both TcSKRs. The interactive residues are presented in Table V-2. A general finding was that these peptides made contact with TcSKR1 differently from how nsSK did, whereas they interacted with TcSKR2 in a similar manner as nsSK did. nsSK bound to TcSKR1 via the EL 2 and the TM III, V and VII. Peptides 2003-2008 bound to TcSKR1 via different regions spanning from the N-terminus to the TM VII. nsSK bound to TcSKR2 via the N-terminus, the EL1 and the EL 2. Peptides 2003-2008 bound to TcSKR2 via the same regions and the EL 3 was also recruited by peptides 2005 and 2006.

In terms of bioactivity, for the activation of TcSKR1, peptides 2003-2006 were inactive and peptides 2007-2008 were active. But it is not conclusive to explain the bioactivity simply from docking result, because all these peptides interacted with a wide range of regions in TcSKR1. However, for the case of TcSKR2, all peptides retained the bioactivity and they contacted with the N-terminus, EL 1 and EL 2 of TcSKR2 in the docking.

4. Discussion

The modeling of the two TcSKRs revealed their structural characteristics for the first time and confirmed their property of being GPCRs. Very interesting is the difference of the outer opening between TcSKR1 and TcSKR2. By far, different results were obtained from the two

TcSKRs assays such as RNAi (Chapter II) and cell-based receptor activation (Chapter IV). The detailed information on their 3D structures will provide clues to interpret their potential different roles.

In the previous experiment, 1 nM of sSK was sufficient to activate TcSKR1 while the activation of TcSKR2 required 10 nM of sSK (Table IV-1), although the EC_{50} value was similar for both TcSKRs (Figure IV-2). The structural property of the TcSKR-sSK complex provides some clues to decipher this observation. The predicted TcSKR1 model exhibits a deeper cavity compared to TcSKR2 model, which allows intrusion and binding of peptides.

As discussed in previous chapters, sulfate moiety on Y^6 has been reported to be important for the activity of SK, which was also observed in the cell-based receptor activation assay (Chapter IV), but not in the feeding assay (Chapter III). We expected to gain more information at the molecular level through modeling and docking. Based on docking data, the (SO_3H) on Y^6 enhanced the binding of sSK and TcSKR1 via two TM residues (D221 in TM V and H432 in TM VII), which contributes to the 1000 to 10000 times more activity of sSK than nsSK (Table IV-1). Conversely, Y^6 in both sSK and nsSK made similar contact with TcSKR2, with the common interactive residue L9 in the N-terminus. In the TcSKR activation bioassay, SKR1 was more sensitive to the sulfate group (Table IV-1). Therefore, sulfate moiety may work as a switch for the activation of TcSKR1 but is less effective to activate TcSKR2.

The docking results showed some insights to explain the observed bioactivity of peptides, for example, the lack of activity in the Ala-substitution series. The amidated F^1 of nsSK interacted with R199 in the EL 2 of TcSKR1. In peptide 2003, this F^1 is replaced by A^1. This modification generated more polar contact with peptide 2003, however, the hydrogen bond formed with R199 was lost. Archer-Lahoul et al. (2005) conducted several site-directed mutation experiments in CCKR1 coupled with docking of CCK. The replacement of R197 (equivalent to R199 in TcSKR1) by M197 caused a 3154-fold reduction in the affinity of CCK to CCK1R. Therefore, we suggest that the lack of interaction with this particular amino acid could abolish the binding affinity of peptide 2003 to TcSKR1. In the remaining Ala-substituted peptides (2004, 2005, 2006, 2007 and 2008) a higher number of hydrogen bounds were found but no one interacting with the Ala seemed crucial for receptor activation (Table V-2).

The Ala-substitution caused a drastic reduction in the peptide activity on TcSKR1 but to a much less extent on TcSRK2 (Table V-2; Chapter VI). The same peptide was docked in TcSKR1 and TcSKR2 in a different manner (Figure V-4), which makes it difficult to correlate the biological activity and the interactive points, as the same peptides probably activate different receptors by making different interactions. Despite of this difficulty, overall, peptides with Ala-substitution made more interactions with the EL regions in TcSKR2 than they did in TcSKR1. The increased interaction of peptides with the EL regions of TcSKR2 seems to be involved in diminishing the impact of Ala-substitution, resulting in less loss of activity of SK peptide on TcSKR2 than on TcSKR1. nsSK bound TcSKR2 via the N-terminus, the EL 1 and the EL 2. We speculate that these three regions are important to the activation of TcSKR2. In addition, the peptide 2009 only interacted with TcSKR2 via the N-terminus and it showed no bioactivity (Table V-s1), which is supportive of the speculation.

Molecular modeling and docking are used to provide substantial structural knowledge about receptor-ligand complexes, from which functional information could be inferred (Smith and Sternberg, 2002). However, modeling and docking are still approximation and optimism. It is difficult to reach the near-native complex because many factors are excluded or optimized in the approach. In addition, the scoring and ranking for the best candidate is not completely objective, which relies very much on the existing information, such as available template and functional motifs. The development of modeling and docking is directed towards the introduction of more parameters. Together with the increasing experimental information, modeling and docking will expand our knowledge of the interaction of SK and SKRs.

In conclusion, the modeling of TcSKRs and the docking of peptides to TcSKRs are indicative to the SAR of SK signaling, although we are aware of the limitations of the approach. TcSKR1 and TcSKR2 display similar characteristics of GPCR but have different outer opening of the cavity, which affects the binding of TcSKRs with ligand peptide. The sulfate moiety contributes to the higher activity of sSK than nsSK by enhancing the binding of sSK to TcSKR1. Also, the diverse bioactivity of peptides with Ala-substitution could be due to the structural differences between the two TcSKRs.

Supplementary data

Table V-s1. Summary of residues in TcSKR1 and TcSKR2 involved in binding of peptides through polar interactions.

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
sSK	FDDY(SO_3H)GHMRF-NH_2	N-term	ARG41	F/O	>4000	N-term	ASP2/OD2	Y/O1H	>800
		EL 2	GLU205/OE1	H/ND1		N-term	THR8/H01	D**D**/OD1	
		TM V	ASN217/ND2	**D**D/O		N-term	LEU9/O	Y/N	
		TM V	ASN217/ND2	D**D**/OD1		EL 2	SER178/O	F/NH2	
		TM V	ASP221/OD2	Y /O3					
		TM VI	HIS432/HD1	Y /O1H					
nsSK	FD**D**YGHMRF-NH_2	TM III	GLN132/0	F/N	100	N-term	LEU9/O	Y/N	100
		EL2	ARG199/O	F/NH2		EL 1	GLU109/OE2	H/ND1	
		TM V	GLN214/OE1	H/ND1		EL 2	LEU177/O	R**F**/NH2	
		TM VI	TYR438/OH	H/N		EL 2	LYS181/H01	H/O	
						EL 2	SER196/H01	D**D**/OD1	

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
2003	FDDYGHMRA-NH_2	N-term	LYS39/0	R/NE	6	N-term	ASP2/OD2	F/NH2	69
		N-term	LYS39/H	A/0		N-term	THR8/H01	D**D**/OD1	
		N-term	LYS40/H	A/0		N-term	LEU9/O	Y/N	
		EL 1	ASN117/OD1	R/NH2		EL 1	GLU109/OE2	H/ND1	
		EL 2	ARG204/O	R/O		EL 2	LEU177/O	A/NH2	
		TM V	ASN217/HD21	D**D**/OD1		EL 2	LYS181/H01	H/O	
		TM VII	SER451/H	A/N		EL 2	SER196/H01	D**D**/OD2	
		TM VII	SER451/OG	A/O					
		TM VII	GLN458/HE21	G/0					
2004	F**DD**YGHMAF-NH_2	EL 1	ASN117/OD1	F/HT1	4	N-term	THR8/OG1	**D**D/NH	48
		TM IV	SER184/O	F/NH2		N-term	THR8/O	F/NH	
		TM IV	SER184/OG	F/NH2		N-term	TRP10/H07	G/O	
		EL 2	ARG204/O	F/HT2		N-term	THR22/OG1	FNH2/O	
		EL 2	GLU205/OE2	**D**D/N		EL 1	GLU109/OE2	H/ND1	
		TM VII	SER451/HG	**D**D/OD1		EL 2	SER196/H01	D**D**/O	
		TM VII	SER455/OG	D**D**/OD2		EL 2	ASP198/OD1	G/N	
		TM VII	GLN458/HE22	D**D**/OD2					

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
2005	FD**D**YGHARF-NH_2	EL 2	GLU205/OE2	F/N	4	N-term	THR8/O	H/N	71
		EL 2	GLU205/OE1	F/NH2		N-term	TRP10/H07	R/O	
		EL 2	GLU205/OE2	R/NH1		N-term	THR22/H01	R/NH1	
		EL 2	GLU206/O	H/ND1		EL 2	SER196/H01	D**D**/OD1	
		TM V	ASN217	Y/O		EL 2	LEU197/H02	D**D**/OD2	
						EL 3	SER342/CA	Y/O	
						EL 3	SER342/OG	Y/NH	
2006	FDDYGAMRF-NH_2	TM III	PRO129/O	**D**D/OD2	2	N-term	THR8/H01	D**D**/OD1	30
		TM V	GLU213/OE2	R/NH2		N-term	LEU9/O	Y/N	
		TM V	ASN217/OD1	F/NH2		EL 1	GLU109/O	R/H2N	
		TM V	ASN217/HD21	D**D**/O		EL 2	LEU177/O	**F**/NH2	
						EL 2	LEU180/O	R/NE	
2007	**F**DDYAHMRF-NH_2	TM VII	GLN458/HE22	H/O	80	N-term	ASN11/H02	D**D**/OD1	57
		TM VII	ALA461/O	R/NH2		N-term	ASN18/H01	**F**D/O	
						EL 2	LYS181/H08	H/NE2	
						EL 2	ASP198/OD2	H/ND1	
2008	FDDAGHMRF-NH_2	TM II	THR109/OG1	G/N	42				30
		EL1	ARG116/O	H/ND1					
		TM V	SER211/OG	F-NH2					

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
2009	DDYGHMRF-NH_2	TM III	GLN132/HE22	H/O	71	N-term	GLU5/OE2	F/NH2	-7
		EL 2	GLU205/OE1	**D**D/OD2		N-term	THR8/H01	H/O	
		EL 2	GLU206/O	G/N		N-term	LEU9/O	G/N	
		TM VII	HIS432/HD1	F/O		N-term	ASN18/H05	Y/OH	
						N-term	THR22/OG1	D**D**/N	
2010	DYGHMRF-NH_2	EL 2	GLU205/OE2	F/N	21				5
		EL 2	GLU205/OE1	F-NH2					
		EL 2	GLU205/OE2	R/NH1					
		EL 2	GLU206/O	H/ND1					
		TM V	ASN217/HD21	Y/O					
2011	YGHMRF-NH_2	EL 2	ARG204/O	F/NH2	2				11
		EL 2	GLU205/OE2	F/NH2					
		EL 2	GLU205/OE2	F/N					
		EL 2	GLU205/OE1	R/NH1					
		TM V	ASN217/HD21	Y/O					
		TM V	ASN217/O	Y/N					
		TM VI	ASN435/OD1	Y/N					

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
2053	GHMRF-NH_2	EL 2	GLU205/OE2	F/HN2	2	EL 3	SER341/H01	F/O	17
		EL 2	GLU205/OE2	F/N		EL 3	SER341/O	R/H2N	
		TM VI	TYR438	R/NH2		EL 3	SER341/O	R/NE	
						EL 3	SER342/H0	F/O	
2052	HMRF-NH_2	EL 2	GLU206/O	R/NH2	2				25
		TM V	ASN217/HD21	H/O					
		TM V	ASN217/O	H/N					
		TM VI	ASN435/OD1	H/N					
		TM VII	GLN458/OE1	R/NH					
		TM VII	GLN458/O	F/NH2					
		TM VII	GLN458/OE1	R/NE					
2051	MRF-NH_2	TM VII	GLN458/OE1	R/NH2	2	EL 1	GLU109/OE1	F/NN2	16
		TM VII	GLN458/OE1	R/NH		EL 1	GLU109/O	F/NH2	
		TM VII	GLN458/O	F/NH2		EL 2	LEU177/O	R/NH2	
2076	FDDYGHMR-NH_2	EL 2	GLU206/O	Y/N	1				-4
		TM VII	GLN458/HE21	Y/O					
1070	FDDAGHMRF-NH_2	EL 2	GLU206/OE2	F/HT3	-2				-9
		TM V	ASN217/HD21	G/O					

