Screening and purification of bioactive peptides with potential to activate the cholecystokinin receptor type 1

ir. Dorien STALJANSSENS

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Chemistry

Promotors:	**Prof. Dr. ir. Guy Smagghe** Department of Crop Protection Faculty of Bioscience Engineering Ghent University, Ghent, Belgium
	Prof. Dr. ir. John Van Camp Department of Food Safety and Food Quality Faculty of Bioscience Engineering Ghent University, Ghent, Belgium
Chair of the examination committee:	**Prof. Dr. ir. Wim Verbeke** Department of Agricultural Economics Faculty of Bioscience Engineering Ghent University, Ghent, Belgium
Members of the examination committee:	**Prof. Dr. Els J.M. Van Damme** Department of Molecular Biotechnology Faculty of Bioscience Engineering Ghent University, Ghent, Belgium
	Prof. Dr. ir. Tom Van de Wiele Department of Biochemical and Microbial Technology Faculty of Bioscience Engineering Ghent University, Ghent, Belgium
	Prof. Dr. ir. Katleen Raes Research Group EnBiChem Department of Industrial Engineering and Technology University College West-Flanders, Kortrijk, Belgium
	Dr. Aart van Amerongen Group Bioactive Food Ingredients Food & Biobased Research Wageningen University and Research Centre Wageningen, The Netherlands
Dean:	**Prof. Dr. ir. Guido Van Huylenbroeck**
Rector:	**Prof. Dr. Paul Van Cauwenberge**

Screening and purification of bioactive peptides with potential to activate the cholecystokinin receptor type 1

ir. Dorien STALJANSSENS

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Chemistry

Titel van het doctoraat in het Nederlands:

Screening en opzuivering van bioactieve peptiden met potentieel om de cholecystokinine receptor type 1 te activeren

Please refer to this thesis as:

Staljanssens, D. (2013). Screening and purification of bioactive peptides with potential to activate the cholecystokinin receptor type 1. PhD thesis. Ghent University, Ghent, Belgium.

ISBN-number: 978-90-5989-645-1

Cover front: woman in front of computer in multiwell plate
Cover back: woman walking away in multiwell plate

This research was supported by the Special Research Fund of Ghent University (project 01J3309).

The research was performed at:

Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Laboratory of Food Chemistry and Human Nutrition, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Department of Internal Medicine, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium

Laboratory of Molecular Biology and Biochemistry, The Rockefeller University, New York, NY

Preface

Als pas afgestudeerde in 2009, mijn leven rustig kabbelend, begon ik aan een doctoraat, vol jeugdig enthousiasme en idealisme, absoluut niets vermoedend van wat er mij te wachten zou staan. Ik probeerde, leerde, herbegon, kanaliseerde mijn frustraties en boekte soms resultaat. Onderzoek loopt zelden van een leien dakje, maar kleine succesjes gaven me de moed om steeds verder te doen. De weg was soms eenzaam, maar je kweekt er een olifantenhuid door en leert dingen in een groter geheel te zetten. De kansen die ik door mijn doctoraat gekregen heb zijn onbetaalbaar en onvergetelijk. Ik ging op congres naar Bali, Turijn en Zürich en ontmoette wetenschappers uit alle uithoeken van de wereld. Ik kon een deel van mijn onderzoek uitvoeren in een labo in New York, waar ik moest knokken voor mijn plaats tussen al die talentvolle en ambitieuze mensen, maar waar ik tegelijk zoveel kon groeien als persoon. Het was een bijzonder verrijkende ervaring die mijn beeld op de wereld en de manier waarop ik in het leven sta voor goed heeft veranderd. Nu ik sinds enkele maanden terug in België ben, mijn heimat waar ik als een vis in het water ben, kom ik opnieuw tot rust en kan enkel met ongelooflijk veel dankbaarheid terugkijken op die woelige periode. Ik ben een ander mens. Ik heb zelfstandig leren denken, ik heb geleerd dat het leven, noch wetenschap, zwart-wit is en dat er in al het grijs heel erg veel schoonheid en waarheid schuilt. Als je natuurlijk je ogen durft te openen om dit te kunnen zien.

Ik dank Prof. Guy Smagghe en Prof. John Van Camp voor de vleugels die ze mij gegeven hebben, voor hun vertrouwen, hun luisterend oor, hun goede raad en hun geloof in mij en mijn onderzoek. Hoewel ze het vaak erg druk hadden, heb ik toch steeds het gevoel gehad dat ik altijd bij hen terecht kon.

I thank Prof. Sakmar from the Rockefeller University in New York for hosting me in his lab and for believing in my new research idea. I thank Prof. Huber for the brainstorming sessions and the cooperative and innovative lab experiments. I thank Dr. Park for teaching me several new techniques, for the guidance througout my project and for the day-to-day follow-up. I also thank Manija Kazmi, Carlos Rico and all the other members of the Sakmar lab who were always providing me the necessary help.

Ik dank Dr. Winnok De Vos voor de vele experimenten die we samen hebben uitgevoerd aan de confocale microscoop, de hulp bij de dataverwerking en het schrijven van de eerste papers. Verder dank ik ook Prof. Kris Gevaert voor de LC/MS analyses, Prof. Boon en Prof. Robrecht Raedt voor het gebruik van het labo voor de dierproeven, Prof. Moises Zotti voor de modellering en docking

experimenten en Geert Meesen voor het ontwikkelen van een automatisch injectiesysteem gekoppeld aan de confocale microscoop.

Ik dank de leden van de examencommissie, nl. Prof. Wim Verbeke , Prof. Els Van Damme, Prof. Tom Van de Wiele, Dr. Aart Van Amerongen en Prof. Katleen Raes voor het lezen van mijn manuscript en de constructieve opmerkingen.

Ik dank mijn thesisstudenten, Nadine Al Shukor, Annelies Billiet, Hanne Vriens, Anneleen Schollier, Jens Van Vynckt en Umman Tanriverdi voor hun hulp en gezelschap in het labo.

Ik dank mijn collega's van het labo agrozoölogie en het labo levensmiddelenchemie en humane voeding voor het uitwisselen van kennis in het labo, de aanmoediging, het uitwisselen van ideeën, het luisteren naar al mijn verhalen tijdens het koffie drinken, het delen van een lach en een traan en gewoon hun aanwezigheid.

Tot slot dank ik ook mijn familie en vrienden voor hun onvoorwaardelijke steun en om mij graag te zien.

Table of Contents

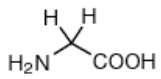
List of abbreviations

α-MSH	α-melanocyt stimulating hormone
AC	affinity chromatography
ACE	angiotensin converting enzyme
AgRP	agouti-related peptide
AP	area postrema
ARC	arcuate nucleus
BMI	body mass index
CART	cocaine and amphetamine-regulated transcript
CCK	cholecystokinin
CCK-8S	sulfated cholecystokinin octapeptide
CCK1R	cholecystokinin receptor type 1
CCK2R	cholecystokinin receptor type 2
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CHO	chinese hamster ovary
CHS	cholesteryl-hemasuccinate
CNS	central nervous system
CV	column volume
CVD	cardiovascular diseases
DVZ	devazepide
DM	dodecyl-β-D-maltoside
DMN	dorsomedial nucleus
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
EMEA	European Medicine Agency
FBS	foetal bovine serum
FDA	U.S. Food and Drug Administration
GBP	gastric bypass
GFP	green fluorescent protein
GHS receptor	growth hormone secretagogue receptor
GI	gastrointestinal tract
GPCR	G-protein coupled receptor
LC-MS	liquid chromatography – mass spectrometry
LHA	lateral hypothalamic area
MCR	melanocortin receptor
MW	molecular weight
MWCO	molecular weight cut-off
NABB	nanoscale apo-lipoprotein bound bilayer
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
OXM	oxyntomodulin
PDL	poly-D-lysine-hydro-bromide
PMSF	phenylmethanesulfonyl fluoride solution
POMC	pro-opiomelanocortin

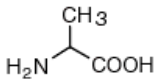
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PP	pancreas polypeptide
PVN	paraventricular nucleus
PYY	peptide tyrosine tyrosine
QSAR	quantative structure-activity relationship
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
VMN	ventromedial hypothalamic nucleus
zap1	zebrafish apolipoprotein 1

Overview of amino acids

Small

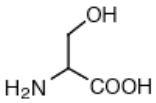


Glycine (Gly, G)
MW: 57.05

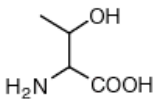


Alanine (Ala, A)
MW: 71.09

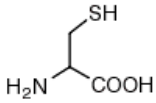
Nucleophilic



Serine (Ser, S)
MW: 87.08, pK_a ~ 16

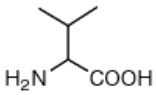


Threonine (Thr, T)
MW: 101.11, pK_a ~ 16

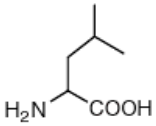


Cysteine (Cys, C)
MW: 103.15, pK_a = 8.35

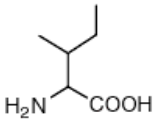
Hydrophobic



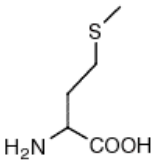
Valine (Val, V)
MW: 99.14



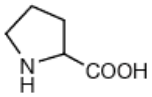
Leucine (Leu, L)
MW: 113.16



Isoleucine (Ile, I)
MW: 113.16

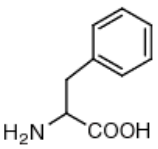


Methionine (Met, M)
MW: 131.19

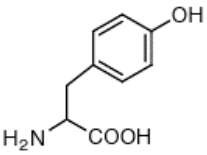


Proline (Pro, P)
MW: 97.12

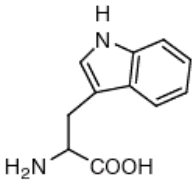
Aromatic



Phenylalanine (Phe, F)
MW: 147.18

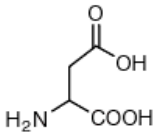


Tyrosine (Tyr, Y)
MW: 163.18

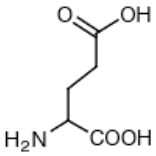


Tryptophan (Trp, W)
MW: 186.21

Acidic

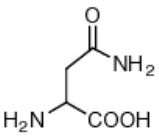


Aspartic Acid (Asp, D)
MW: 115.09, pK_a = 3.9

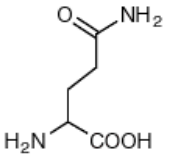


Glutamic Acid (Glu, E)
MW: 129.12, pK_a = 4.07

Amide

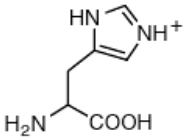


Asparagine (Asn, N)
MW: 114.11

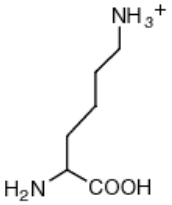


Glutamine (Gln, Q)
MW: 128.14

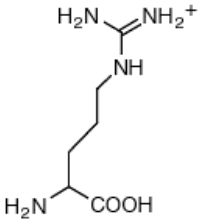
Basic



Histidine (His, H)
MW: 137.14, pK_a = 6.04

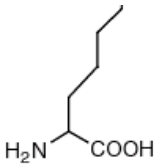


Lysine (Lys, K)
MW: 128.17, pK_a = 10.79



Arginine (Arg, R)
MW: 156.19, pK_a = 12.48

Unnatural amino acid



Norleucine (Nle)
MW: 131.17

Aim and outline of the thesis

Obesity is a worldwide health problem. One of the systems involved in appetite and weight regulation is the cholecystokinin (CCK)-signaling system. In this thesis, we tried to develop a novel method to prevent and treat obesity by influencing the CCK-signaling system. CCK, a hormone and neuropeptide, is produced by the intestines upon ingestion of food. When it binds to the cholecystokinin receptor type 1 (CCK1R), which is localized on vagal afferents in the gastrointestinal tract, it induces a feeling of satiety. Bioactive peptides are peptides that can be released from food protein following enzymatic hydrolysis and that have an effect on physiological processes. These peptides could also be used to exert an effect on the CCK-signaling system, they are promising candidates to mimic the effect of CCK. The **goal of this thesis** is to discover novel bioactive peptides with the ability to activate the CCK1R, thereby inducing a feeling of satiety. Such peptides can be used as satiating ingredients for the development of functional foods as an aid in the battle against obesity.

To reach this goal, four major steps are crucial: method development to assess *in vitro* CCK1R activity, screening of different protein hydrolysates, purification and identification of the active peptides and evaluation of the *in vivo* effect on food intake. Peptides are produced by enzymatic hydrolysis from well-known protein sources such as milk and soy protein and the potential of these hydrolysates to activate the CCK1R is measured. The active protein hydrolysates are separated in different molecular weight classes and purification and identification of the peptides takes place. Also, a new technique for peptide isolation and identification is proposed. Finally, the *in vivo* effect on food intake is assessed with rat experiments.

The outline of this work is schematically represented in Figure 0.1. More specifically, the goals of this work were:

- To set up a cell-based bioassay using CHO cells expressing the CCK1R to screen for compounds with CCK1R activity. The cell-based bioassay was validated using pure compounds such as the natural hormone (CCK), a partial agonist (JMV-180) and an antagonist (lorglumide) **(Chapter 2)**.
- To evaluate the potential of different protein hydrolysates from several food sources to activate the CCK1R and compare the reliability and specificity of measurements with a fluorescence plate reader and a confocal laser scanning microscope **(Chapter 3)**.

- To purify and to identify the active peptides by separation of the hydrolysates showing CCK1R activity in different molecular weight fractions using size exclusion gel filtration chromatography **(Chapter 4)**.
- To test the effect of the active peptide fractions on food intake *in vivo* in rats **(Chapter 4)**.
- To develop a novel pull-down technique using nanoscale apo-lipoprotein bound bilayer particles (NABBs) in which the CCK1R can be incorporated **(Chapter 5)** to identify CCK1R binding peptides

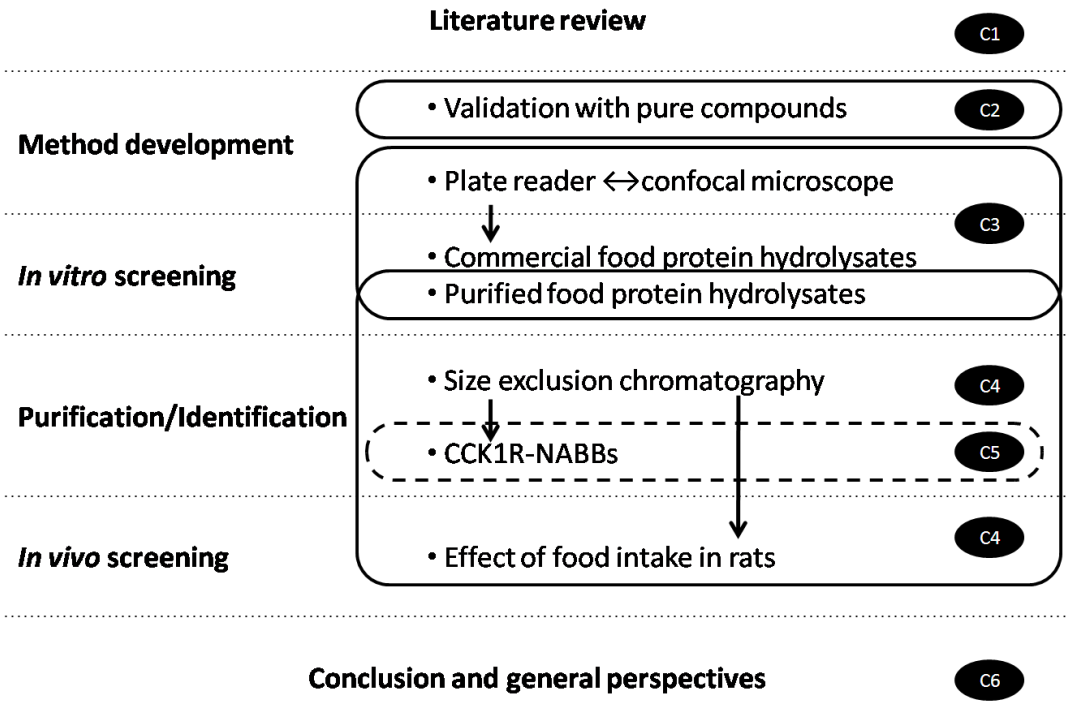


Figure 0.1. Outline of this study. C= chapter.

Chapter 1

Literature review on food intake and obesity in relation to CCK and the CCK1 receptor

Parts of this chapter have been published in Staljanssens, D.; Azari, E. K.; Christiaens, O.; Beaufays, J.; Lins, L.; Van Camp, J.; Smagghe, G., The CCK(-like) receptor in the animal kingdom: Functions, evolution and structures. *Peptides* **2011,** 32, (3), 607-619 and Staljanssens, D.; Smagghe, G.; Van Camp, J., Protein-derived bioactives affecting CCK-induced satiety. Agro Food Ind. Hi Tec 2012, 23, (2), 6-8

Chapter 1 Literature review on food intake and obesity in relation to CCK and the CCK1 receptor

1.1 Introduction

Obesity, defined as a body mass index (BMI) greater than 30 kg/m², and overweight, defined as a BMI greater than 25 kg/m² , is a world-wide epidemic as it was estimated that in 2008 nearly 500 million people were obese and in total 1.4 billion people were overweighed. Obesity and overweight form a serious health problem, globally being the fifth leading risk for death. Furthermore, obesity and overweight are a major risk factor for diabetes type 2, cardiovascular diseases (CVD), musculoskeletal disorders and some cancers (*293*). In recent decades, the mechanisms for appetite regulation and their potential interaction points have gained a lot of attention in view of the continuous rise in the prevalence of obesity (*22*). One important mechanism involves the hormone cholecystokinin (CCK) and its interaction with the cholecystokinin receptor type 1 (CCK1R), upon activation of this receptor, satiation and satiety is induced. (*52, 102, 185*).

In this chapter, the scientific literature concerning mechanisms that regulate food intake and therapies against overweight and obesity will be briefly described. As cholecystokinin receptor type 1 (CCK1R) interactions are the main subject of this PhD thesis, the functions, structure, evolution and the ligands of this receptor will be pointed out. As a final point, bioactive peptides, protein hydrolysates and proteins that influence food intake through the CCK-signaling system will be discussed.

1.2 Mechanisms affecting food intake

Food intake regulation is based on a complex balance between several mechanisms affecting appetite and satiety at short and long term (*13, 56, 102, 128*). When food is ingested, the stomach expands and this physical alteration can be sensed by mechanoreceptors on neurons enervating the stomach, giving a signal of satiety to the brain (*234*). Next to the physical change in our body due to ingestion of food, the gastrointestinal tract (GI) constantly produces different hormones depending on the presence of nutrients in the stomach and intestines. These hormones, also called gut peptides, affect appetite and satiety on the short term (within hours) (*298*). On the short term, a distinction should be made between satiation, the termination of a meal, and satiety, the time after a meal when a feeling of fullness is perceived and there is no desire to eat (*28*). However, even in scientific literature this distinction is not always made and the words satiation or satiety are used to indicate both satiation and satiety, for example in the study of Moran et al. (*185*), Verbaeys et al.