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
1658	DDY(SO_3H)GH(NIe)R F-NH_2	EL 2	ARG204/HE	Y/O2	53				109
		EL 2	GLU205/OE1	Y/O1H					
		EL 2	GLU206/O	G/HN					
		TM III	GLN132/HE22	H/O					
		TM VII	SER451/HB3	**D**D/HN					
		TM VII	GLN458/HE22	D**D**/OD1					
		TM VII	ALA461/O	R/NH2					
		TM VII	SER465/OG	R/NH2					
1591-1	EAY(SO_3H)GH(NIe)K F-NH_2	EL 1	ARG116/O	Y/O1H	57				1
		TM III	GLN132/HE22	H/O					
		EL 2	GLU206/O	G/NH					
		TM VII	SER451/OG	E/HT3					
		TM VII	ALA461/O	K/HZ1					
		TM VII	TYR462/O	K/HZ1					
		TM VII	SER465/OG	K/HZ2					

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
1432-2	SPVDYDRPIMAF-NH_2	EL 1	ARG116/O	R/NH1	2				11
		TM V	SER211/O	F/NH2					
		TM V	SER211/OG	Y/OH					
		TM V	SER212/O	V/N					
		TM V	GLN214/H	Y/OH					
		TM V	ASN217/OD1	F/NH2					
		TM VI	TYR438/HH	D/OD1					
		TM VII	GLN458/HE21	I/0					
1567	SPVDY(SO_3H)DRPIM F-NH_2	N-Tterm	THR40/OG1	R/NH1	3				-4
		TM III	ARG126/HH21	Y/O3					
		EL 2	GLY200/H	Y/O1					
		EL 2	TRP207/HE1	D/O					
		TM V	ASN217/HD21	P/O					
		TM VI	ASN435/HD21	R/O					

(Table V-s1 continued)

Peptides	AA seq*	TcSKR1 region	TcSKR1 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay	TcSKR2 region	TcSKR2 AA	Peptide AA interaction**	Relative activity (% nsSK) in bioassay
sNLP-12b	DGY(SO_3H)RPLQF-NH_2	N-term	ANS38/HD21	Y/OH					
		EL 1	ARG116/O	R/NE					
		EL 1	ARG116/O	F/NH					
		EL 2	GLU205/OE2	R/NH2					
		TM V	GLN214/OE1	D/NH2					
		TM V	ASN217/HD22	Q/OD1					
		EL 3	GLU443/OE2	Y/O3					
sNLP-12a	DY(SO_3H)RPLQF-NH_2	TM V	SER211/O	Y/O3					
		TM V	ASN217/OD1	D/N					
		TM VII	SER451/O	Q/NH2					
		TM VII	GLN458/HE21	L/O					
NLP-12b	DGYRPLQF-NH_2	N-Term	LYS39/HZ3	Y/O					
		N-Term	SER46/O	D/NH2					
		TM II	GLN113/O	D/N					
		TM III	GLN132/HE22	Q/OD1					
		EL 2	ARG204/O	R/NE					
		EL 2	GLU205/OE2	R/NH2					
		TM V	ASN217/OD1	F/NH2					

***Underlined residues refer to those interacting with peptides.**

**** In the case where the same amino acid occurs twice (e.g. DD), the interaction amino acid is bold and underlined.**

Chapter VI. General conclusions and future perspectives

1. General conclusions

1.1. Sulfakinin signaling inhibits feeding in *Tribolium castaneum*

Insect sulfakinins are structurally and functionally homologous to the vertebrate cholecystokinin (CCK), one of the neuropeptides that can reduce appetite. SKs have been reported to be involved in multiple physiological processes including inhibiting feeding in a few species. However, not much is known about SK signaling, which consists of SK peptide and SK receptors in insects, in terms of function and mechanism in the regulation of feeding in the red flour beetle *Tribolium castaneum*.

First, genes encoding one SK prepropeptide and two SKRs (SKR1 and SKR2) were cloned and characterized from *T. castaneum*. The *Tcsk* and *Tcskr1* were upregulated when the supply of food was poor, suggesting that SK signaling is related to the regulation of feeding.

Next, the function of SK signaling in the regulation of feeding was revealed by the dsRNA-mediated gene silencing. Individual silencing of *Tcsk* and *Tcskr2* resulted in a dramatic increase in the larval food intake of *T. castaneum*. This observation indicates that SK signaling inhibits feeding in *T. castaneum*. In addition, direct injection of a synthetic sulfated SK peptide exerted a drastic reduction on the larval food intake by enhancing the SK signaling, supporting that SK signaling inhibits feeding in *T. castaneum*.

Remarkably, the silencing of *Tcskr1* did not exert an effect as strong as that of silencing of *Tcsk* or *Tcskr2*, suggesting that TcSKR2 functions predominantly in the feeding-inhibiting function. However, other possibilities cannot be excluded, such as the turnover of TcSKR1, the functional threshold concentration of TcSKR1 and the compensating mechanisms to the loss of TcSKR1.

1.2. Mechanism of SK in feeding remains to be determined due to the complexity of the regulation of feeding

To study the mechanism of SK signaling, structure-activity relationship (SAR) study was carried out in combination of *in vivo* and *in vitro* experiments. SK-related peptides were synthesized with either substitution or deletion of amino acids compared to the native SK peptide. The effect of these peptides on feeding was examined by injecting peptides *in vivo*; the affinity of these peptides to the individual SKRs was examined in a cell-based receptor

system *in vitro*. Some results are consistent in both experiments while the others require further investigation.

Particularly, nsSK-II exerted a similar inhibitory effect as sSK-II on feeding *in vivo*, suggesting that the sulfate moiety is not essential for the regulation of feeding in *T. castaneum*. This is in contrast to the previous studies in desert locust (Wei et al., 2000) and cockroach (Maestro et al., 2001), where nsSK showed no activity on inhibiting feeding. *In vitro*, sSK-II activated TcSKR1 and TcSKR2 at concentrations of 1 nM and 10 nM, respectively, while nsSK-II was only active at a concentration of 10 μM. Data reported by Zels et al. (2014) on the two TcSKRs did however show differences with our results as some activity for nsSK at 1 uM was observed. Nevertheless, sulfate moiety is proven to be important for SK-II to activate TcSKRs. This is consistent with previous studies on *Drosophila* SKRs (Chen et al., 2012; Kubiak et al., 2002). Only one, relatively large dose was applied to compare the SK analogs in the *in vivo* (16 μM) and *in vitro* (10 μM) experiments, which does not distinguish between peptides that have widely divergent potencies. Therefore, the nsSK injected *in vivo* might be at a concentration high enough to activate the SKRs and cause an effect similar to sSK.

Most of SK-related peptides retained the activity on inhibiting feeding compared to SK-II, but most of these peptides lost the activity on TcSKRs. The activation of TcSKR1 required DYGHMRF-NH_2 with strict match for amino acid content, especially the tetrapeptide HMRF-NH_2. The activation of TcSKR2 required the entire length of FDDYGHMRF-NH_2 but was tolerant to C-terminal substitution and the length of nsSK-II seemed to be more important than the exact amino acid content. Cyclic peptides were inactive in our assay at 10 uM. However, Zels et al. (2014) reported significant activity of cyclic peptides on both receptors at 1 uM, a possible indication that the higher concentrations led to desensitization of the receptors. Regardless, the conformational interaction between SK-II analogs and TcSKRs is important.

The *C. elegans* NLP-13 peptides showed similar activity as nsSK-II on feeding, although they only share the Y, M^3 and F^1. However, NLP-13 peptides showed low affinity to TcSKRs, probably because the rest of the C-terminal core differed too much from the insect SKs to reach a response level comparable to YGHMRF-NH_2 (Zels et al., 2014). *C. elegans* NLP-12 peptides, especially the NLP-12b, exhibited an opposite activity to nsSK-II by increasing feeding, although NLP-12 did not affect the food intake in nematodes (Janssen et al., 2008).

NLP-12 peptides were not as active as SKs, suggesting that they may act on other receptors. This requires further studies.

The mechanism of SK signaling on feeding is not conclusive because the complex situation inside the insect body and the non-optimized receptor system. Many factors can lead to the discrepancies between experiments *in vivo* and *in vitro*. For instance, it is not clear whether these SK analogs were further processed *in vivo*, and what influence these modifications will make on the activity of peptides. In addition, the potency of a peptide can not be determined based on one dose.

1.3. Two TcSKRs are predicted to interact with ligand differently due to their distinct structures

In order to investigate the interaction of SK and TcSKRs at the molecular level, *in silico* modeling of TcSKRs and docking of SK analogs were carried out. Both TcSKRs were predicted to exhibit structural characteristics of GPCRs with seven transmembrane domains. However, the cavities built with the seven transmembrane α-helices in the predicted models of the two TcSKRs are with different outer opening. TcSKR1 model possesses a wider outer opening allowing peptides to be docked deeper into the cavity, which is not the case in TcSKR2 model. As a consequence, the extracellular region and the transmembrane region in TcSKR1 can be involved in the binding of ligand while only the extracellular region in TcSKR2 is involved in the contact with ligand. Therefore, the two TcSKRs likely display a different affinity to various SK and SK analogs.

The model of SK-SKR complex predicted that the sulfate moiety in sSK contributes to the high activity of sSK to SKR. The peptides with the Ala-substitution of SK were modeled differently to interact with the two TcSKRs. In general, peptides with Ala-substitution make more interactions with the extracellular regions in TcSKR2 than they do in TcSKR1. The increased interaction of peptides with the extracellular regions of TcSKR2 seems to be involved in diminishing the impact of Ala-substitution, resulting in less loss of the activity of SK peptide on TcSKR2 than on TcSKR1 *in vitro*, although it is not conclusive to simply correlate the docking data with the bioactivity.

The combination of data from feeding (*in vivo*), cell-based receptor activation (*in vitro*), and modeling and docking (*in silico*) provides some ideas on the mechanism of SK signaling.

First, the sulfate moiety on Y^6 in SK is important for the activation of TcSKRs given that sSK is at least 1000-fold more potent than nsSK. But, it is not sure yet what occurs to nsSK in the organism, because similar activity of nsSK and sSK was observed on inhibiting feeding in *T. castaneum*, although at a relatively high concentration. Second, the C-terminal tetrapeptide HMRF-NH_2 is important for SK, although YGHMRF-NH_2 is more potent. Third, the distinct outer opening of the cavity in the model of TcSKR1 and TcSKR2 offers a different binding affinity to peptides.

2. Future perspectives

2.1. Evolution of sulfakinin(-like) signaling in Metazoa

Sulfakinin (SK) and its homologs and their receptors are widely spread in metazoans, but the knowledge we have of their evolutionary relationships remains unclear. To advance future study on the SK(-like) signaling, several topics can be discussed.

First, the consensus nomenclature and classification of peptides and receptors could be further clarified. Coast and Schooley (2011) brought forward a consensus nomenclature for insect neuropeptides. The receptors should also be classified especially when there is more than one receptor. By then, the identification and study of SK signaling will be less confusing.

Second, the information on the presence or absence of SK signaling can be complemented. The SKR or SK signaling system is missing in many insect species (Chapter I). Absence of SK signaling or the components in some insects could be due to the incomplete search or annotation in their genomes. For instance, SK peptides or SK precursor genes have been identified in several Crustaceans, but the SKR gene has only been found in the water flea *Daphnia pulex* (Johnsen et al., 2000; Torfs et al., 2002). Determining the presence of the SK signaling is a precondition to understand the function and evolution of SK signaling among animals.

Third, the evolutionary relationship of SK(-like) signaling within Metazoa can be further studied. Generally, SK signaling is a conserved neuropeptidergic signaling system among Metazoa. SK(-like) peptides with their receptors have been recognized as coevolving in lineages to human, nematodes and arthropods (Mirabeau and Joly, 2013; Taghert and Nitabach, 2012). The accumulating genome information among Metazoa offers a good

opportunity to conduct the evolutionary study in a comprehensively comparative way. For example, phylogenetic analyses will promote the recognition of the origin of SK(-like) signaling and the gene duplication/deletion event during evolution; research on the neighboring genes and rearrangements of chromosomal regions will also obtain some clues for the evolution of SK(-like) signaling.

2.2. Mechanism of SK signaling

The mechanism of SK signaling can be further investigated as a continuation of this research.

First, the cell-based TcSKR system can be optimized for a sensitive performance. The properness of the CRE- and NFAT-driven reporter system should be examined carefully. For example, the upperstream factors in the CRE binding protein signaling pathway in Sf9 cells should be present in order to directly link the change of the intracellular cAMP level to the transcription of reporter gene. Change of the intracellular calcium mobilization was not detectable in the present study, which could be due to either no intracellular calcium flux induced by the activation of TcSKR or the dysfunction of the reporter vector in insect cells. Therefore, it is important to find out the reason. For example, a positive control, where the calcium mobilization is increased, can be included to determine if the reporter vector functions properly in insect cells. In addition, the TcA cell line derived from *T. castaneum* provides another option for host cell line. But the naturally occurring TcSKR in TcA cells should be taken into consideration before the cells are employed.

Second, more molecules involved in the SK signaling could be determined with the improved precision of the assay system. Among these molecules, Gα-proteins are the most important. This study only focused on $G\alpha_s$-and $G\alpha_q$-proteins, while other types of Gα-protein can be studied in the follow-up research.

Third, the interaction between SK and TcSKRs is also interesting to explore. On one hand, the active core of SK to activate TcSKRs can be determined by screening more peptides with single or multiple amino acid substitution or deletion. On the other hand, the specific residues in TcSKRs that interact with SK can be studied by mutating the exogenously expressed TcSKRs. The studies are most likely to be performed in cell-based systems. Therefore, researchers should be aware of the limitations of *in vitro* study and to combine the data obtained *in vitro* and *in vivo* if possible.

2.3. Application of SK signaling in pest control

The regulation of feeding is essential for an organism. Understanding of regulation of feeding by the SK signaling in pests such as *T. castaneum* provides a potential pest control strategy. For instance, the active core of SK can be formulated to become a reagent that inhibits feeding in pests, which will eventually disturb the development of target pest. nsSK-II exhibits similar activity as sSK-II in the present study when they are applied at a relatively high concentration, although the exact role and mechanism of the two forms of SK in SK signaling is not clear yet. nsSK seems to be a practical candidate of potential pesticide, because it is a short peptide and that it does not have the sulfate group, making the synthesis and modification of peptide easier and more cost effective than sSK. In addition, because SK signaling is conserved in insects, it may be useful for controlling a group of pests; while specificity can be obtained after more efforts such as figuring out the diverse N-terminal extensions of SK peptides and the species-specific interaction between SK and SKR.

Many neuropeptides are involved in the regulation of feeding via stimulatory or inhibitory activity. Knowledge is hardly complete on these neuropeptides, for example, how these neuropeptide signaling pathways function and interact with each other. To obtain more information, researchers can apply similar methodologies as to the study of SK signaling. Also, new techniques will be developed at the same time, which can be utilized to improve the preciseness and sensitivity.

In conclusion, the current study on SK signaling in *T. castaneum* provides new primary information on its function in the regulation of feeding. The whole picture of SK signaling or even neuropeptide networks in the regulation of feeding could be completed with continuous effort.

References

Aguilar, R., Maestro, J.L., Vilaplana, L., Chiva, C., Andreu, D., Bellés, X., 2004. Identification of leucomyosuppressin in the German cockroach, *Blattella germanica*, as an inhibitor of food intake. Regulatory peptides 119, 105-112.

Aguilar, R., Maestro, J.L., Vilaplana, L., Pascual, N., Piulachs, M.D., Bellés, X., 2003. Allatostatin gene expression in brain and midgut, and activity of synthetic allatostatins on feeding-related processes in the cockroach *Blattella germanica*. Regulatory peptides 115, 171-177.

Ahmed, T., Frey, J.U., 2005. Plasticity-specific phosphorylation of CaMKII, MAP-kinases and CREB during late-LTP in rat hippocampal slices in vitro. Neuropharmacology 49, 477-492.

Alvarado, A.S., Newmark, P.A., 1999. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proceedings of the national academy of sciences 96, 5049-5054.

Amare, A., Sweedler, J.V., 2007. Neuropeptide precursors in *Tribolium castaneum*. Peptides 28, 1282-1291.

Antonio, D.S.M., Guidugli-Lazzarini, K.R., Do Nascimento, A.M., Simões, Z.L.P., Hartfelder, K., 2008. RNAi-mediated silencing of vitellogenin gene function turns honeybee (*Apis mellifera*) workers into extremely precocious foragers. Naturwissenschaften 95, 953-961.

Arakane, Y., Li, B., Muthukrishnan, S., Beeman, R.W., Kramer, K.J., Park, Y., 2008. Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle, *Tribolium castaneum*. Mechanisms of development 125, 984-995.

Arakane, Y., Muthukrishnan, S., Beeman, R.W., Kanost, M.R., Kramer, K.J., 2005. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proceedings of the national academy of sciences of the united states of america 102, 11337-11342.

Aronstein, K., Pankiwi, T., Saldivar, E., 2006. SID-I is implicated in systemic gene silencing in the honey bee. Journal of apicultural research 45, 20-24.

Arthur, F., 2010. Residual efficacy of aerosols to control *Tribolium castaneum* and *Tribolium confusum*. Julius-Kühn-Archiv, S. 789.

Arthur, F.H., Puterka, G.J., 2002. Evaluation of kaolinite-based particle films to control *Tribolium* species (Coleoptera: Tenebrionidae). Journal of stored products research 38, 341-348.

Audsley, N., Matthews, H., Down, R.E., Weaver, R.J., 2011. Neuropeptides associated with the central nervous system of the cabbage root fly, *Delia radicum* (L). Peptides 32, 434-440.

Audsley, N., Weaver, R., 2009. Neuropeptides associated with the regulation of feeding in insects. General and comparative endocrinology 162, 93-104.

Banner, S., Osborne, R., 1989. Modulation of 5-HT and proctolin receptors by FMRFamide in the foregut of the locust *Schistocerca gregaria*. Journal of insect physiology 35, 887-892.

Beggs, K.T., Mercer, A.R., 2009. Dopamine receptor activation by honey bee queen pheromone. Current biology 19, 1206-1209.

Bellés, X., 2010. Beyond *Drosophila*: RNAi *in vivo* and functional genomics in insects. Annual review of entomology 55, 111-128.

Bentley, P.H., Kenner, G.W., Sheppard, R.C., 1966. Human gastrin: isolation, structure and synthesis: structures of human gastrins I and II. Nature 209, 583-585.

Bertaccini, G., 1976. Active polypeptides of nonmammalian origin. Pharmacological reviews 28, 127-177.

Bi, S., Moran, T.H., 2002. Actions of CCK in the controls of food intake and body weight: lessons from the CCK-A receptor deficient OLETF rat. Neuropeptides 36, 171-181.

Bloch Qazi, M.C., Aprille, J.R., Lewis, S.M., 1998. Female role in sperm storage in the red flour beetle, *Tribolium castaneum*. Comparative biochemistry and physiology part A: molecular & integrative physiology 120, 641-647.

Bucher, G., 2009. *Tribolium* beetle book version 1-2.

Bucher, G., Scholten, J., Klingler, M., 2002. Parental RNAi in *Tribolium* (Coleoptera). Current Biology 12, R85-R86.

Caers, J., Verlinden, H., Zels, S., Vandersmissen, H. P., Vuerinckx, K., Schoofs, L., 2012. More than two decades of research on insect neuropeptide GPCRs: an overview. Frontiers in endocrinology 3, 151.

Cao, Z., Yu, Y., Wu, Y., Hao, P., Di, Z., He, Y., Chen, Z., Yang, W., Shen, Z., He, X., 2013. The genome of *Mesobuthus martensii* reveals a unique adaptation model of arthropods. Nature communications 4, 2602.

Cardoso, J.C., Félix, R.C., Fonseca, V.G., Power, D.M., 2012. Feeding and the rhodopsin family G-protein coupled receptors in nematodes and arthropods. Frontiers in endocrinology 3, 157.

Galés, C., Kowalski-Chauvel, A., Dufour, M. N., Seva, C., Moroder, L., Pradayrol, L., Vaysse, N., Fourmy, D., Silvente-Poirot, S., 2000. Mutation of Asn-391 within the conserved NPXXY motif of the cholecystokinin B receptor abolishes Gq protein activation without affecting its association with the receptor. The journal of biological chemistry 275, 17321-17327.

Chapman, R.F., 1998. The insects: structure and function. Cambridge university press.

Chen, X., Peterson, J., Nachman, R.J., Ganetzky, B., 2012. Drosulfakinin activates CCKLR-17D1 and promotes larval locomotion and escape response in *Drosophila*. Fly (Austin) 6, 290-297.

Cheng, Z., Garvin, D., Paguio, A., Stecha, P., Wood, K., Fan, F., 2010. Luciferase reporter assay system for deciphering GPCR pathways. Current chemical genomics 4, 84-91.

Choe, E.S., Wang, J.Q., 2002. Regulation of transcription factor phosphorylation by metabotropic glutamate receptor-associated signaling pathways in rat striatal neurons. Neuroscience 114, 557-565.

Christiaens, O., Swevers, L., Smagghe, G., 2014. DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. Peptides 53, 307-314.

Christie, A.E., 2008a. *In silico* analyses of peptide paracrines/hormones in Aphidoidea. General and comparative endocrinology 159, 67-79.

Christie, A.E., 2008b. Neuropeptide discovery in Ixodoidea: An *in silico* investigation using publicly accessible expressed sequence tags. General and comparative endocrinology 157, 174-185.

Christie, A.E., 2014. Prediction of the first neuropeptides from a member of the *Remipedia* (Arthropoda, Crustacea). General and comparative endocrinology 201, 74-86.

Christie, A.E., Cashman, C.R., Brennan, H.R., Ma, M., Sousa, G.L., Li, L., Stemmler, E.A., Dickinson, P.S., 2008. Identification of putative crustacean neuropeptides using *in silico*

analyses of publicly accessible expressed sequence tags. General and comparative endocrinology 156, 246-264.

Christie, A.E., McCoole, M.D., Harmon, S.M., Baer, K.N., Lenz, P.H., 2011a. Genomic analyses of the *Daphnia pulex* peptidome. General and comparative endocrinology 171, 131-150.

Christie, A.E., Nolan, D.H., Ohno, P., Hartline, N., Lenz, P.H., 2011b. Identification of chelicerate neuropeptides using bioinformatics of publicly accessible expressed sequence tags. General and comparative endocrinology 170, 144-155.

Coast, G.M., Schooley, D.A., 2011. Toward a consensus nomenclature for insect neuropeptides and peptide hormones. Peptides 32, 620-631.

Currie, P.J., Coscina, D.V., 1995. Dissociated feeding and hypothermic effects of neuropeptide Y in the paraventricular and perifornical hypothalamus. Peptides 16, 599 – 604.

De Carvalho, F., Gheysen, G., Kushnir, S., Van Montagu, M., Inze, D., Castresana, C., 1992. Suppression of beta-1, 3-glucanase transgene expression in homozygous plants. The EMBO journal 11, 2595-2602.

De Tullio, P., Delarge, J., Pirotte, B., 2000. Therapeutic and chemical developments of cholecystokinin receptor ligands. Expert opinion on investigational drugs 9, 129-146.

Deweerth, A., Pisegna, J., Huppi, K., Wank, S., 1993. Molecular cloning, functional expression and chromosomal localization of the human cholecystokinin type A receptor. Biochemical and biophysical research communications 194, 811-818.

Dickinson, P.S., Stevens, J.S., Rus, S., Brennan, H.R., Goiney, C.C., Smith, C.M., Li, L., Towle, D.W., Christie, A.E., 2007. Identification and cardiotropic actions of sulfakinin peptides in the American lobster *Homarus americanus*. Journal of experimental biology 210, 2278-2289.

Dillen, S., Zels, S., Verlinden, H., Spit, J., Van Wielendaele, P., Broeck, J.V., 2013. Functional characterization of the short neuropeptide F receptor in the desert locust, Schistocerca gregaria. PLoS One 8, e53604.

Dimaline, R., Dockray, G.J., 1994. 1 Evolution of the gastrointestinal endocrine system (with special reference to gastrin and CCK). Baillière's clinical endocrinology and metabolism 8, 1-24.

Dockray, G., 1977. Molecular evolution of gut hormones: application of comparative studies on the regulation of digestion. Gastroenterology 72, 344-358.

Dockray, G., Gregory, R., Hutchison, J., Harris, J.I., Runswick, M., 1978. Isolation, structure and biological activity of two cholecystokinin octapeptides from sheep brain. Nature 274, 711-713.

Dockray, G., Varro, A., Dimaline, R., Wang, T., 2001. The gastrins: their production and biological activities. Annual review of physiology 63, 119-139.

Dong, Y., Friedrich, M., 2005. Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper. BMC biotechnology 5, 25.

Dönitz, J., Grossmann, D., Schild, I., Schmitt-Engel, C., Bradler, S., Prpic, N.-M., Bucher, G., 2013. TrOn: An anatomical ontology for the beetle *Tribolium castaneum*. PLoS One 8, e70695.

Donovan, M.J., Paulino, G., Raybould, H.E., 2007. CCK(1) receptor is essential for normal meal patterning in mice fed high fat diet. Physiology and behavior 92, 969-974.

Douris, V., Swevers, L., Labropoulou, V., Andronopoulou, E., Georgoussi, Z., Iatrou, K., 2006. Stably transformed insect cell lines: tools for expression of secreted and membrane-anchored proteins and high-throughput screening platforms for drug and insecticide discovery. Advances in virus research 68, 113-156.

Downer, K., Haselton, A., Nachman, R., Stoffolano Jr, J., 2007. Insect satiety: Sulfakinin localization and the effect of drosulfakinin on protein and carbohydrate ingestion in the blow fly, *Phormia regina* (Diptera: Calliphoridae). Journal of insect physiology 53, 106-112.