(*284*) and Lateef et al. (*157*). The search on the topic "cholecystokinin AND satiety" yields 1255 results in ISI Web Of Knowledge, whereas the "cholecystokinin AND satiation" only yields 212 hits, therefore in the rest of this work, the word satiety will be used to indicate both terms.

The production of hormones acting on the long term (within weeks) depends on the amount of adipose tissue in the body (*265*). These short and long term determinants for food intake together with stimuli like smell, sight and social context of food are integrated by the hypothalamus which finally controls eating behavior (*128*).

1.2.1 Central pathways regulating food intake

A schematic representation of the peripheral and central pathways regulation food intake is shown in Figure 1.1. The hypothalamus, and more specifically the arcuate nucleus (ARC) is the most important part of the brain for regulation of food intake. It is thought that peripheral signals (like gut peptides) can reach the hypothalamus via the median eminence, a circumventricular organ with an incomplete blood-brain barrier lying close to the hypothalamus (*53*). Within the ARC, two distinct groups of neurons exist of which one enhances food intake and the other reduces food intake. The group of neurons responsible for reducing food intake contains pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). The group of neurons that enhances food intake contains neuropeptide Y (NPY) and agouti-related peptide (AgRP). POMC is the precursor for α-melanocyt stimulating hormone (α-MSH) that binds on melanocortin receptors (MC3R and MC4R) to suppress food intake. The receptor for CART is unknown (*53, 265*). NPY acts on Y receptors (Y1 and Y5) to increase food intake and AgRP is an antagonist of MC3R/MC4R. POMC/CART and NPY/AgRP neurons project to the paraventricular nucleus (PVN). These neurons also communicate with other second order hypothalamic neuropeptide neurons. POMC/CART also projects to the ventromedial hypothalamic nucleus (VMN) and NPY/AgRP to the lateral hypothalamic area (LHA) and dorsomedial nucleus (DMN) (*245, 265*). The LHA used to be considered as the hunger centre and the VMN as the satiety centre (*175*).

The dorsal vagal complex (DVC) within the brainstem comprises the nucleus tractus solitarius (NTS) and the area postrema (AP). Peripheral signals can reach the NTS by crossing the incomplete blood-brain-barrier in the AP. Vagal afferents from the gut convey information from the gut directly to the NTS. It is well established that there are neural connections between the hypothalamus and the brain stem to modify appetite (*53, 265*).

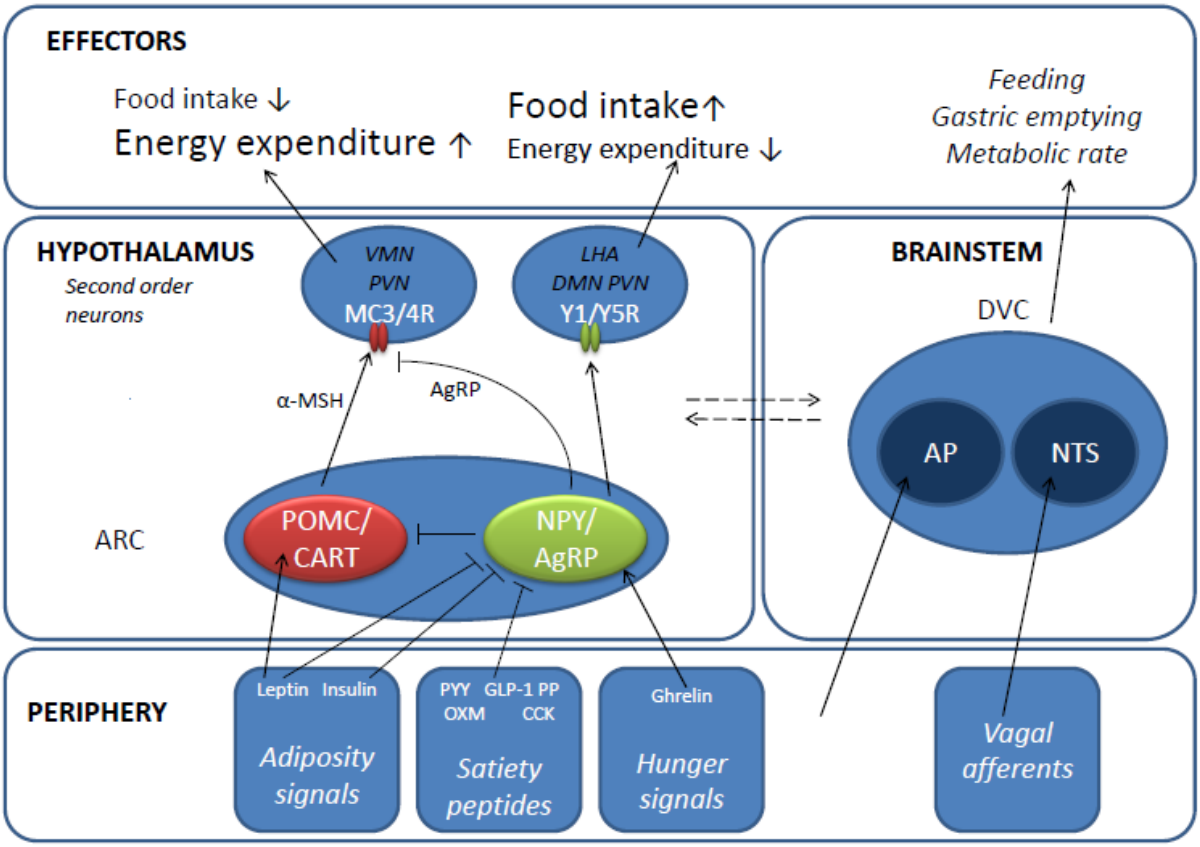


Figure 1.1. Simplified representation of central regulation of food intake and integration of signals from the periphery. Neurons inducing satiety are shown in red. Neurons inducing hunger are shown in green. → : direct stimulatory, ⊣ : direct inhibitory, -≫ : indirect pathways. Redrafted after Badman & Flier (*13*).

1.2.2 Gut peptides: affecting food intake at short term

Ghrelin

To date, ghrelin is the only known gut peptide that induces hunger. It is secreted by endocrine cells in the fundus of the stomach. Plasma concentrations are elevated before meals and fall back to low levels 60 to 120 min after eating. Ghrelin signals via the growth hormone secretagogue receptor (GHS receptor) in the hypothalamus and on vagal afferents. To a limited extent, ghrelin might be produced within the hypothalamus itself where it can activate NPY/AgRP neurons and neurons in the LHA directly (*128*). Some evidence exists that antagonists of the ghrelin receptor can inhibit food intake and might be useful as therapeutics in the treatment of obesity (*9, 128*).

Peptide YY (PYY)

PYY is produced by L-cells in the distal small intestine and colon upon stimulation with nutrients, especially fat reaching the ileum (*55*). It may play a role in the "ileal brake": gastric emptying and jejunal motility is slowed down, resulting in enhanced satiety (*171*). PYY has a high affinity for the Y2 receptor in the hypothalamus. Central injection of an agonist of the Y2 receptor resulted in reduced food intake (*18*). Gastric bypass (GBP) surgery increases the levels of PYY, suggesting that pharmacologically stimulating this effect might be effective to induce weight loss (*128*).

Pancreas polypeptide (PP)

PP is produced in F-cells of pancreatic islets of Langerhals upon ingestion of food, exercise and hypoglycaemia (*140*). It binds to Y4 receptors in the hypothalamus, AP and adjacent brainstem areas (*234, 265*). PP would cause a moderate food intake reduction by decreasing gastric emptying rate through direct interaction with receptors in the brainstem and hypothalamus (*140*). It might also exert its diminishing effect on food intake via the vagus nerve, as vagatomy counteracts the anorectic effects of PP (*9*). Intravenous injection of PP resulted in a 25% reduction in a 24 h food intake in humans with a normal weight, but is rapidly degraded in circulation (*18*). Y4 agonists might form another strategy for the development of anti-obesity therapeutics (*265*).

Glucagon-like peptide 1 (GLP-1)

Like PYY, GLP-1 is secreted by the L-cells of the small and large intestines as a response to nutrients. It is also expressed in neurons of the NTS. It is an incretin hormone, meaning that it stimulates insulin release after an oral glucose load (*128, 265*). GLP-1 binds to the GLP-1 receptor which is localized in pancreatic islets, the ARC and PVN in the hypothalamus and the AP in the brainstem (*53*), but might also exert its effect through vagal afferents (*132*). Together with PYY, GLP-1 may induce an 'ileal brake', enhancing the feeling of fullness and satiety (*128*). Several studies report that peripheral administration of GLP-1 causes a reduction in food intake (*287*). GLP-1 analogues can be useful in the treatment of overweight patients with diabetes type 2, but also promising results concerning a GLP-1 analogue have been found for the treatment of obese non-diabetic individuals (*10, 53*).

Oxyntomodulin (OXM)

OXM is produced by L-cells in the intestine proportional to the caloric intake. It exerts an anorectic effect via the GLP-1 receptor. However, affinity of OXM for GLP-1 receptor is much lower than GLP-1

itself, so it is possible that it also signals via another receptor (*128, 265*). OXM analogues resistant to dipetidyl peptidase IV are being developed as anti-obesity therapeutics (*67*).

Cholecystokinin (CCK)

One of the most important hormones for induction of a feeling of satiation and satiety is the hormone CCK (*69*). CCK release, receptor interaction, function and structure are extensively discussed later on in this chapter.