Dufresne, M., Seva, C., Fourmy, D., 2006. Cholecystokinin and gastrin receptors. Physiological reviews 86, 805-847.

Dupré, D., Tostivint, H., 2014. Evolution of the gastrin–cholecystokinin gene family revealed by synteny analysis. General and comparative endocrinology 195, 164-173.

Duve, H., Rehfeld, J.F., East, P., Thorpe, A., 1994. Localisation of sulfakinin neuronal pathways in the blowfly *Calliphora vomitoria*. Cell and tissue research 275, 177-186.

Duve, H., Thorpe, A., Scott, A.G., Johnsen, A.H., Rehfeld, J.F., Hines, E., East, P.D., 1995. The sulfakinins of the blowfly *Calliphora Vomitoria*. European journal of biochemistry 232, 633-640.

Dyrløv Bendtsen, J., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. Journal of molecular biology 340, 783-795.

Dzitoyeva, S., Dimitrijevic, N., Manev, H., 2001. Intra-abdominal injection of double-stranded RNA into anesthetized adult *Drosophila* triggers RNA interference in the central nervous system. Molecular psychiatry 6, 665-670.

East, P., Hales, D., Cooper, P., 1997. Distribution of sulfakinin-like peptides in the central and sympathetic nervous system of the American cockroach, *Periplaneta americana* (L.) and the field cricket, *Teleogryllus commodus* (Walker). Tissue and cell 29, 347-354.

Eipper, B.A., Mains, R.E., 1988. Peptide α-amidation. Annual review of physiology 50, 333-344.

Eipper, B.A., Stoffers, D.A., Mains, R.E., 1992. The biosynthesis of neuropeptides: peptide alpha-amidation. Annual review of neuroscience 15, 57-85.

Eysselein, V.E., Eberlein, G.A., Schaeffer, M., Grandt, D., Goebell, H., Niebel, W., Rosenquist, G.L., Meyer, H.E., Reeve, J., 1990. Characterization of the major form of cholecystokinin in human intestine: CCK-58. American journal of physiology-gastrointestinal and liver physiology 258, G253-G260.

Fan, F., Paguio, A., Garvin, D., Wood, K.V., 2005. Using luciferase assays to study G-protein-coupled receptor pathways and screen for GPCR modulators. Luminescence (RLU) 10, 1000.

Fan, Y., Sun, P., Wang, Y., He, X., Deng, X., Chen, X., Zhang, G., Chen, X., Zhou, N., 2010. The G protein-coupled receptors in the silkworm, *Bombyx mori*. Insect biochemistry and molecular biology 40, 581-591.

Farrell, P.J., Lu, M., Prevost, J., Brown, C., Behie, L., Iatrou, K., 1998. High-level expression of secreted glycoproteins in transformed lepidopteran insect cells using a novel expression vector. Biotechnology and bioengineering 60, 656-663.

Farrell, P.J., Behie, L.A., Iatrou, K., 1999. Transformed Lepidopteran insect cells: new sources of recombinant human tissue plasminogen activator. Biotechnology and bioengineering 64, 426-433.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.

Fónagy, A., Schoofs, L., Proost, P., Van Damme, J., De Loof, A., 1992. Isolation and primary structure of two sulfakinin-like peptides from the fleshfly, *Neobellieria bullata*. Comparative biochemistry and physiology part C: Comparative pharmacology 103, 135-142.

Fraga, A., Ribeiro, L., Lobato, M., Santos, V., Silva, J.R., Gomes, H., da Cunha Moraes, J.L., de Souza Menezes, J., de Oliveira, C.J.L., Campos, E., 2013. Glycogen and glucose metabolism are essential for early embryonic development of the red flour beetle *Tribolium castaneum*. PLoS One 8, e65125.

Geiselman, P.J., 1996. Control of food intake: a physiologically complex, motivated behavioral system. Endocrinology and metabolism clinics of North America 25, 815-829.

Gigoux, V., Escrieut, C., Silvente-Poirot, S., Maigret, B., Gouilleux, L., Fehrentz, J.A., Gully, D., Moroder, L., Vaysse, N., Fourmy, D., 1998. Met-195 of the cholecystokinin-A receptor interacts with the sulfated tyrosine of cholecystokinin and is crucial for receptor transition to high affinity state. Journal of biological chemistry 273, 14380-14386.

Gigoux, V., Maigret, B., Escrieut, C., Silvente-Poirot, S., Bouisson, M., Fehrentz, J.A., Moroder, L., Gully, D., Martinez, J., Vaysse, N., Fourmy, D., 1999. Arg-197 of the cholecystokinin-A receptor binding site interacts with the sulfate of the peptide agonist cholecystokinin. Protein science 8, 2347-2354.

Goodman, C.L., Stanley, D., Ringbauer Jr, J.A., Beeman, R.W., Silver, K., Park, Y., 2012. A cell line derived from the red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae). In vitro cellular & developmental biology-animal 48, 426-433.

Goto, A., Blandin, S., Royet, J., Reichhart, J.M., Levashina, E.A., 2003. Silencing of Toll pathway components by direct injection of double-stranded RNA into *Drosophila* adult flies. Nucleic acids research 31, 6619-6623.

Gregory, H., Hardy, P., Jones, D., Kenner, G., Sheppard, R., 1964. The antral hormone gastrin. Structure of the gastrin. Nature 204, 931-933.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-98.

Haliscak, J.P., Beeman, R.W., 1983. Status of malathion resistance in five genera of beetles infesting farm-stored corn, wheat, and oats in the United States. Journal of economic entomology 76, 717-722.

Hannon, G.J., 2002. RNA interference. Nature 418, 244-251.

Harshini, S., Nachman, R., Sreekumar, S., 2002. In vitro release of digestive enzymes by FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina arenosella* (Walk.). Peptides 23, 1759-1763.

Hauser, F., Cazzamali, G., Williamson, M., Blenau, W., Grimmelikhuijzen, C.J., 2006a. A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. Progress in neurobiology 80, 1-19.

Hauser, F., Cazzamali, G., Williamson, M., Park, Y., Li, B., Tanaka, Y., Predel, R., Neupert, S., Schachtner, J., Verleyen, P., 2008. A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum*. Frontiers in neuroendocrinology 29, 142-165.

Hauser, F., Neupert, S., Williamson, M., Predel, R., Tanaka, Y., Grimmelikhuijzen, C.J., 2010. Genomics and peptidomics of neuropeptides and protein hormonespresent in the parasitic wasp *Nasonia vitripennis*. Journal of proteome research 9, 5296-5310.

Hauser, F., Williamson, M., Cazzamali, G., Grimmelikhuijzen, C.J., 2006b. Identifying neuropeptide and protein hormone receptors in *Drosophila melanogaster* by exploiting genomic data. Briefings in functional genomics and proteomics 4, 321-330.

Hawkins, P.C., Skillman, A.G., Warren, G.L., Ellingson, B.A., Stahl, M.T., 2010. Conformer generation with OMEGA: algorithm and validation using high quality structures from the protein databank and Cambridge structural database, Journal of chemical information and modeling 50, 572-584.

He, Z.B., Cao, Y.Q., Yin, Y.P., Wang, Z.K., Chen, B., Peng, G.X., Xia, Y.X., 2006. Role of hunchback in segment patterning of Locusta migratoria manilensis revealed by parental RNAi. Development, growth and differentiation 48, 439-445.

Hergarden, A.C., Tayler, T.D., Anderson, D.J., 2012. Allatostatin-A neurons inhibit feeding behavior in adult Drosophila. Proceedings of the national academy of sciences 109, 3967-3972.

Hoffmann, W., Bach, T., Seliger, H., Kreil, G., 1983. Biosynthesis of caerulein in the skin of *Xenopus laevis*: partial sequences of precursors as deduced from cDNA clones. The EMBO journal 2, 111-114.

Hummon, A.B., Richmond, T.A., Verleyen, P., Baggerman, G., Huybrechts, J., Ewing, M.A., Vierstraete, E., Rodriguez-Zas, S.L., Schoofs, L., Robinson, G.E., 2006. From the genome to the proteome: uncovering peptides in the *Apis* brain. Science 314, 647-649.

Huppi, K., Siwarski, D., Pisegna, J., Wank, S., 1995. Chromosomal localization of the gastric and brain receptors for cholecystokinin (CCKAR and CCKBR) in human and mouse. Genomics 25, 727-729.

Huvenne, H., Smagghe, G., 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. Journal of insect physiology 56, 227-235.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., Linsley, P.S., 2003. Expression profiling reveals off-target gene regulation by RNAi. Nature biotechnology 21, 635-637.

Janssen, T., Lindemans, M., Meelkop, E., Temmerman, L., Schoofs, L., 2010. Coevolution of neuropeptidergic signaling systems: from worm to man. Annals of the New York academy of sciences 1200, 1-14.

Janssen, T., Meelkop, E., Lindemans, M., Verstraelen, K., Husson, S.J., Temmerman, L., Nachman, R.J., Schoofs, L., 2008. Discovery of a cholecystokinin-gastrin-like signaling system in nematodes. Endocrinology 149, 2826-2839.

Janssen, T., Meelkop, E., Nachman, R.J., Schoofs, L., 2009. Evolutionary conservation of the cholecystokinin/gastrin signaling system in nematodes. Annals of the New York academy of sciences 1163, 428-432.

Jaubert-Possamai, S., Le Trionnaire, G., Bonhomme, J., Christophides, G.K., Rispe, C., Tagu, D., 2007. Gene knockdown by RNAi in the pea aphid *Acyrthosiphon pisum*. BMC biotechnology 7, 63.

Jayawickreme, C.K., Kost, T.A., 1997. Gene expression systems in the development of high-throughput screens. Current opinion in biotechnology 8, 629-634.

Jensen, J., 2001. Regulatory peptides and control of food intake in non-mammalian vertebrates. Comparative biochemistry and physiology part A: molecular and integrative physiology 128, 469-477.

Jensen, R.T., 2002. Involvement of cholecystokinin/gastrin-related peptides and their receptors in clinical gastrointestinal disorders. Pharmacology and toxicology 91, 333-350.

Jinek, M., Doudna, J.A., 2009. A three-dimensional view of the molecular machinery of RNA interference. Nature 457, 405-412.

Johnsen, A.H., 1998. Phylogeny of the cholecystokinin/gastrin family. Frontiers in neuroendocrinology 19, 73-99.

Johnsen, A.H., Duve, H., Davey, M., Hall, M., Thorpe, A., 2000. Sulfakinin neuropeptides in a crustacean. European journal of biochemistry 267, 1153-1160.

Johnsen, A.H., Rehfeld, J., 1990. Cionin: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the neural ganglion of the protochordate *Ciona intestinalis*. Journal of biological chemistry 265, 3054-3058.

Joyce, K.A., Atkinson, A.E., Bermudez, I., Beadle, D.J., King, L.A., 1993. Synthesis of functional $GABA_A$ receptors in stable insect cell lines. FEBS 335, 61-64.

Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., Kramer, S.J., 1989. Identification of an allatotropin from adult *Manduca sexta*. Science 243, 1481-1483.

Kelley, L.A., Sternberg, M.J., 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nature protocols 4, 363-371.

Kennedy, J., Bradwejn, J., Koszycki, D., King, N., Crowe, R., Vincent, J., Fourie, O., 1999. Investigation of cholecystokinin system genes in panic disorder. Molecular psychiatry 4, 284-285.

Kim, H.S., Murphy, T., Xia, J., Caragea, D., Park, Y., Beeman, R.W., Lorenzen, M.D., Butcher, S., Manak, J.R., Brown, S.J., 2010. BeetleBase in 2010: revisions to provide comprehensive genomic information for *Tribolium castaneum*. Nucleic acids research 38, D437-D442.

Knight, P.J., Grigliatti, T.A., 2004. Diversity of G proteins in Lepidopteran cell lines: partial sequences of six G protein alpha subunits. Archives of insect biochemistry and physiology 57, 142-150.

Knight, P.J., Pfeifer, T.A., Grigliatti, T.A., 2003. A functional assay for G-protein-coupled receptors using stably transformed insect tissue culture cell lines. Analytical biochemistry 320, 88-103.

Knorr, E., Bingsohn, L., Kanost, M.R., Vilcinskas, A., 2013. *Tribolium castaneum* as a model for high-throughput RNAi screening, Yellow biotechnology II. Springer, pp. 163-178.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., Li, J.P., Carney, R.L., Schooley, D.A., 1991. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. Proceedings of the national academy of sciences 88, 9458-9462.

Krogh, A., Larsson, B., Von Heijne, G., Sonnhammer, E.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of molecular biology 305, 567-580.

Kubiak, T.M., Larsen, M.J., Burton, K.J., Bannow, C.A., Martin, R.A., Zantello, M.R., Lowery, D.E., 2002. Cloning and functional expression of the first *Drosophila melanogaster* sulfakinin receptor DSK-R1. Biochemical and biophysical research communications 291, 313-320.

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N., Mathey-Prevot, B., 2006. Evidence of off-target effects associated with long dsRNAs in Drosophila melanogaster cell-based assays. Nature methods 3, 833-838.

Lal, S., McLaughlin, J., Barlow, J., D'Amato, M., Giacovelli, G., Varro, A., Dockray, G.J., Thompson, D.G., 2004. Cholecystokinin pathways modulate sensations induced by gastric distension in humans. American journal of physiology: gastrointestinal and liver physiology 287, G72-79.

Larsson, L.I., Rehfeld, J., 1977. Evidence for a common evolutionary origin of gastrin and cholecystokinin. Nature 269, 335-338.

Laskowski, R. A., MacArthur, M. W., Moss, D. S., Thornton, J. M., 1993. Procheck: a program to check the stereochemical quality of protein structures. Journal of applied crystallography 26, 283-291.

Lee, K.S., You, K.H., Choo, J.K., Han, Y.M., Yu, K., 2004. *Drosophila* short neuropeptide F regulates food intake and body size. Journal of biological chemistry 279, 50781-50789.

Lee, K.Y., Horodyski, F.M., 2002. Restriction of nutrient intake results in the increase of a specific *Manduca sexta* allatotropin (Manse-AT) mRNA in the larval nerve cord. Peptides 23, 653-661.

Lee, K.Y., Horodyski, F.M., Chamberlin, M., 1998. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* FLRFamides in the tobacco hornworm *Manduca sexta*. Journal of experimental biology 201, 3067-3074.

Li, B., Predel, R., Neupert, S., Hauser, F., Tanaka, Y., Cazzamali, G., Williamson, M., Arakane, Y., Verleyen, P., Schoofs, L., 2008. Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium castaneum*. Genome research 18, 113-122.

Li, C., Yun, X., Hu, X., Zhang, Y., Sang, M., Liu, X., Wu, W., Li, B., 2013. Identification of G protein-coupled receptors in the pea aphid, *Acyrthosiphon pisum*. Genomics 102, 345-354.

Lord, J.C., Hartzer, K., Toutges, M., Oppert, B., 2010. Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. Journal of microbiological methods 80, 219-221.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., 1995. Identification of two allatostatins from the cricket, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae): additional members of a family of neuropeptides inhibiting juvenile hormone biosynthesis. Regulatory peptides 57, 227-236.

Luo, Y., Wang, X., Yu, D., Kang, L., 2012. The SID-1 double-stranded RNA transporter is not required for systemic RNAi in the igratory locust. RNA biology 9, 663-671.

Lwalaba, D., Hoffmann, K.H., Woodring, J., 2010. Control of the release of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda*. Archives of insect biochemistry and physiology 73, 14-29.

Maestro, J.L., Aguilar, R., Pascual, N., Valero, M.L., Piulachs, M.D., Andreu, D., Navarro, I., Bellés, X., 2001. Screening of antifeedant activity in brain extracts led to the identification of sulfakinin as a satiety promoter in the German cockroach. European journal of biochemistry 268, 5824-5830.

Maleszka, J., Foret, S., Saint, R., Maleszka, R., 2007. RNAi-induced phenotypes suggest a novel role for a chemosensory protein CSP5 in the development of embryonic integument in the honeybee (*Apis mellifera*). Development genes and evolution 217, 189-196.

Mao, J., Zeng, F., 2012. Feeding-based RNA intereference of a gap gene is lethal to the pea aphid, Acyrthosiphon pisum. PLoS One 7, e48718.

Marciniak, P., Kuczer, M., Rosinski, G., 2011. New physiological activities of myosuppressin, sulfakinin and NVP-like peptide in *Zophobas atratus* beetle. Journal of comparative physiology B 181, 721-730.

Mårvik, R., Johnsen, A., Rehfeld, J., Sandvik, A., Waldum, H., 1994. Effect of cionin on histamine and acid secretion by the perfused rat stomach. Scandinavian journal of gastroenterology 29, 591-594.

Maule, A., Shaw, C., Halton, D., Thim, L., Johnston, C., Fairweather, I., Buchanan, K., 1991. Neuropeptide F: a novel parasitic flatworm regulatory peptide from *Moniezia expansa* (Cestoda: Cyclophyllidea). Parasitology 102, 309-316.

May, R.C., Plasterk, R.H., 2005. RNA Interference Spreading in *C. elegans*. Methods in enzymology 392, 308-315.

Maynard, B.F., Bass, C., Katanski, C., Thakur, K., Manoogian, B., Leander, M., Nichols, R., 2013. Structure-activity relationships of FMRF-NH_2 peptides demonstrate a role for the conserved C-terminus and unique N-terminal extension in modulating cardiac contractility. PLoS One 8, e75502.

McClintock, T.S., Lerner, M.R., 1997. Functional analysis by imaging of melanophore pigment dispersion of chimeric receptors constructed by recombinant polymerase chain reaction. Brain research protocols 2, 59-68.

McGann, M., 2011. FRED pose prediction and virtual screening accuracy, Journal of chemical information and modeling 51, 578-596.

McKay, R.M., McKay, J.P., Suh, J.M., Avery, L., Graff, J.M., 2007. Tripeptidyl peptidase II promotes fat formation in a conserved fashion. EMBO reports 8, 1183-1189.

McVeigh, P., Leech, S., Marks, N.J., Geary, T.G., Maule, A.G., 2006. Gene expression and pharmacology of nematode NLP-12 neuropeptides. International journal for parasitology 36, 633-640.

Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., Nachman, R.J., Huybrechts, R., De Loof, A., Schoofs, L., 2002. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. Proceedings of the national academy of sciences 99, 15363-15368.

Meister, G., Tuschl, T., 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343-349.

Mello, C.C., Conte, D., 2004. Revealing the world of RNA interference. Nature 431, 338-342.

Merkler, D.J., 1994. C-Terminal amidated peptides: Production by the *in vitro* enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. Enzyme and microbial technology 16, 450-456.

Meyering-Vos, M., Müller, A., 2007a. RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*. Journal of insect physiology 53, 840-848.

Meyering-Vos, M., Müller, A., 2007b. Structure of the sulfakinin cDNA and gene expression from the Mediterranean field cricket *Gryllus bimaculatus*. Insect molecular biology 16, 445-454.

Miller, L.J., Gao, F., 2008. Structural basis of cholecystokinin receptor binding and regulation. Pharmacology and therapeutics 119, 83-95.

Miller, S.C., Brown, S.J., Tomoyasu, Y., 2008. Larval RNAi in *Drosophila*? Development genes and evolution 218, 505-510.

Miller, S.C., Miyata, K., Brown, S.J., Tomoyasu, Y., 2012. Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: parameters affecting the efficiency of RNAi. PLoS One 7, e47431.

Mirabeau, O., Joly, J.S., 2013. Molecular evolution of peptidergic signaling systems in bilaterians. Proceedings of the national academy of sciences 110, E2028-E2037.

Mizuguchi, K, Deane, C.M., Blundell, T.L., Overington, J.P., 1998. HOMSTRAD: a database of protein structure alignments for homologous families. Protein Science 7:2469-2471.

Mlotshwa, S., Voinnet, O., Mette, M.F., Matzke, M., Vaucheret, H., Ding, S.W., Pruss, G., Vance, V.B., 2002. RNA silencing and the mobile silencing signal. The plant cell online 14, S289-S301.

Montgomery, M.K., Xu, S., Fire, A., 1998. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proceedings of the national academy of sciences 95, 15502-15507.

Morris, K., Lorenzen, M.D., Hiromasa, Y., Tomich, J.M., Oppert, C., Elpidina, E.N., Vinokurov, K., Jurat-Fuentes, J.L., Fabrick, J., Oppert, B., 2009. *Tribolium castaneum* larval gut transcriptome and proteome: a resource for the study of the coleopteran gut. Journal of proteome research 8, 3889-3898.

Mutti, N.S., Park, Y., Reese, J.C., Reeck, G.R., 2006. RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. Journal of insect science 6, 1-7.

Nachman, R., Holman, G., Haddon, W., Hayes, T., 1989. Structure-activity relationships for myotropic activity of the gastrin/cholecystokinin-like insect sulfakinins. Peptide research 2, 171-177.

Nachman, R.J., Giard, W., Favrel, P., Suresh, T., Sreekumar, S., Holman, G., 1997. Insect myosuppressins and sulfakinins stimulate release of the digestive enzyme α-amylase in two invertebrates: the scallop *Pecten maximus* and insect *Rhynchophorus ferrugineus*. Annals of the New York academy of sciences 814, 335-338.

Nachman, R.J., Holman, G.M., Cook, B.J., 1986a. Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure-function studies. Biochemical and biophysical research communications 137, 936-942.

Nachman, R.J., Holman, G.M., Cook, B.J., Haddon, W.F., Ling, N., 1986b. Leucosulfakinin-II, a blocked sulfated insect neuropeptide with homology to cholecystokinin and gastrin. Biochemical and biophysical research communications 140, 357-364.

Nachman, R.J., Holman, G.M., Haddon, W.F., 1988. Structural aspects of gastrin/CCK-like insect leucosulfakinins and FMRF-amide. Peptides 9, 137-143.

Nachman, R.J., Holman, G.M., Haddon, W.F., Ling, N., 1986c. Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. Science 234, 71-73.

Nachman, R.J., Holman, G.M., Hayes, T.K., Beier, R.C., 1993. Structure-activity relationships for inhibitory insect myosuppressins: contrast with the stimulatory sulfakinins. Peptides 14, 665-670.

Nachman, R.J., Vercammen, T., Williams, H., Kaczmarek, K., Zabrocki, J., Schoofs, L., 2005. Aliphatic amino diacid Asu functions as an effective mimic of Tyr(SO_3H) in sulfakinins for myotropic and food intake-inhibition activity in insects. Peptides 26, 115-120.

Nakayama, K., Watanabe, T., Nakagawa, T., Kim, W., Nagahama, M., Hosaka, M., Hatsuzawa, K., Kondoh-Hashiba, K., Murakami, K., 1992. Consensus sequence for precursor processing at mono-arginyl sites. Evidence for the involvement of a Kex2-like endoprotease in precursor cleavages at both dibasic and mono-arginyl sites. Journal of biological chemistry 267, 16335-16340.

Napoli, C., Lemieux, C., Jorgensen, R., 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. The plant cell online 2, 279-289.

Nässel, D.R., 2002. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Progress in neurobiology 68, 1-84.

Nässel, D.R., Wegener, C., 2011. A comparative review of short and long neuropeptide F signaling in invertebrates: any similarities to vertebrate neuropeptide Y signaling? Peptides 32, 1335-1355.

Nathoo, A.N., Moeller, R.A., Westlund, B.A., Hart, A.C., 2001. Identification of neuropeptide-like protein gene families in *Caenorhabditiselegans* and other species. Proceeding of the national academy of sciences U S A. 98, 14000-14005.

Neupert, S., Russell, W.K., Predel, R., Russell, D.H., Strey, O.F., Teel, P.D., Nachman, R.J., 2009. The neuropeptidomics of *Ixodes scapularis* synganglion. Journal of proteomics 72, 1040-1045.

Nichols, R., 1992. Isolation and expression of the *Drosophila* drosulfakinin neural peptide gene product, DSK-I. Molecular and cellular neuroscience 3, 342-347.

Nichols, R., 2007. The first nonsulfated sulfakinin activity reported suggests nsDSK acts in gut biology. Peptides 28, 767-773.

Nichols, R., Egle, J.P., Langan, N.R., Palmer, G.C., 2008a. The different effects of structurally related sulfakinins on *Drosophila melanogaster* odor preference and locomotion suggest involvement of distinct mechanisms. Peptides 29, 2128-2135.

Nichols, R., Manoogian, B., Walling, E., Mispelon, M., 2008b. Plasticity in the effects of sulfated and nonsulfated sulfakinin on heart contractions. Frontiers in bioscience (Landmark edition) 14, 4035-4043.

Nichols, R., Schneuwly, S., Dixon, J., 1988. Identification and characterization of a *Drosophila* homologue to the vertebrate neuropeptide cholecystokinin. Journal of biological chemistry 263, 12167-12170.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein engineering 10, 1-6.

Nilsson, I., Svensson, S.P., Monstein, H.J., 2003. Molecular cloning of a putative *Ciona intestinalis* cionin receptor, a new member of the CCK/gastrin receptor family. Gene 323, 79-88.

Noble, F., Roques, B.P., 1999. CCK-B receptor: chemistry, molecular biology, biochemistry and pharmacology. Progress in neurobiology 58, 349-379.

Nygaard, S., Zhang, G., Schiøtt, M., Li, C., Wurm, Y., Hu, H., Zhou, J., Ji, L., Qiu, F., Rasmussen, M., 2011. The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. Genome research 21, 1339-1348.