1.2.3 Adiposity signals: affecting food intake at long term

Leptin

Leptin is produced by adipocytes, proportional to the amount of fat mass (*48*). The concentration of leptin in the blood does not seem to be dependent on food intake (*150*). This hormone exerts its anorectic effect via leptin receptors on NPY/AgRP (inhibiting effect) and POMC/CART (activating effect) neurons in the ARC (*265*). It informs the brain about changes in energy balance and the amount of fat stored in the body (*102*), therefore having an effect on food intake on the long term. Administration of leptin to obese children with leptin deficiency results in a decrease of fat mass (*80*). Still, obesity in humans can also be related to resistant leptin receptors (*188*).

Insulin

Insulin is produced by β-cells of the pancreas. It stimulates glycogen and lipid synthesis (*103*). It acts through receptors in the ARC (*265*). Administration of insulin to the brain proved to reduce appetite in rodents and primates. It also potentiates other satiety factors such as CCK (*13*).

1.3 Treatment strategies for overweight and obesity

1.3.1 Behavioral therapy

The most obvious treatment for obesity and overweight is lifestyle modification. The diet must be adapted and the physical activity must be increased. Total energy intake should be reduced, e.g. a deficit of 500 kcal per day results in a weight loss of 0.5 kg after one week (*38*). An energy intake goal must be set dependent on the individual's baseline weight. Furthermore, it is important that the physical activity is increased and to create an exercise routine resulting in an energy expenditure of about 1000 kcal/week. To obtain these goals, obese and overweighed people must change their eating and exercise behavior and therefore might get help from a healthcare provider (*38*).

1.3.2 Pharmacotherapy

Although caloric restriction and increased physical activity can induce substantial weight loss, the majority of the people relapse in old habits and weight loss is not sustained (*13*). In the latter case, obese individuals might combine lifestyle modification with medication, however not many obesity drugs are available on the market. One well-known obesity drug is **orlistat;** it is a lipase inhibitor and therefore reduces fat absorption in the gastrointestinal tract (*207*). It is marketed under the brandnames Alli (over-the-counter, GlaxoSmithCline) or Xenical (under prescription, Roche). After 4 years, lifestyle modification in combination with orlistat results in 2.7 kg more weight reduction than lifestyle modification alone (*276*). Gastro-intestinal side effects like flatulence and fecal incontinence occur often and can be decreased by lowering fat-intake (*207*).

Sibutramine, originally developed as an antidepressant, is an appetite suppressant by inhibiting the reuptake of norepinephrine and serotonine (*38, 207*). After one year, on average a weight loss of 5 kg is obtained by sibutramine. Most important side effects are obstipation, insomnia, nausea and dry mouth (*207*). It used to be marketed under various brandnames such as Meridia (Abbott Laboratories), Reductil, and Sibutrex. The FDA (U.S. Food and Drug Administration) asked in October 2012 to Abbott Laboratories to voluntarily withdraw Meridia from the market because of clinical data indicating increased risk of cardiovascular adverse effects (*81*). In 2010, the EMEA (European Medicines Agency) also decided in 2010 to take sibutramine from the market (*76*) as the risks of this medicine were higher than the benefits.

Another appetite suppressant is **rimonabant** and was marketed as Acomplia (Sanofi-Aventis). It acts centrally and peripherally by antagonizing the cannabinoid-1 receptors which are involved in the regulation of energy balance (*13*). It can induce a weight loss of 4.6 kg compared to placebo treatment. Side effects include nausea, dizziness, diarrhea and insomnia (*207*). The EMEA also decided to withdraw the marketing authorization for this product in 2007 (*75*).

Novel anti-obesity targets, for example melanocortin receptor agonists, are under development, however it can be concluded that the pharmacological approach to treat obesity thus far has not been very successful (*207, 305*). Therefore, natural products form an interesting and safe alternative strategy for treatment of obesity (*305*).

1.3.3 Natural products and functional foods

Natural products might be used for the treatment of obesity via several mechanisms including (i) lipase inhibition, (ii) suppression of food intake, (iii) stimulation of energy expenditure, (iv) inhibition of adipocyte differentiation and regulation of lipid metabolism (*305*).

(i) Natural products exist that have a similar mode of action as orlistat i.e. the reduction of fat absorption by inhibiting lipases in the gastrointestinal tract. Some examples are ellagic acid from *Acanthopanax senticosus* (stem bark) and a crude ethanolic extract from the leaves from *Nelumbo nucifera*. Also different types of tea (e.g. oolong, black and gree tea) are widely studied in this context (*108, 163, 191, 271, 305*), different types of polyphenols showed strong lipase inhibitory activity.

(ii) Food intake suppressants alter the peripheral peptide satiety signals and levels of the neuropeptides in the CNS (*305*). Some examples of products that alter satiety probably through peripheral signals are soybean beta-conglycinin hydrolysate (*122*), pea protein hydrolysate (*59*), whey protein (*306*) and yellow pea protein (*255*). *Hoodia gordonii*, a succulent from South African countries, is an example of a natural appetite suppressant probably acting at the CNS (*282, 283*).

(iii) An excess in energy can be dissipated via thermogenesis, in which UCP1 is a key player as it discharges the proton gradient that is produced during oxidative phosphorylation, thereby transforming energy into heat (*305*). Important natural components for thermogenesis are caffeine (*70, 220, 292*), capsaicin ((*224*), catechins and green tea extract (*184, 297*).

(iv) Adipogenesis might be inhibited by polyunsaturated fatty acids (*167, 206*). Furthermore, phytochemicals such as resveratrol, esculetin, quercetin, genistein, capsaicin and conjugated linoleic acids can induce apoptosis in maturing pre-adipocytes (*110, 125, 131, 301, 302*). The adipocyte cell cycle is thus an important target in the treatment of obesity (*305*). Also stimulation of lipolysis can be useful to combat obesity, however this requires that the released fatty acids are oxidized (*155*). This can be ameliorated by activation of the β_3-adrenergic receptor, natural components such as flavonoids in the leaves of *Nelumbo nucifera* can have this effect (*205, 305*).

An extensive list with natural anti-obesity components can be found in a review by Yun (*305*). Natural products can be used for the development of functional foods and food supplements that enhance satiety. Doyon & Labrecque (*66*) propose the following definition for a functional food:

> "A functional food is, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions."

Food supplements also contain natural products, but come in the form of pills, tablets, powders, ampoules or drop dispensing bottles and other similar forms (*274*). There is a huge range of weight control products available on the market, e.g. products containing herbal extracts as described above, fatty acid-based products to reduce abdominal fat, fibre-based supplements to prevent fat absorption and various proteins. However these products do not make specific claims on appetite, but rather specific health claims that promise consumers that they will feel full longer, stay satisfied, reduce hunger and so forth. Considerable appetite-control health claims are submitted to EFSA, but only few get approved because most studies fail to meet the requirements to demonstrate sustained and enduring effects of foods on appetite (*106*).

1.3.4 Bariatric surgery

Some obese people do not succeed to lose weight via the methods described above. In that case, the only way out might be bariatric surgery. Criteria for surgery are having a BMI of at least 40 kg/m² or a BMI of at least 35 kg/m² together with sleep apnea, diabetes, CVD or hampered daily functioning because of the excess weight (*37*). Substantial and long term weight loss can be obtained by bariatric surgery and it can also reduce diabetes, hypertension, hyperlipidemia and sleeping disorders. Although bariatric surgery has proven its positive effect on weight loss, serious risks like malabsorption should be taken into account (*13*).

1.4 The CCK(-like) receptor in the animal kingdom: function, evolution and structure

The cholecystokinin (CCK) receptor is a G-protein coupled receptor (GPCR) with the typical seven transmembrane (TM) domains (Figure 1.2), which is mainly expressed in the brain and the alimentary tract.

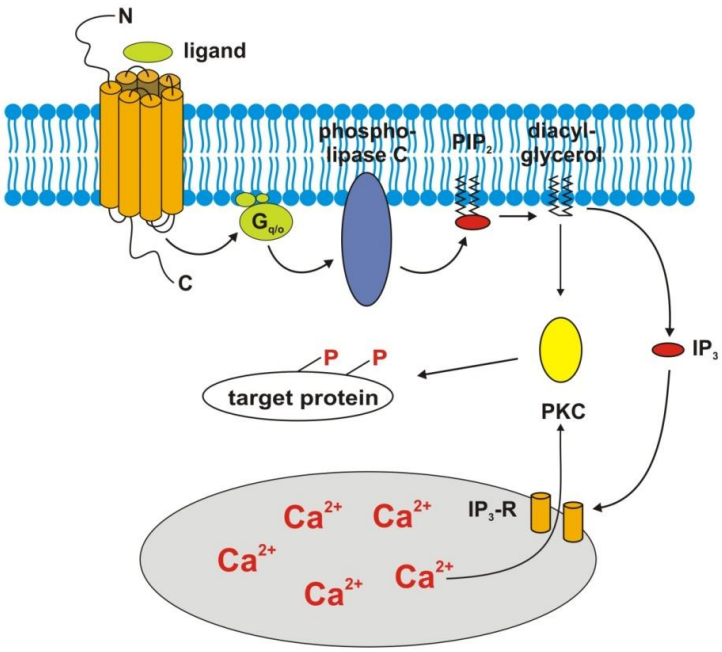


Figure 1.2. Signaling pathway of the CCK1R via the Gq type of G-protein coupled to the IP_3/DAG cascade reaction (modified from Blenau and Baumann (*27*)).