Oldham, W.M., Hamm, H.E., 2008. Heterotrimeric G protein activation by G-protein-coupled receptors. Nature reviews molecular cell biology 9, 60-71.

Omasits, U., Ahrens, C.H., Müller, S., Wollscheid, B., 2013. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics, btt607.

Ons, S., Richter, F., Urlaub, H., Pomar, R.R., 2009. The neuropeptidome of *Rhodnius prolixus* brain. Proteomics 9, 788-792.

Ons, S., Sterkel, M., Diambra, L., Urlaub, H., Rivera-Pomar, R., 2011. Neuropeptide precursor gene discovery in the Chagas disease vector *Rhodnius prolixus*. Insect molecular biology 20, 29-44.

Owyang, C., Logsdon, C.D., 2004. New insights into neurohormonal regulation of pancreatic secretion. Gastroenterology 127, 957-969.

Paluzzi, J.-P., Park, Y., Nachman, R.J., Orchard, I., 2010. Isolation, expression analysis, and functional characterization of the first antidiuretic hormone receptor in insects. Proceedings of the national academy of sciences 107, 10290-10295.

Parthasarathy, R., Tan, A., Bai, H., Palli, S.R., 2008. Transcription factor broad suppresses precocious development of adult structures during larval–pupal metamorphosis in the red flour beetle, *Tribolium castaneum*. Mechanisms of development 125, 299-313.

Patel, A., Fondrk, M.K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., Amdam, G.V., 2007. The making of a queen: TOR pathway is a key player in diphenic caste development. PLoS One 2, e509.

Pausch, M.H., 1997. G-protein-coupled receptors in *Saccharomyces cerevisiae*: high-throughput screening assays for drug discovery. Trends in biotechnology 15, 487-494.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature methods 8, 785-786.

Pitino, M., Coleman, A.D., Maffei, M.E., Ridout, C.J., Hogenhout, S.A., 2011. Silencing of aphid genes by dsRNA feeding from plants. PLoS One 6, e25709.

Poels, J., Franssens, V., Van Loy, T., Martinez, A., Suner, M.M., Dunbar, S.J., De Loof, A., Vanden Broeck, J., 2004. Isoforms of cyclic AMP response element binding proteins in Drosophila S2 cells. Biochemical and biophysical research communications 320, 318-324.

Posnien, N., Schinko, J., Grossmann, D., Shippy, T.D., Konopova, B., Bucher, G., 2009. RNAi in the red flour beetle (*Tribolium*). Cold Spring Harbor Protocols 2009, pdb. prot5256.

Pratt, G.E., Farnsworth, D.E., Siegel, N.R., Fok, K.F., Feyereisen, R., 1989. Identification of an allatostatin from adult *Diploptera punctata*. Biochemical and biophysical research communications 163, 1243-1247.

Predel, R., Brandt, W., Kellner, R., Rapus, J., Nachman, R.J., Gäde, G., 1999. Post-translational modifications of the insect sulfakinins. European journal of biochemistry 263, 552-560.

Predel, R., Rapus, J., Eckert, M., 2001. Myoinhibitory neuropeptides in the American cockroach. Peptides 22, 199-208.

Price D.A., Greenberg M.J., 1977. The structure of a molluscan cardioescitatory neuropeptide. Science 197, 670–671.

Radhakrishnan, I., Pérez-Alvarado, G.C., Parker, D., Dyson, H.J., Montminy, M.R., Wright, P.E., 1999. Structural analyses of CREB-CBP transcriptional activator-coactivator complexes by NMR spectroscopy: implications for mapping the boundaries of structural domains. Journal of molecular biology 287, 859-865.

Rahman, M.M., Neupert, S., Predel, R., 2013. Neuropeptidomics of the Australian sheep blowfly *Lucilia cuprina* (Wiedemann) and related *Diptera*. Peptides 41, 31-37.

Ramos, E.J., Meguid, M.M., Campos, A.C., Coelho, J.C., 2005. Neuropeptide Y, alpha-melanocyte-stimulating hormone, and monoamines in food intake regulation. Nutrition, 269-279.

Raybould, H.E., 2007. Mechanisms of CCK signaling from gut to brain. Current opinion in pharmacology 7, 570–574.

Rehfeld, J.F., Friis-Hansen, L., Goetze, J.P., Hansen, T.V., 2007. The biology of cholecystokinin and gastrin peptides. Current topics in medicinal chemistry 7, 1154-1165.

Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R., Bucher, G., Friedrich, M., Grimmelikhuijzen, C.J., 2008. The genome of the model beetle and pest *Tribolium castaneum*. Nature 452, 949-955.

Romano, N., Macino, G., 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Molecular microbiology 6, 3343-3353.

Ron, D., Gilon, C., Hanani, M., Vromen, A., Selinger, Z., Chorev, M., 1992. N-methylated analogs of Ac[Nle28, 31]CCK (26-33): synthesis, activity, and receptor selectivity. Journal of medicinal chemistry 35, 2806-2811.

Saleh, M.-C., van Rij, R.P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P.H., Andino, R., 2006. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nature cell biology 8, 793-802.

Sander, C., Schneider, R., 1991. Database of homology-derived protein structures and the structural meaning of sequence alignment. Proteins 9, 56-68.

Savard, J., Tautz, D., Lercher, M.J., 2006. Genome-wide acceleration of protein evolution in flies (*Diptera*). BMC evolutionary biology 6, 7.

Schjoldager, B., Jørgensen, J.C., Johnsen, A.H., 1995. Stimulation of rainbow trout gallbladder contraction by cionin, an ancestral member of the CCK/gastrin family. General and comparative endocrinology 98, 269-278.

Schneider, E.H., Seifert, R., 2010. Sf9 cells: a versatile model system to investigate the pharmacological properties of G protein-coupled receptors. Pharmacology and therapeutics 128, 387-418.

Schoofs, L., Holman, M., Hayes, T., De Loof, A., 1991. Isolation and identification of a sulfakinin-like peptide, with sequence homology to vertebrate gastrin and cholecystokinin, from the brain of *Locusta migratoria*, Chromatography and isolation of insect hormones and pheromones. Springer, pp. 231-241.

Schoofs, L., Veelaert, D., Vanden Broeck, J., De Loof, A., 1997. Peptides in the Locusts, *Locusta migratoria* and Schistocerca gregaria. Peptides 18, 145-156.

Schucht, R., Lydford, S., Andzinski, L., Zauers, J., Cooper, J., Hauser, H., Wirth, D., May, T., 2011. Rapid establishment of G-protein-coupled receptor-expressing cell lines by site-specific integration. Journal of biomolecular screening 16, 323-31.

Sekiguchi, T., Ogasawara, M., Satake, H., 2012. Molecular and functional characterization of cionin receptors in the ascidian, *Ciona intestinalis*: the evolutionary origin of the vertebrate cholecystokinin/gastrin family. Journal of endocrinology 213, 99-106.

Sousa, S.F., Fernandes, P.A., Ramos, M. J., 2006. Protein-ligand docking: current status and future challenges. Proteins: structure, function, and bioinformatics 65, 15-26.

Shukla, J.N., Palli, S.R., 2012. Sex determination in beetles: Production of all male progeny by parental RNAi knockdown of transformer. Scientific reports 2, 602.

Shukla, J.N., Palli, S.R., 2013. *Tribolium castaneum* Transformer-2 regulates sex determination and development in both males and females. Insect biochemistry and molecular biology 43, 1125-1132.

Simon, M.I., Strathmann, M.P., Gautam, N., 1991. Diversity of G proteins in signal transduction. Science 252, 802-808.

Simpson, S., Bernays, E., 1983. The regulation of feeding: locusts and blowflies are not so different from mammals. Appetite 4, 313-346.

Singh, M., Webster, P., 1978. Neurohormonal control of pancreatic secretion. A review. Gastroenterology 74, 294-309.

Smith, G. R., Sternberg, M. J. E., 2002. Prediction of protein-protien interactions by docking methods. Current opinion in structural biology 12, 28-35.

Söderberg, J.A., Carlsson, M.A., Nässel, D.R., 2012. Insulin-producing cells in the *Drosophila* brain also express satiety-inducing cholecystokinin-like peptide, drosulfakinin. Frontiers in endocrinology 3.

Soderstrom, E.L., Brandl, D.G., Mackey, B., 1992. High temperature combined with carbon dioxide enriched or reduced oxygen atmospheres for control of *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae). Journal of stored products research 28, 235-238.

Sokoloff, A., 1974. The biology of Tribolium, with special emphasis on genetic aspects. Oxford University Press.

Sonnhammer, E.L., von Heijne, G., Krogh, A., 1998. A hidden Markov model for predicting transmembrane helices in protein sequences, Ismb, pp. 175-182.

Sossin, W.S., Fisher, J.M., Scheller, R.H., 1989. Cellular and molecular biology of neuropeptide processing and packaging. Neuron 2, 1407-1417.

Spit, J., Badisco, L., Verlinden, H., Van Wielendaele, P., Zels, S., Dillen, S., Vanden Broeck, J., 2012. Peptidergic control of food intake and digestion in insects. Canadian journal of zoology 90, 489-506.

Staljanssens, D., Azari, E.K., Christiaens, O., Beaufays, J., Lins, L., Van Camp, J., Smagghe, G., 2011. The CCK (-like) receptor in the animal kingdom: functions, evolution and structures. Peptides 32, 607-619.

Strong, D.R., Lawton, J.H., Southwood, S.R., 1984. Insects on plants. Community patterns and mechanisms. Blackwell Scientific Publicatons.

Tabara, H., Grishok, A., Mello, C.C., 1998. RNAi in *C. elegans*: soaking in the genome sequence. Science 282, 430-431.

Taghert, P.H., Nitabach, M.N., 2012. Peptide neuromodulation in invertebrate model systems. Neuron 76, 82-97.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28, 2731-2739.

Tanaka, Y., Suetsugu, Y., Yamamoto, K., Noda, H., Shinoda, T., 2013. Transcriptome analysis of neuropeptides and G-protein coupled receptors (GPCRs) for neuropeptides in the brown planthopper *Nilaparvata lugens*. Peptides.

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J.L., Barthel, A., 2011. RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. Journal of insect physiology 57, 231-245.

The international aphid genomics consortium, 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS biology 8, e1000313.

Thomsen, W., Frazer, J., Unett, D., 2005. Functional assays for screening GPCR targets. Current opinion in biotechnology 16, 655-665.

Thorndyke, M., Dockray, G., 1986. Identification and localization of material with gastrin-like immunoreactivity in the neural ganglion of a protochordate, *Ciona intestinalis*. Regulatory peptides 16, 269-279.

Tijsterman, M., May, R.C., Simmer, F., Okihara, K.L., Plasterk, R.H., 2004. Genes Required for Systemic RNA Interference in *Caenorhabditis elegans*. Current Biology 14, 111-116.

Timmons, L., Court, D.L., Fire, A., 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263, 103-112.

Tomoyasu, Y., Denell, R.E., 2004. Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Development genes and evolution 214, 575-578.

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D., Bucher, G., 2008. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome Biology 9, R10.

Torfs, P., Baggerman, G., Meeusen, T., Nieto, J., Nachman, R., Calderon, J., De Loof, A., Schoofs, L., 2002. Isolation, identification, and synthesis of a disulfated sulfakinin from the central nervous system of an arthropods the white shrimp *Litopenaeus vannamei*. Biochemical and biophysical research communications 299, 312-320.

Torfs, H., Shariatmadari, R., Guerrero, F., Parmentier, M., Poels, J., Van Poyer, W., Swinnen, E., De Loof, A., Akerman, K., Vanden Broeck, J., 2000. Charaterization of a receptor for insect tachykinin-like peptide agonist by functional expression in a stable *Drosophila* Schneider 2 cell line. Journal of neurochemistry 74, 2182-2189.

Tsang, P.W., Orchard, I., 1991. Distribution of FMRFamide-related peptides in the blood-feeding bug *Rhodnius prolixus*. Journal of comparative neurology 311, 17-32.

Turner, C., Davy, M., MacDiarmid, R., Plummer, K., Birch, N., Newcomb, R., 2006. RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect molecular biology 15, 383-391.

Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R.A., Kocks, C., Rämet, M., 2006. Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in *Drosophila* S2 cells. Journal of biological chemistry 281, 14370-14375.

Van Wielendaele, P., Dillen, S., Zels, S., Badisco, L., Vanden Broeck, J., 2013. Regulation of feeding by Neuropeptide F in the desert locust, *Schistocerca gregaria*. Insect biochemistry and molecular biology 43, 102-114.

Vanden Broeck, J., Vulsteke, V., Huybrechts, R., De Loof, A., 1995. Charaterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. Journal of neurochemistry 64, 2387-2395.

Veenstra, J., 1989. Isolation and structure of two gastrin/CCK-like neuropeptides from the American cockroach homologous to the leucosulfakinins. Neuropeptides 14, 145-149.

Verlinden, H., Lismont, E., Bil, M., Urlacher, E., Mercer, A., Vanden Broeck, J., Huybrechts, R., 2013. Characterisation of a functional allatotropin receptor in the bumblebee, *Bombus terrestris* (Hymenoptera, Apidae). General and comparative endocrinology 193, 193-200.

Vigna, S.R., 1986. Evolution of hormone and receptor diversity: Cholecystokinin and gastrin. American zoologist 26, 1033-1040.

Vigna, S.R., 2000. Evolution of the cholecystokinin and gastrin peptides and receptors. American zoologist 40, 287-295.

Vilaplana, L., Pascual, N., Perera, N., Leira, D., Bellés, X., 2008. Antifeeding properties of myosuppressin in a generalist phytophagous leafworm, *Spodoptera littoralis* (Boisduval). Regulatory peptides 148, 68-75.

Vleugels, R., Lenaerts, C., Baumann, A., Broeck, J.V., Verlinden, H., 2013. Pharmacological characterization of a 5-HT1-type serotonin receptor in the red flour beetle, *Tribolium castaneum*. PLoS One 8, e65052.

Voinnet, O., 2005. Non-cell autonomous RNA silencing. FEBS letters 579, 5858-5871.

Von Heijne, G., 1985. Signal sequences: the limits of variation. Journal of molecular biology 184, 99-105.

Vuerinckx, K., Verlinden, H., Lindemans, M., Broeck, J.V., Huybrechts, R., 2011. Characterization of an allatotropin-like peptide receptor in the red flour beetle, *Tribolium castaneum*. Insect biochemistry and molecular biology 41, 815-822.

Wang, L., Wang, S., Li, Y., Paradesi, M.S., Brown, S.J., 2007. BeetleBase: the model organism database for *Tribolium castaneum*. Nucleic acids research 35, D476-D479.

Wechselberger, C., Kreil, G., 1995. Structure of two cDNAs encoding cholecystokinin precursors from the brain of *Xenopus laevis*. Journal of molecular endocrinology 14, 357-364.

Wei, Z., Baggerman, G., J Nachman, R., Goldsworthy, G., Verhaert, P., De Loof, A., Schoofs, L., 2000. Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*. Journal of insect physiology 46, 1259-1265.

Weinshilboum, R.M., Otterness, D., Aksoy, I.A., Wood, T.C., Her, C., Raftogianis, R., 1997. Sulfation and sulfotransferases 1: Sulfotransferase molecular biology: cDNAs and genes. The FASEB journal 11, 3-14.

Whangbo, J.S., Hunter, C.P., 2008. Environmental RNA interference. Trends in genetics 24, 297-305.

Whited, K.L., Thao, D., Lloyd, K.C., Kopin, A.S., Raybould, H.E., 2006. Targeted disruption of the murine CCK1 receptor gene reduces intestinal lipid-induced feedback inhibition of gastric function. American journal of physiology: gastrointest and liver physiology 291, G156-162.

Winston, W.M., Molodowitch, C., Hunter, C.P., 2002. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science 295, 2456-2459.

Woodhead, A., Stay, B., Seidel, S., Khan, M., Tobe, S., 1989. Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. Proceedings of the national academy of sciences 86, 5997-6001.

Wu, S., Zhang, Y., 2007. LOMETS: A local meta-threading-server for protein structure prediction. Nucleic acids research 35, 3375-3382.

Wu, S., Zhang, Y., 2009. Protein Structure Prediction. In: D. Edwards et al., editors. Bioinformatics: tools and applications, Springer-London, chapter 11, p.225-242.

Wyder, S., Kriventseva, E.V., Schröder, R., Kadowaki, T., Zdobnov, E.M., 2007. Quantification of ortholog losses in insects and vertebrates. Genome Biology 8, R242.

Xie, Q., Guo, H.S., 2006. Systemic antiviral silencing in plants. Virus research 118, 1-6.

Xu, J., Feng, J., Yu, L., 2008. Protein structure prediction using threading. In: Zaki, M., Bystroff, C., editors. Methods in molecular biology, 2nd edition. 413, p. 91-121.

Xu, J., Sheng, Z., Palli, S.R., 2013. Juvenile hormone and insulin regulate trehalose homeostasis in the red flour beetle, *Tribolium castaneum*. PLoS genetics 9, e1003535.

Yang, H., He, X., Yang, J., Deng, X., Liao, Y., Zhang, Z., Zhu, C., Shi, Y., Zhou, N., 2013. Activation of cAMP-response element-binding protein is positively regulated by PKA and calcium-sensitive calcineurin and negatively by PKC in insect. Insect biochemistry and molecular biology 43, 1028-1036.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., Smagghe, G., 2013. Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect science 20, 4-14.

Yule, D.I., Tseng, M., Williams, J.A., Logsdon, C.D., 1993. A cloned CCK-A receptor transduces multiple signals in response to full and partial agonists. American journal of physiology 265, G999-G999.

Zels, S., Verlinden, H., Dillen, S., Vleugels, R., Nachman, R.J., Broeck, J.V., 2014. Signaling properties and pharmacological analysis of two sulfakinin receptors from the red flour beetle, *Tribolium castaneum*. PLoS One 9, e94502.

Zoephel, J., Reiher, W., Rexer, K.-H., Kahnt, J., Wegener, C., 2012. Peptidomics of the agriculturally damaging larval stage of the cabbage root fly *Delia radicum* (Diptera: Anthomyiidae). PLoS One 7, e41543.

Summary

Feeding is one of the most basic behaviors for animals, by which food is obtained to provide energy and essential nutrients. The regulation of feeding in insects is a complex system in which different mechanisms are involved. One of these mechanisms is the neuropeptidergic signaling, which has been found to be active in the process. The neuropeptidergic signaling systems are conserved during evolution, which facilitates the study of a neuropeptide signaling in different species. For example, the cholecystokinin (CCK) is one of the neuropeptides that can reduce appetite in veterbrates. A homolog of CCK has been identified in insects as sulfakinin (SK) based on the similarity on sequence and function. Insect SK proves to be a neuropeptide involved in multiple physiological processes including inhibiting feeding in species such as the desert locust and cricket. However, not much is known about SK and SK signaling in insects such as the model insect *Tribolium castaneum* when this project started. Besides, understanding the regulation of feeding in *T. castaneum* may shed light on the development of new pest control strategies, because *T. castaneum* is a notorious pest causing damage to stored grain. Therefore, this project focused on the function and mechanism of the SK signaling in the regulation of feeding in *T. castaneum*.

The study consists of four parts. First, the characterization and function of SK signaling on feeding in *T. castaneum* were studied. The neuropeptide SK and its receptor (SKR) are the most important constituents of the SK signaling. One SK precursor gene and two SKR genes were characterized via cloning, sequence analysis and transcript profile analysis. Two SK peptides were present in the SK precursor gene: Trica-SK-I (QTSDDYGHLRF) and Trica-SK-II (GEEPFDDYGHMRF). Two TcSKRs (TcSKR1 and TcSKR2) were characterized as G-protein coupled receptors (GPCRs) with the typical seven transmembrane domains. They were all found in the three developmental stages (larva, pupa and adult) with the most abundance in head. *Tcsk* and *Tcskr1* were upregulated when the food supply was poor. The function of SK signaling was investigated with dsRNA-mediated gene silencing. SK and SKR genes were silenced by specific dsRNAs and a drastic increase in feeding was observed, suggesting that SK signaling inhibits feeding in *T. castaneum*. Remarkably, the silencing of *Tcskr2* affected the feeding dramatically while the silencing of *Tcskr1* was less effective in altering feeding. Therefore, TcSKR2 was believed to be more involved in the regulation of feeding. In parallel, a direct injection of a sulfated SK peptide reduced feeding, confirming the regulatory function of SK on feeding.

Second, the activity-structure relationship (SAR) of SK in feeding was primarily investigated with a series of synthetic SK-related peptides with amino acid substitution in the C-terminus or deletion from the N-terminus. SKs have a conserved C-terminal YGHM/LRF-NH_2 sequence with diverse N-terminal extensions. SKs are present in two forms: sulfated SK (sSK) and nonsulfated SK (nsSK), depending on whether there is a sulfate group (SO_3H) on Y^6 or not. *T. castaneum* SK-II[1-9] (SK-II for short) contains the sequence FDDYGHMRF-NH_2. Surprisingly, nsSK-II was as active as sSK-II in inhibiting feeding in *T. castaneum*. This suggests either that the sulfate moiety is not critical for the activity with the dose of sSK and nsSK used in the *in vivo* study, which were high enough to activate TcSKRs at least to some extent, or that further processing occurs to nsSK-II *in vivo*. Substitutions such as $G^5 \rightarrow A^5$ and $M^3 \rightarrow Nle^3$ in SK led to inactive compounds. The C-terminal tetrapeptides HMRF-NH_2 seems to be important for SK-II although YGHMRF-NH_2 is more potent. *C. elegans* NLP-13 peptides exerted similar effect as SK-II while NLP-12 peptides caused an opposit effect to SK-II by increasing food intake. However, the active core of SK-II in inhibiting feeding is not clear yet, which calls for further efforts.

Although there was a clear effect *in vivo* with whole insects, it is hard to interpret the observation with the SK(-related) peptides in feeding, because the data obtained are a result of a complex of processes more than SK signaling in the organism. It is of interest to ascertain if the TcSKRs are capable of stimulating similar effects to that were seen in the whole insect and specifically to determine if a particular receptor is capable of differentially activating signal transduction on the stimulation by different SK analogs. Therefore, a cell-based receptor bioassay was established to investigate the two TcSKRs individually *in vitro*. TcSKR is a G-protein coupled protein and its proper function involves the activation of certain Gα-proteins. In the established bioassay, Gαs- and Gαq-proteins were the candidates to determine the involved Gα-protein. Intracellular cAMP level and calcium motility were used as the indicator of the activated Gαs- and Gαq-protein, respectively. It turned out that Gαs-protein was active in the SK signaling, leading to the accumulation of intracellular cAMP upon the activation of SKRs by SK. No intracellular calcium flux was detected in the bioassay. The sulfate moiety on Y^6 was of importance for SK to activate TcSKR, as sSK-II was about 1000-fold more potent than nsSK-II to both TcSKRs in the bioassay. The two TcSKRs responded to sSK-II in a dose-response manner, with an EC_{50} at nanomolar concentration. Moreover, the SAR of SK in activating TcSKRs was studied in the bioassay with the synthetic SK-related

peptides. The activation of TcSKR1 required DYGHMRF-NH_2 with strict match for amino acid content, especially the tetrapeptide HMRF-NH_2. The activation of TcSKR2 required the entire length of FDDYGHMRF-NH_2 but was tolerant to the C-terminal substitution and the length of nsSK-II seemed to be more important than the exact amino acid content.

Lastly, the SAR of SK was studied *in silico*, with the modeling of TcSKRs and docking of peptides to the modeled TcSKRs. The three-dimensional structures of both TcSKRs were modeled via a modified protein threading strategy. They were similar in the typical seven transmembrane structure of GPCR but different in the width of the outer opening of the cavity built with the seven α-helices. The predicted structure of TcSKR1 possesses a wider outer opening than TcSKR2, which allows peptides to be docked deeper into the cavity. Therefore, the extracellular region and the transmembrane region in TcSKR1 are predicted to be involved in the binding of ligand while only the extracellular region in TcSKR2 is involved in the contact with ligand, which provides an explanation to the different potentials of the two TcSKRs to SK analogs. The docking of peptides to TcSKRs shows some insights to explain the observed bioactivity of peptide. For example, the Ala-substituted peptides were modeled to make more interactions with the extracellular regions in TcSKR2 than they do in TcSKR1. The increased interaction of peptides with the extracellular regions of TcSKR2 seems to be involved in diminishing the impact of Ala-substitution, resulting in less loss of activity of SK peptide on TcSKR2 than on TcSKR1, although it is not conclusive to simply correlate the modeling data with the bioactivity.

The aim of this project was to increase our understanding of the function and mechanism of SK signaling in the regulation of feeding in *T. castaneum*. Further research could continue to dig more details on the mechanism of SK signaling, the evolution of SK signaling as well as the crosstalk between different neuropeptide signaling pathways. The insights into the regulation of insect feeding provide the proper background for an in-depth discussion of improving existing and developing new pest control methods such as the potential of nsSK as a pesticide.