Binding of this receptor with its ligand, the neuropeptide hormone cholecystokinin (CCK) and/or gastrin, regulates diverse interesting physiological functions. To date most information is collected in vertebrates, specifically in humans. Of interest are that the binding of CCK on its receptor induces satiety, stimulates gall bladder contraction and bile secretion, plays a role in gastric juice secretion and gastrointestinal motility, and it should also pose an effect on nociception, panic, anxiety and memory and learning processes (*20, 21, 54, 212*). Next to vertebrates, Kawada et al. (*141*) reported in another chordate species, namely *Ciona intestinalis,* a CCK-like receptor (CioR protein) and peptide (cionin). Also, a CCK-like peptide has recently been demonstrated in Nematoda and this peptide, NLP-12, mediates its activity via a specific receptor as well, namely the CK receptor (*133*). Moreover, in the large phylum of Arthropoda, that is expected to represent 95% of the animal kingdom diversity, a peptide with similar structure and function to CCK, called sulfakinin (SK), is present and an

orthologous receptor, namely the SK receptor, exists (*153*). These observations indicate strong evidence that it is most likely that the CCK-like signaling system exists throughout the entire animal kingdom and this system has probably diverged from one common ancestor of the CCK and gastrin family of receptors over chordates, nematodes and arthropods/insects. In this section, a detailed overview is provided of the diversity of functions and the tissue-dependent expression of the CCK(-like) receptors and the natural ligands, supporting the large repertoire of interesting physiological mechanisms and interactions of the CCK signaling in animal behavior, growth and development. Here, the human (*Homo sapiens*), a nematode (*Caenorhabditis elegans*) and the fruitfly (*Drosophila melanogaster*) are employed as representative model organisms for vertebrates (Chordata), nematodes (Nematoda) and arthropods/insects (Arthropoda), respectively. Furthermore, the evolution of this intriguing family of CCK-like and gastrin receptors, supporting the above given hypothesis that the signaling system may have diverged over the animal kingdom from one common ancestor receptor, is reviewed in detail. With the recent availability of a large number of genomes in these phyla of the animal kingdom (Chordata, Nematoda, Arthropoda, Echinodermata, Acanthocephala, Annelida, Brachiopoda, Plathyelmintes, Rotifera etc.), this knowledge can bring new insights on the molecular evolution of this interesting family of CCK/SK and gastrin receptors.

1.4.1 Types, ligands and genetic background

Table 1.1 provides an overview of the different CCK(-like) receptors with their natural ligand, their functions and tissue-dependent expression on the basis of three model organisms, *H. sapiens*, *C. elegans* and *D. melanogaster*, respectively, overspanning three important animal phyla as Chordata, Nematoda and Arthropoda. Evidently also attention goes to fishes (Pisces with *Danio rerio*), birds (Aves with *Gallus gallus*), frogs (Amphibia with *Xenopus laevis*), tunicates (Ascidiacea with *C. intestinalis*), urchins (Echinodermata with *Stronglylocentrotus purpuratus*) and acorn worms (Hemicordata with *Saccologussus kowalevskii*) as the whole genome of several representatives has been sequenced in the last years. CCK (…DYMGWMDFamide) is a well-studied amidated peptide (*69*) of which in this PhD thesis the receptors are of special interest.

In humans two major types of the CCK receptor exist: namely the **CCK1 receptor** (**CCK1R**, previously called CCKA receptor, as mainly present in the alimentary tract) and the **CCK2 receptor** (**CCK2R**, previously called CCKB receptor, as mainly present in the brain). As other vertebrates like rats, chimpanzees, rhesus monkeys, dogs, rabbits, cows, birds and fish show good homology with the human CCK1R and human CCK2R, ranging between 87%-100% and 89%-100%, respectively, humans can be used as a model organism for the Vertebrata group.

Recently, Janssen et al. (*133*) discovered a CCK-like signaling system in nematodes. The *C. elegans* genome has two genes (*i.e*., T23B3.4 and Y39A3B.5) with high identity to the genes of the vertebrate CCK receptors. For the first gene, T23B3.4, the encoded protein did not have the necessary seven TM domains to be a GPCR, and so in turn, this gene was not further pursued. The other gene, Y39A3B.5, was predicted to encode four isoforms, but actually only 2 isoforms which are a combination of two of the predicted isoforms seem to exist and have seven putative TM domains; these two receptors are designated CKR-2a and CKR-2b. Then the same authors (*133*) used the *C. elegans* CCK-like receptors as a fishing hook and could identify two CCK-like neuropeptides: NLP-12a or also called CK-I (DYRPLQFamide) and NLP-12b or CK-II (DGYRPLQFamide).

As representative of arthropods/insects, the *D. melanogaster* genome contains two genes that have been identified as putative drosulfakinin receptors, DSK-R1 and DSK-R2 (*35, 121*). Kubiak et al. (*153*) cloned the DSK-R1 and brought it functionally to expression; DSK-R2 remains however to be cloned. Sulfakinins are a group of peptides which contain the consensus structure XDYGHMRFamide, where X is D or E (*196*). Three *D. melanogaster* sulfakinins or drosulfakinins (DSK) have been identified in recent years: DSK-1, DSK-2 and DSK-0. DSK-1 (FDDYGHMRFamide) was isolated from adult *D. melanogaster* (*194*) and DSK-2S (sulfated DSK-2; GGDDQFDDY(SO_3H)GHMRFamide) has been identified in the larval central nervous system (*14*). A third one, DSK-0 (NQKTMSFamide), has an atypical structure and was found inactive to the DSK-R1 (drosulfakinin receptor 1) (*153, 199*). The DSK receptors show a relatively good homology with SK receptors of other insects like the mosquitoes *Culex quinquifasciatus*, *Anopheles gambiae* and *Aedes aegypti,* and also the *cockroach Periplaneta americana*, being representatives of the Arthropoda phylum. Homology percentages ranged between 49% and 100%. Finally, it has to be noted that this CCK(-like) receptor seems unique for the animal kingdom as it cannot be found in plants or fungi.

Table 1.1 Overview of CCK-(like) receptors in *Homo sapiens*, *Caenorhabditis elegans* and *Drosophila melanogaster*: the name of the receptor, the natural ligand, function and tissue expression

	H. sapiens		***C. elegans***			***D. melanogaster***	
Name of receptor	Human-CCK1R	Human- CCK2R	Ce_CKR-1	Ce_CKR-2a	Ce_CKR-2b	DSK-R1	DSK-R2
Number of gene			T23B3.4	Y39A3B.5c/b	Y39A3B.5c/d	CG32540, CG6881	CG6857
Acc. Nr.	NP_000721.1	NP_795344.1	NP_491918.2	ACA81683.1	ACA81684.1	NP_001097023.1	NP_001097021.1
% similarity to human CCK1R	100	88	75	77		81	81
Natural ligand	Sulfated cholecystokinin (CCK-S)	(Sulfated) gastrin/ (sulfated) cholecystokinin (CCK-(S))	-	CKI (NLP-12a) CKII (NLP-12b)		Sulfated drosulfakinin (DSK-S)	Drosulfakinin? (DSK)
C-terminal structure	…DY(SO_3H)MGWMDF-NH_2	…YMGWMDF-NH_2/ …DYMGWMDF-NH_2	-	DYRPLQF-NH_2 DGYRPLQF-NH_2		…DY(SO_3H)GHMRF-NH_2	…DYGHMRF-NH_2 ?
Function	induce satiety; slowing down of GI motility; inhibition of gastric acid secretion; gall bladder contraction; exocrine and endocrine pancreas secretion; regulation of pancreas growth;	stimulation of nociception, memory and learning processes and endocrine pancreas secretion; stimulation of gastric acid secretion, induce panic and anxiety	-	amylase secretion, fat storage, contraction of innervated dorsal and ventral *Ascaris suum* body wall muscle preparations		increase the frequency of adult and larval gut contractions, increased the frequency of larval, pupal, and adult heart contractions	increase the frequency of adult animal foregut and larval anterior midgut contractions, increase the frequency of larval heart contractions
Tissue expression	mainly in GI; chief cells, D-cells and mucus cells in gastric mucosa; glucagon secreting cells, smooth muscle cells of GI: gall bladder, pylorus, intestines, sphincter of Oddi; vagal afferent fibers;	mainly in brain; parietal, ECL-, D-, and chief cells of gastric mucosa; glucagon secreting cells, insulin secreting cells and pancreatic acinar cells; smooth muscle cells of GI	-	(only information available about ligand expression)		(only information available about ligand expression)	(only information available about ligand expression)
Reference	(*20, 21, 54, 69, 135, 173, 187, 203, 204, 212, 227, 231, 244, 270*)		(*130, 133, 176, 177, 204*)			(*72, 121, 153, 194, 195, 197, 198, 204*)	

1.4.2 Tissue-dependent expression

The expression pattern of the CCK receptors in mammals seems to be comparable for different species (*69*). The CCK1R is mainly found in the gastrointestinal tract and, in humans, is expressed in chief cells, D-cells and mucus cells of the gastric mucosa, in glucagon secreting cells of the pancreas, in smooth muscle cells of the gall bladder, the pyloric sphincter, the intestines and the sphincter of Oddi. The CCK1R is also present on vagal afferent fibers and nerve cells of the myenteric plexus. The CCK2R is especially located in the brain, including the cerebellum, cerebral cortex, basal ganglia, and amygdala, but is also found on parietal, ECL, D and chief cells of the gastric mucosa, on glucagon secreting cells, insulin secreting cells and acinar cells of the pancreas and on smooth muscle cells of the gastrointestinal tract (*69, 158, 203, 225*).

In contrast to CCK receptors, to our knowledge no information is available on the tissue-dependent expression of CK and SK receptors in nematodes and insects. Few data are available about the tissue expression of the natural ligands for these receptors. Janssen et al. (*133*) demonstrated the presence of CK in nematodes in the ring interneuron DVA (interneuron with cell body in dorsal rectal ganglion and process extending to nerve ring) and in the anteriorly directed process of DVA, running along the ventral nerve cord, toward and around the circumpharyngeal nerve ring. As in vertebrates, CK peptides seem to play a neurotransmitter role.

In *D. melanogaster*, sulfakinins are observed in the larval superior protocerebrum, medial protocerebrum, thoracic ganglia and the posterior most abdominal ganglion [62]. Also in other insects, sulfakinins are found in the brain and the stomatogastric neurons, supporting the role of sulfakinins as a neurotransmitter in feeding behavior (*45, 65, 72, 73*). SK immunoreactivity is also found in the CNS of Crustacea (*137*). As well in vertebrates as in nematodes and arthropods, the peptide hormones CCK, CK and SK and their CCK(-like) receptors are present in the ganglia, which might illustrate their evolutionary kinship and might point at a neuromodulatory role for these peptides and their receptors.

1.4.3 Functions

As depicted in Figure 1.2, signaling through the CCK(-like) receptors happens via a G_q type of G-protein (*69, 133, 153*). *In vitro* studies on second messengers revealed the following cascade pathway: agonistic binding on the receptor results in activation of phospholipase C. This enzyme hydrolyzes phosphatidyl-inositol-4,5-biphosphate into the second messengers: inositol-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of Ca^{2+} from the endoplasmic reticulum, which

in its turn together with DAG will activate protein kinase C (PKC). PKC is responsible for the phosphorylation of other proteins which leads to different cellular responses (*69, 203*).

CCK is produced in the gastrointestinal tract and can exert an endocrine effect via receptors in the brain or a paracrine effect via receptors in the gut (*20, 261*). In humans, CCK which acts through the CCK1R can induce satiety, slow down gastrointestinal motility, stimulate secretion of pepsinogen, inhibit gastric acid secretion by stimulation of the production of fundic somatostatin, stimulate gall bladder contraction and relaxation of the sphincter of Oddi, and induce endocrine and exocrine pancreatic secretion. CCK that binds with the CCK2R can stimulate nociception, memory and learning processes, panic and anxiety, endocrine pancreas secretion, gastric acid secretion and gastric mucosa growth. The CCK receptor can also be involved in the development of cancer (*159, 256*). Extensive reviews about the functions of CCK and its receptors in humans can be found in the recent medical literature (*20, 54, 69, 203, 212, 227*) and an overview of these functions is shown in Figure 1.3. CCK would also be partially involved in the regulation of the cardiovascular function (*166*), and the effects of CCK on the exocrine pancreas are mediated by CCK1R on the vagus nerve (*160*). In general it is believed that most of the above mentioned functions are mediated via binding of CCK on the CCK1Rs of the vagal afferent fibers (*225*).

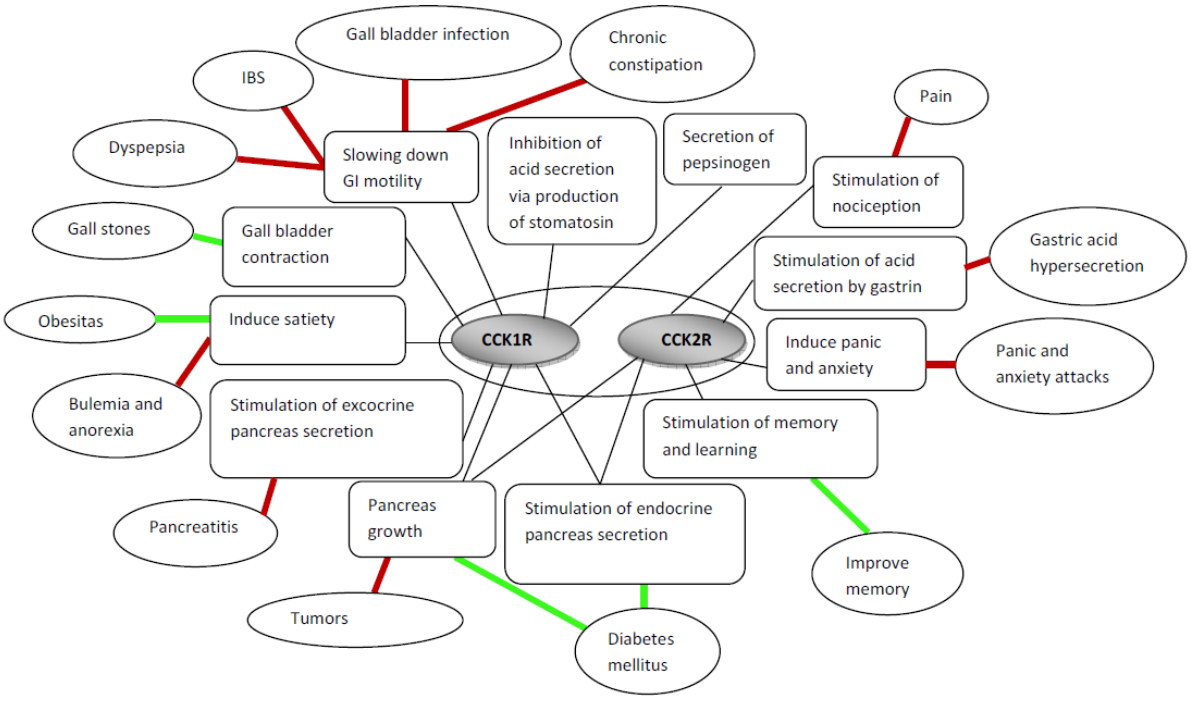


Figure 1.3. Overview of the different biological functions of the CCK1R and CCK2R. In the rectangles, different functions of CCK when it binds to the CCK1R or CCK2R are shown. In the ovals, diseases for which agonistic (green line) or antagonistic (red line) ligands could be used as therapeutics (*20, 21, 54, 173, 212*).

For invertebrates, Janssen et al. (*133*) could not show that CK peptides have an influence on the myotropic activity in a cockroach hindgut assay. The reason for this discrepancy is most likely that the structure of CK peptides is too different from that of sulfakinins to activate the SK receptor. In parallel, these authors could not demonstrate any effect of CK peptides on food intake or defecation rhythm in *C. elegans*. However, it should be mentioned that CKR-2 mutated *C. elegans* displayed a decrease in intestinal amylase activity and appear to have a higher fat storage. As a consequence, more research is needed to elicit the functions of CK peptides in nematodes and other invertebrates.

Like CCK, sulfakinins affect several biological processes. In the Arthropoda phylum it is shown that SK can induce satiety in *e.g.* the desert locust, *Schistocerca gregaria* (*289*), the German cockroach, *Blattella germanica* (*168*), the cricket, *Gryllus bimaculatus* (*179*), and reduced the carbohydrate feeding in the blowfly, *Phormia regina* (*65*). Moreover, sulfakinins induced myotropic activity of foregut and hindgut in *D. melanogaster* and cockroaches including *B. germanica* (*168, 195, 218*), increased the frequency of heart contractions in the American Lobster, *Homarus americanus,* and *D. melanogaster* (*58, 198*), and stimulated the secretion of α-amylase in the scallop, *Pecten maximus,* and the red palm weevil, *Rhynchophorus ferrugineus* (*116, 189*). In summary, the current experiments demonstrated that for vertebrates and arthropods, CCK and SK seem to share similar effects on feeding behavior. We believe that the latter phenomenon opens certain interesting avenues for research and potential applications in obesity or metabolic syndrome treatment and/or pest control.

1.4.4 Classification and evolution

The GPCRs can be divided into seven clans named A to F (*11, 83, 148*). A subfamily of clan A, clan D, E and F does not exist in humans. Therefore Fredriksson et al. (*85*) made a classification of all GPCRs present in humans based on five main families. The CCK receptors belong to the β-group of rhodopsin receptors in the latter classification or to the subfamily A6 in the A-F classification.

The predicted phylogenetic relationship of the different CCK(-like) receptors of different species in the animal kingdom which were obtained by blasting is shown in Figure 1.4. In this phylogenetic tree, five phyla can be distinguished: Chordata, Echinodermata, Arthropoda, Hemichordata and Nematoda. In the phylum of Chordata, next to a large selection of vertebrate species expressing CCK receptors, the CioR protein in the invertebrate vase tunicate *(C. intestinalis*) was found. Interestingly, a clear separation between the CCK1R and the CCK2R could be made in the vertebrate group. This could also be done for the phylum of the Nematoda, but here one group contains proteins which are similar to the two splice isoforms CKR-2a and CKR-2b of the *C. elegans* gene Y39A3B.5, and the other

group contains proteins similar to the predicted expression product of T23B3.4 *i.e.* CKR-1. However, as discussed in the introduction, the encoded protein for T23B3.4 did not yield the necessary 7 TM.

So in the phylum of the Nematoda, one group contains functional receptors and the other one probably does not, which implies that only one real group is present in Nematoda. Furthermore, in the phylum of Arthropoda, no clear distinction between type 1 or type 2 receptors could be made. Remarkably, while blasting the human CCK1R for the echinoderms, the CioR protein was also found in the urchin *Strongylocentrotus pupuratus*. Also for the silkmoth (*Bombyx mori*) and the hemicordata *Saccoglossus kowalevskii*, atypical proteins were found that are called A9 receptor and orexin-like receptor, respectively.

Even more remarkable is that some well known species showed no good homology or could not be found at all by blasting as for example: the hemimetabolous pea aphid *Acyrthosiphon pisum* (Hemiptera; (*232*); AphidBase; and also own unpublished data), the two holometabolous lepidopterans, the silkmoth *B. mori* ((*183*); SilkBase) and the African cotton leafworm *Spodoptera littoralis*, the honeybee *Apis mellifera* ((*290*); BeeBase) and the waterflea *Daphnia pulex* (wFleaBase). No crustacean SK receptors could be found by blasting, while some studies report about the presence of sulfakinins or sulfakinin DNA in Crustacea (*58, 88, 137, 275*). However, to the best of our knowledge, no studies describe SK receptors in Crustacea. So more research will be necessary to reveal the receptors for sulfakinins in Crustacea.