Samenvatting

De opname van voedsel, met als doel het voorzien in de energiebehoefte, is één van de meest basale processen bij dieren. De regulatie van die proces bij insecten is een complex systeem waarbij verschillende mechanismen betrokken zijn. Eén van deze mechanismen is de neuropeptiden-signalisatie, dat geactiveerd wordt tijdens het voedingsproces. Deze signalisatie-routes zijn evolutionair geconserveerd, wat de studie van neuropeptide-signalisatie over verschillende soorten mogelijk maakt. Bij vertebraten is cholecystokinine (CCK) één van de gekende neuropeptides die het hongergevoel kunnen onderdrukken. Een homoloog van CCK dat bij insecten is teruggevonden, is sulfakinine (SK), dat zowel qua eiwitsequentie als qua functie sterke gelijkenissen vertoont met de vertebrate CCK. Het is een neuropeptide dat betrokken is bij meerdere fysiologische processen waaronder de inhibitie van voedselopname in insecten zoals de woestijnsprinkhaan en de krekel. Bij de start van dit project was echter nog niet veel bekend over SK bij andere insecten, inclusief in het modelorganisme *Tribolium castaneum*, de kastanjebruine rijstmeelkever. Meer inzicht in de regulatie van de voedselopname in deze voorraadbeschadiger kan ons meer inzicht verschaffen met het oog op mogelijke plaagbestrijdingsstrategieën. Dit project focust zich daarom op de functie en mechanismen van SK signalisatie in de regulatie van voedselopname in *T. castaneum*.

Deze thesis omvat vier delen. In een eerste deel werd een SK gekarakteriseerd en werd de rol van dit neuropeptide bij de signalisatie bij deze voedselopname bestudeerd. Het neuropeptide SK en zijn receptor (SKR) zijn de belangrijkste elementen van deze signalisatieroute. Eén gen coderend voor de SK precursor en 2 genen coderend voor de receptor SKR werden via klonering, sequentieanalyse en transcript profile analyse gekarakteriseerd. Twee SK peptides werden teruggevonden in het SK precursor gen: Trica-SK-I (QTSDDYGHLRF) en Trica-SK-II (GEEPFDDYGHMRF). Twee SKRs (TcSKR1 and TcSKR2) werden gekarakteriseerd als G-proteïne gekoppelde receptoren met het typische 7-transmembraan domein. De genen kwamen tot expressie in de drie levensstadia (larve, pop en adult) en de hoogste expressieniveaus werden geregistreerd in de kop van het insect. Expressie van *Tcsk* en *Tcskr 1* waren beiden opgereguleerd wanneer de voedselvoorraad schaars was. De functie van SK signalisatie werd verder onderzocht met behulp van dsRNA-gemedieerde gensilencing. De genen coderend voor SK en SKR werden gesilenced door specifieke dsRNAs en een drastische toename aan voedselopname werd opgemerkt, wat suggereert dat SK signalisatie de voedselopname in *T. castaneum* inhibeert. Opmerkelijk was dat ook het uitschakelen van

skr2 expressie de voedselopname beïnvloedde , terwijl het uitschakelen van *Tcskr1* een kleiner effect had. Een directe injectie van het SK peptide reduceerde ook de voedselopname en bevestigde dus de inhibitorische functie van SK op de voedselopname.

In het tweede luik van dit onderzoek werd de activiteit-structuur relatie (SAR) van SK in de voedselopname onderzocht met behulp van een reeks synthetische SK (-gerelateerde) peptiden waarbij aminozuren werden vervangen in de C-terminus of verwijderd werden ter hoogte van de N-terminus. SKs hebben een geconserveerde C-terminus YGHMR/LF-NH2 sequentie en een variabele N-terminale extensie. SKs kunnen gesulfateerd (sSK) of niet-gesulfateerd (nsSK) zijn, afhankelijk of er een sulfaatgroep op de Y^6 zit. *T. castaneum* SK-II$^{[1-9]}$ (kortweg SK-II) bevat de sequentie FDDYGHMRF-NH_2.

Opvallend was dat snSK-II net zo actief bleek te zijn in de inhibitie van voedselopname als sSK-II. Dit suggereert ofwel dat de sulfaatgroep niet kritiek is voor de normale activiteit van het peptide bij de sSK- en nsSK-dosissen die gebruikt zijn in het experiment en die hoog genoeg waren om TcSKRs tot op zekere hoogte te activeren, of dat verdere modificaties zoals de sulfatatie verder *in vivo* nog plaatsvinden. Substituties zoals $G^5 \rightarrow A^5$ en $M^3 \rightarrow Nle^3$ leidden tot inactieve molecule. De C-terminale tetrapeptides HMRF-NH_2 lijken belangrijk te zijn voor de werking SK, hoewel YGHMRF- NH_2 meer effect heeft. *C. elegans* NLP-13 peptiden oefenen een gelijkaardig effect uit als SK terwijl NLP-12 peptiden een tegenovergesteld effect hebben als SK, aangezien de voedselopname net verhoogd wordt. De actieve kern van SK in het inhiberen van de voedselopname is echter nog niet gekend, wat voor verder onderzoek vraagt.

Het is niet eenvoudig om de observatie in het *in vivo* experiment met SK (-gerelateerde) peptiden te interpreteren, aangezien de bekomen data uiteindelijk het gevolg zijn van complexe processen in het organisme die veel meer inhouden dan enkel de SK signalisatie. Daarom is het ook interessant om te onderzoeken of de effecten die op het niveau van het hele insect te zien zijn, ook te zien zijn op het niveau van de receptor SKR, en meer specifiek om te onderzoeken of één bepaalde receptor in staat is om de signaaltransductie, geactiveerd door binding van het peptide met de receptor, verder te zetten. Om dit te doen werd een biologische toets ontworpen om deze twee SKRs *in vitro* verder te kunnen onderzoeken. In deze biotoets werd een Sf9-cellijn gebruikt als alternatief voor zoogdier-cellijnen. SKR is een G-proteïne gekoppeld eiwit. Bij de werking van deze klasse receptoren is de activatie van bepaalde Gα-

eiwitten cruciaal. Met deze biotoets werd eerst het functionele Gα-eiwit geïdentificeerd, uit twee kandidaat-eiwitten Gαs en Gαq. Intracellulaire cAMP-niveaus en calcium-fluxen werden gebruikt als indicator voor respectievelijk het geactiveerde Gαs- of geactiveerde Gαq-eiwit. Op basis van de accumulatie van intracellulair cAMP na activatie van SKR door SK bleek het Gαs-eiwit uiteindelijk de actieve component te zijn in de SK-signalisatie. Geen intracellulaire calcium-flux werd gedetecteerd tijdens het experiment. Verder bleek ook de sulfaatgroep op Y^6 belangrijk te zijn bij activatie van de receptor door SK, aangezien sSK 1000 maal effectiever was dan nsSK, voor beide SKRs. De respons van beide SKRs volgde een dosis-respons verhouding, met een nanomolaire concentratie als EC_{50}. Daarnaast werd met deze biotoets, en gebruik makend van de synthetische SK-peptiden, ook de structuur-activiteitsrelatie van SK bij de activatie van SKR bestudeerd. De activatie van beide SKRs bleek een zekere aminozuurlengte binnenin de geconserveerde regio DYGHMRF-NH_2 van SK te vereisen.

Uiteindelijk werd de SAR van SK *in silico* bestudeerd, met de modellering van TcSKRs en het dokken van peptiden op de modelstructuur van de receptor. De 3D-structuren van beide TcSKRs werden gemodeleerd via de eiwit threading strategie. Beiden vertoonden de typische seven-transmembraan-structuur van GPCRs maar verschilden in de diameter van de buitenopening van de holte gecreëerd door de 7 alfa-helices. De voorspelde structuur van TcSKR1 vertoont een bredere opening dan TcSKR2, wat peptiden toestaat om dieper in de holte te binden. Hieruit blijkt dat bij SKR1 zowel de extracellulaire regio, als het transmembranaire domein voorspeld worden betrokken te zijn bij de binding van het ligand, terwijl dat bij SKR2 enkel de extracellulaire regio blijkt te zijn. We kunnen dus besluiten dat beide TcSKRs een verschillend potentieel vertonen voor binding met SK en SK-gerelateerde peptiden. Het dokken van peptiden op TcSKR modellen leverde ook inzichten die de bioactiviteit van het peptide hielpen verklaren. De sulfaatgroep in sSK bijvoorbeeld, draagt bij tot de hoge activiteit van sSK door de binding van het peptide met TcSKR1 te bevorderen. Bovendien toonde het dokken van de peptiden van de Ala-substitutie reeks aan dat deze meer interacties vertonen met EL regio's in SKR2 dan in SKR1. De verhoogde interactie van peptiden met EL regio's bij SKR2 blijkt gelinkt te zijn aan het minimaliseren van de impact van Ala-substituties, wat resulteert in een verminderd verlies van activiteit van het SK peptide op SKR2 in vergelijking met SKR1. Deze docking experimenten kunnen echter niet zomaar gelinkt worden aan bioactiviteit, die nog steeds experimenteel bevestigd dienen te worden.

Het doel van dit onderzoek was om onze kennis omtrent de functie en mechanismen van SK-signalisatie en hun betrokkenheid bij regulatie van voedingsopname in *T. castaneum*, uit te breiden. Verder onderzoek kan ons nog meer informatie opleveren omtrent de mechanismen en evolutie van SK-signalisatie, alsook de crosstalk tussen verschillende neuropeptide signalisatiepathways. Inzichten in de regulatie van het voedingsgedrag van insecten leveren een goeie basis voor de ontwikkeling van nieuwe gewasbeschermingsstrategieën, zoals het potentieel van nsSK als pesticide, en verbeteringen van de reeds bestaande methoden.

Curriculum Vitae

Personal Information

Name	Na YU
Gender	Female
Nationality	Chinese
Place of birth	Shandong, China
Date of birth	September 27, 1986

Education

2010 - 2014	PhD in Applied Biological Sciences - Ghent University
2009-2010	Master study in Marine Biology - Ocean University of China, China
2005 - 2009	Bachelor in Biology - Ocean University of China, China

Scientific Contribution

A1 peer reviewed publications

1. **Yu, N.**, Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., Smagghe, G., 2013. Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Science 20, 4-14.

2. **Yu, N.**, Benzi, V., Zotti. M.J., Staljanssens, D., Kaczmarek, K., Zabrocki, J., Nachman, R.J., Smagghe, G., 2013. Analogs of sulfakinin-related peptides demonstrate reduction in food intake in the red flour beetle, *Tribolium castaneum*, while putative antagonists increase consumption. Peptides 41, 107-112.

3. **Yu, N.**, Nachman, R.J., Smagghe, G., 2013. Characterization of sulfakinin and sulfakinin receptor and their roles in food intake in the red flour beetle *Tribolium castaneum*. General and Comparative Endocrinology 188, 196-203.

4. **Yu, N.**, Smagghe, G., 2014. Characterization of sulfakinin receptor 2 and its role in food intake in the red flour beetle, *Tribolium castaneum*. Peptides 53, 232-237.

5. **Yu, N.**, Swevers, L., Nachman, R. J., Smagghe, G., 2014. Development of cell-based bioassay with Sf9 cells expressing TcSKR1 and TcSKR2 and differential activation by sulfated and non-sulfated SK peptides. Peptides 53, 238-242.

6. **Yu, N.**, Smagghe, G., 2014. CCK(-like) and receptors: structure and phylogeny in a comparative perspective. General and Comparative Endocrinology, in press.

7. Christiaens, O., Delbare, D., Van Neste, C., Cappelle, K., **Yu, N.**, De Wilde, R., Van Nieuwerburgh, F., Deforce, D., Cooreman, K., Smagghe, G., 2014. Differential transcriptome analysis of the common shrimp *Crangon crangon*: special focus on the nuclear receptors and RNAi-related genes. General and Comparative Endocrinology, in press.

International conferences

1. **Yu, N.**, Benzi, V., Nachman, R. J., Smagghe, G. Expression profile and role of sulfakinin peptide and sulfakinin receptor in Tribolium castaneum. The 26th Conference of European Comparative Endocrinologists (CECE), Zürich, Switzerland, 22 – 25 August, 2012. (oral presentation)
2. **Yu, N.**, Nachman, R. J., Smagghe, G. Sulfakinin and sulfakinin receptor regulate food intake in *Tribolium castaneum*. iBeetle Symposium "New Horizons in Molecular Zoology, Göttingen, Germany, 25 March, 2013. (oral presentation)
3. **Yu, N.** G-protein coupled receptors and their application in pest management. The 1st Brazil-Belgium Conference of Innovative Technologies Applied to Pest Control, Pelotas, Brazil, 4 - 6 December, 2013. (oral presentation)
4. **Yu, N.**, Lin, X., Nachman, R. J., Smagghe, G. Sulfakinin signaling regulates food intake in the red flour beetle *Tribolium castaneum*. The 2014 Belgian Peptide Group Meeting, Gent, Belgium, 10 - 11 February, 2014. (poster presentation)
5. **Yu, N.**, Zotti, M. J., Swever, L., Nachman, R. J., Smagghe, G. Sulfakinin signalling regulates food intake in the red flour beetle *Tribolium castaneum*. The Seventh International Symposium on Molecular Insect Science, Amsterdam, Netherland, 13 – 16 July, 2014. (poster presentation)

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