This phylogenetic tree points at the existence of one common ancestor gene for all CCK(-like) receptors. In vertebrates, the CCK receptors encoding genes lie on two separate chromosomes (*129*), but the two CCKR-like receptors in *D. melanogaster*, DSK-R1 and DSK-R2, are encoded by two closely linked genes, namely CG6881 and CG6857, that are only about 30 kb apart from each other. Therefore, Hewes and Taghert (*121*) postulated that CG6881 and CG6857 arose from a duplication of an ancestor gene rather than independently from two different genes. Moreover, the conservation of the same introns in CG6881 and CG6857, as in genes coding for CCK receptors, may indicate that these genes are members of the same subgroup (*121*). Most likely this implies that CCK1R and CCK2R, and SK-R1 and SK-R2 evolved from the same duplicated ancestor genes, as has also been suggested by Janssen et al. (*133*).

In contrast, in nematodes the two different receptors are encoded by two splice isoforms of the same gene. So probably the gene duplication happened before the divergence of deuterostomes and protostomes (*133*). This is in concordance with growing evidence that nematodes have split off the evolutionary tree before the arthropods (*178, 213, 296*). In contrast with the above theory, Kawada

et al. (*141*) postulated the hypothesis that sulfakinins and CK peptides do not belong to the CCK family because sulfakinins fail to activate the vertebrate CCK receptor and the precursors for CK peptides and sulfakinins code for several peptides. The latter authors hypothesize that the cionin receptor in the chordate *C. intestinalis* is the ancestor for both CCK receptors (*141*).

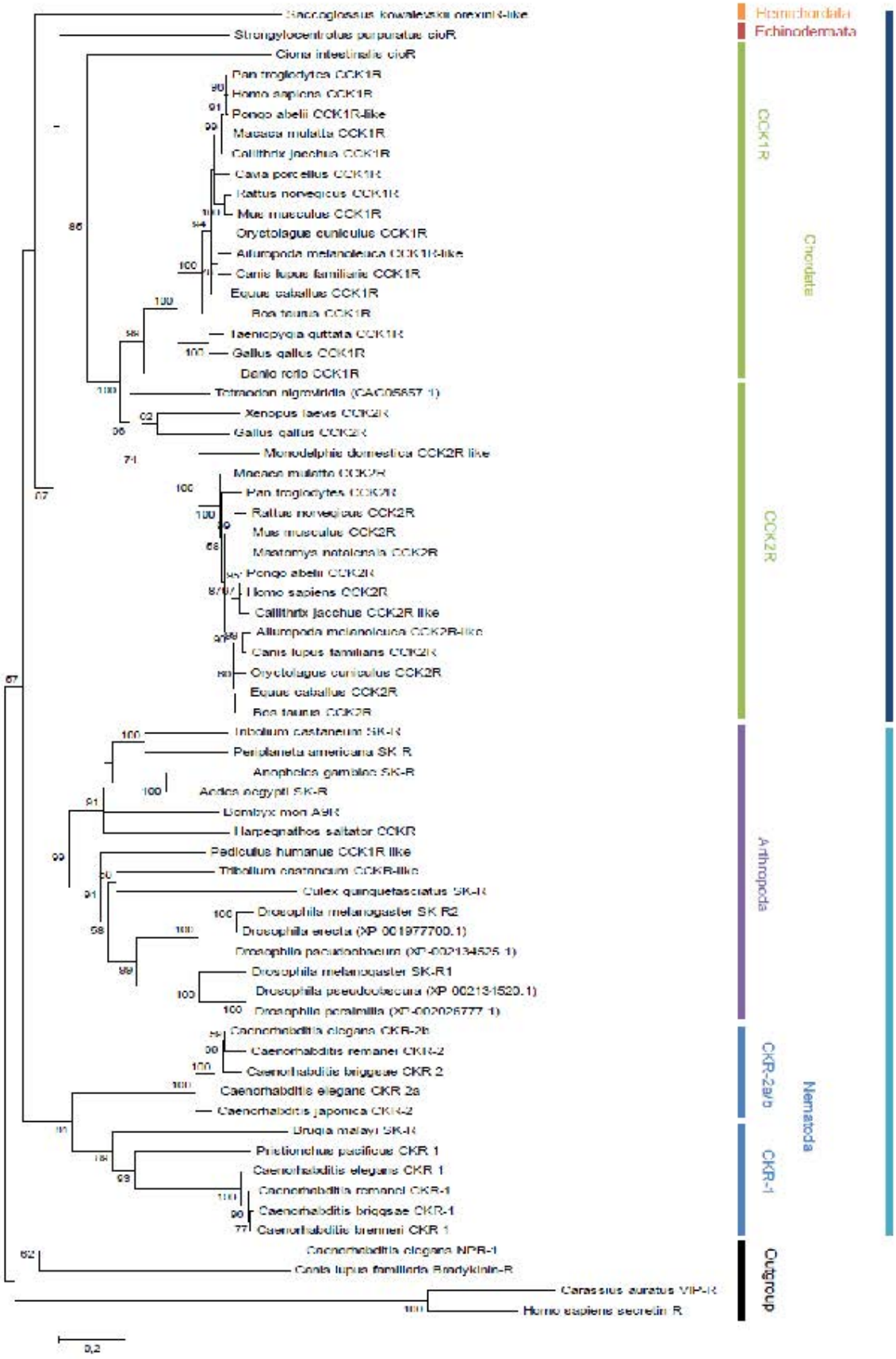

Hemichordata
Echinodermata
CCK1R
CCK2R
CKR-2a/b
CKR-1
Chordata
Arthropoda
Nematoda
Outgroup
Deuterostomia
Protostomia
Saccoglossus kowalevskii orexin/A-like
Strongylocentrotus purpuratus cioR
Ciona intestinalis cioR
Pan troglodytes CCK1R
Homo sapiens CCK1R
Pongo abelii CCK1R-like
Macaca mulatta CCK1R
Callithrix jacchus CCK1R
Cavia porcellus CCK1R
Rattus norvegicus CCK1R
Mus musculus CCK1R
Oryctolagus cuniculus CCK1R
Allmopoda melanoleuca CCK1R-like
Canis lupus familiaris CCK1R
Equus caballus CCK1R
Bos taurus CCK1R
Taeniopygia guttata CCK1R
Gallus gallus CCK1R
Danio rerio CCK1R
Tetraodon nigroviridis (CAG05857.1)
Xenopus laevis CCK2R
Gallus gallus CCK2R
Monodelphis domestica CCK2R like
Macaca mulatta CCK2R
Pan troglodytes CCK2R
Rattus norvegicus CCK2R
Mus musculus CCK2R
Meriones hotolensis CCK2R
Pongo abelii CCK2R
Homo sapiens CCK2R
Callithrix jacchus CCK2R like
Allmopoda melanoleuca CCK2R-like
Canis lupus familiaris CCK2R
Oryctolagus cuniculus CCK2R
Equus caballus CCK2R
Bos taurus CCK2R
Tribolium castaneum SK-R
Periplaneta americana SK R
Anopheles gambiae SK-R
Aedes aegypti SK-R
Bombyx mori ASK
Harpegnathos saltator CCKR
Pediculus humanus CCK1R like
Tribolium castaneum CCKR-like
Culex quinquefasciatus SK-R
Drosophila melanogaster SK R2
Drosophila erecta (XP 001977700.1)
Drosophila pseudoobscura (XP 002134525.1)
Drosophila melanogaster SK-R1
Drosophila pseudoobscura (XP 002013520.1)
Drosophila persimilis (XP 002026777.1)
Caenorhabditis elegans CKR-2b
Caenorhabditis remanei CKR-2
Caenorhabditis briggsae CKR 2
Caenorhabditis elegans CKR 2a
Caenorhabditis japonica CKR-2
Brugia malayi SK-R
Pristionchus pacificus CKR 1
Caenorhabditis elegans CKR 1
Caenorhabditis remanei CKR-1
Caenorhabditis briggsae CKR-1
Caenorhabditis brenneri CKR 1
Caenorhabditis elegans NPR-1
Canis lupus familiaris Bradykinin-R
Carassius auratus VIP-R
Homo sapiens secretin R
0,2

Figure 1.4. Phylogenetic tree of the vertebrate, nematode and insect CCK(-like) receptors, constructed using the neighbor-joining method. Bootstrap values as a percentage of 1000 replicates > 50 are indicated on the tree. Following sequences were selected by blasting and included in the tree: *Aedes aegypti* (XP_001654357.1), *Ailuropoda melanoleuca* (XP_002924347.1 and XP_002925026.1), *Anopheles gambiae* (AAR28375.1), *Bombyx mori* (NP_001127744.1), *Bos taurus* (NP_001095335.1 and NP_776687.2), *Brugia malayi* (XP_001902606.1), *Caenorhabditis brenneri* (CBN10364), *Caenorhabditis briggsae* (XP_002640196.1 and XP_002642853.1), *Caenorhabditis elegans* (NP_491918.2, ACA81683.1 and ACA81684.1), *Caenorhabditis japonica* (CJA02945), *Caenorhabditis remanei* (XP_003114892.1 and XP_003103966.1), *Callithrix jacchus* (XP_00274597 and ABQ22347), *Canis lupus familiaris* (AAX12114.1 and NP_001013868.1), *Cavia porcellus* (Q63931.1), *Ciona intestinalis* (NP_001027945.1), *Culex quinquefasciatus* (XP_001866738.1), *Danio rerio* (XP_697493.2), *Drosophila erecta* (XP_001977700.1), *Drosophila melanogaster* (NP_001097023.1 and NP_001097021.1), *Drosophila persimilis* (XP_002026777.1), *Drosophila pseudoobscura* (XP 002134525.1 and XP_002134520.1), *Equus caballus* (XP_001504633.2 and XP_001499250.1), *Gallus gallus* (NP_001074970.1 and NP_001001742.1), *Harpegnathos saltator* (EFN85362.1), *Homo sapiens* (NP_000721.1 and NP_795344.1), *Macaca mulatta* (XP_001084186.1 and XP_001102094.1), *Mastomys natalensis* (AB41677.1), *Monodelphis domestica* (XP_001380242.1), *Mus musculus* (NP_033957.1 and NP_031653.1), *Oryctolagus cuniculus* (NP_001075852.1 and NP_001164594.1), *Pan troglodytes* (XP_526545.1 and XP_521813.1), *Pediculus humanus corporis* (XP_002433137.1), *Periplaneta americana* (AAX56942.1), *Pristionchus pacificus* (PPA24381), *Pongo abelii* (NP_001127690.1 and XP_002814697.1), *Rattus norvegicus* (NP_036820.1 and NP_037297.1), *Saccoglossus kowalevskii* (NP_001161621.1), *Strongylocentrotus purpuratus* (XP_001193733.1), *Taeniopygia guttata* (XP_002191034.1), *Tetraodon nigroviridis* (CAG05857.1), *Tribolium castaneum* (XP_975226.2 and XP_972750.1), and *Xenopus laevis* (NP_001079277.1). *C. elegans* NPR-1 (NP_508816.1), *C. familiaris* bradykinin receptor (NP_001014306.1), *Carassius auratus* vasoactive intestinal polypeptide receptor (AAB05459.1) and *H. sapiens* secretin receptor (AAA64949.1) were used as an outgroup.

1.5 Structure of CCK and CCK receptor binding

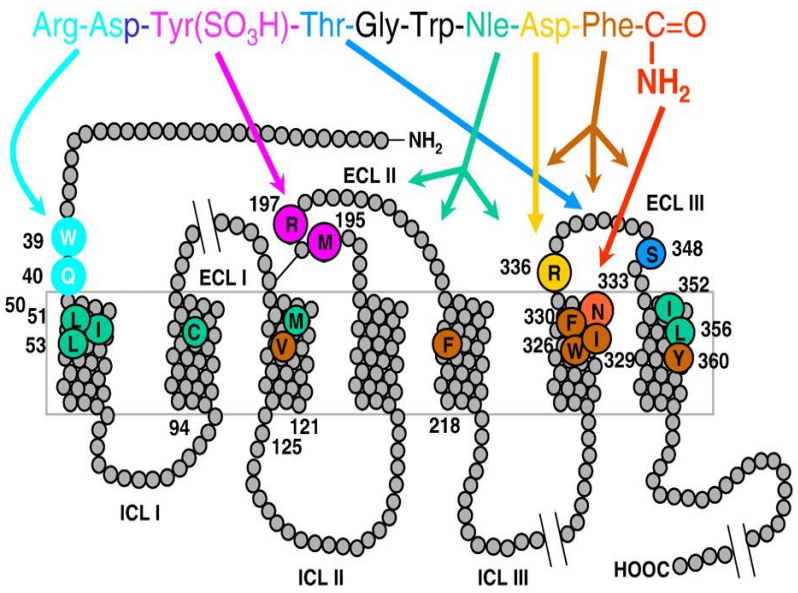
CCK exists in different lengths with 58, 33, 8 or 4 AA residues as the molecule can be prolonged at its N-terminus. It is amidated at the C-terminus. Interestingly, the CCK1R requires (at least) the C-terminal heptapeptide, and the CCK2R only needs the C-terminal tetrapeptide as a requisite for binding and biological activity. The CCK2R also binds to gastrin since this peptide has the same C-terminal tetrapeptide-amide as CCK (*69*). The tyrosine in position 2 can be sulfated (*79, 228*) (See Figure 1.5 for indication of the positions). This sulfated CCK (CCK-S) has a 500 to 1000 fold higher affinity for the CCK1R than for the CCK2R. The CCK2R binds to gastrin and CCK with the same potency and only has a slightly (3- to 10-fold) higher affinity for the sulfated natural ligands (*68, 126*).

As depicted in Figure 1.5b, the CCK receptor is a typical GPCR as it has seven TM domains (*148*). It has a conserved disulfide bond linking between the first and second extracellular loop (ECL I and ECL II) (*181*) and it also contains another disulfide bond within its amino terminus (*61, 210*). According to a model primarily based on mutagenesis data (*7, 93-95, 142*), the two most important interactions which account for the selectivity of the CCK1R for sulfated versus nonsulfated CCK are M195 and R197 in ECL II of the CCKR1. Other key interactions for binding of CCK with the CCK1R included juxtaposition of the N-terminus of CCK with the extracellular residues of the CCK1R on top of TM I (W39 en Q40), contacts between D in position 7 of CCK with R336 in TM VI and proximity of the amidated C-terminus with N333 in TM VI. This model for the human CCK receptors is reviewed in detail by Foucaud et al. (*84*). An overview of the contact points following this model is given in Figure 1.5b and c.

(a)

Asp – Tyr(SO_3H) – Met – Gly – Trp – Met – Asp – Phe – NH_2
1 2 3 4 5 6 7 8

(b)



(c)

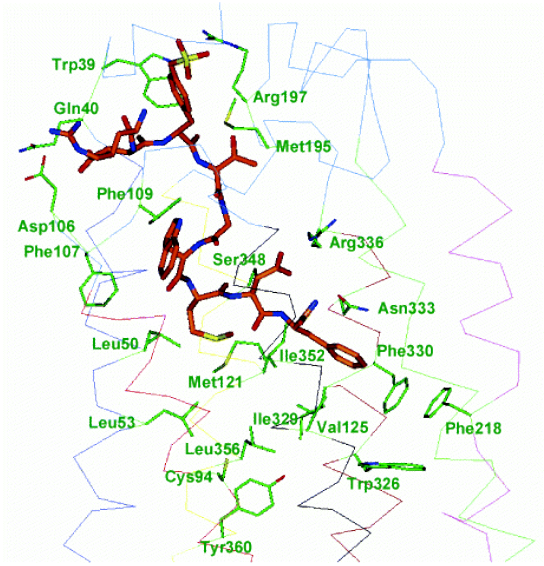


Figure 1.5. (a) Sulfated cholecystokinin octapeptide (CCK-8S), numbers under the amino acids indicate their position. (b) Detail of primary structure of the human CCK1R with binding sites of CCK-9S (RDY(SO_3H)MGWMDF-CONH2 in which the two methionine residues are substituted with threonine and norleucine to increase stability). Contact points between amino acids of CCK and the CCK1R have the same color ECL: extracellular Loop, ICL: intracellular Loop, TM: transmembrane domain (*7*). Side view of the three-dimensional refined model of the active high-affinity CCK1R binding site. Only the side chains of the identified amino acids of the CCK1R (in green) that are in interaction with CCK-8S (orange) are shown (*7*).

Multidimensional NMR studies have also been used in the development of a CCK1R receptor binding model (*97, 99, 210*). Important features included: contacts between D in position 7 of CCK8 and Y338 of the CCK1R, M in position 6 and N333, W in position 5 and A334, M in position 3 and W39, and Y in position 2 and also W39. But these studies are complicated by the use of a nonsulfated CCK ligand. As mentioned above, nonsulfated CCK has a low affinity for the CCK1R, and so it is possible that the experimental constraints from these studies are not relevant to the high affinity CCK ligand complexes.

Next, it is of interest that another model has been developed based on photoaffinity labeling studies (*8, 60, 63, 64, 104, 105, 136, 181*). In this, binding of CCK-S with the CCK1R involved F107, K105 and L99 in ECL I, L199 in ECL II, E344 and R346 in ECL III, and W39 in the N-terminus of the CCK1R. This model also showed that R197 is important in the binding of the sulfated tyrosine at position 2 of CCK. Fluorescence spectroscopy is another powerful tool to define the occupation of different microenvironments of the ligand-bound CCK receptors (*112-114*). A number of fluorescent CCK and gastrin analogues has been developed, some of which pose high affinity to the receptors and subsequently exert high biological activity (*111, 115*). Hence, these data provided evidence for a differential docking of CCK in the CCK1R and the CCK2R.

The first 3D-model of the CCK receptor was created upon the crystal structure of the rhodopsin GPCR (*6*). Recently, in 2009, based on the data of photoaffinity labeling and fluorescence spectroscopy, two homology models of the CCK receptor were built with regard to the crystal structures of the β2-adrenergic receptor and A2a-adenosine receptor (*64*). Interestingly, these new agonist ligand-occupied receptor models agree with the existing experimental data. An overview of the different contact points between CCK and the CCK1R found in the current literature are given in Table 1.2 and is compared to the molecular model built by Archer-Lahlou et al. (*7*) (Figure 1.5c). Table 1.2 demonstrates that these contact points could not or could only be partially confirmed by the use of other techniques as multidimensional NMR and photoaffinity labeling. Therefore, it is recommended that future research should elucidate these discrepancies to converge to a model covering the different sets of data.

Table 1.2 Overview of contact points between CCK and CCK1R

Ligand residue	Contact point in receptor	Place	Ref.	Confirmation by molecular model as depicted in Figure 1.5c. (*7*)
Primarily based on mutagenesis				
N-terminus	W39	N-terminus	(*142*)	Yes
	Q40			Yes
Sulfated Y2	M195	ECL II	(*94, 95*)	Yes
	R197			Yes
Nle 6	L50	TM I	(*7*)	Yes
(Met→Nle)	I51			Yes
	L53			Yes
	C94	TM II		Yes
	I352	TM VII		
	L356			Yes
	M121		(*77*)	Yes
				Yes
D7	R336	TM VI	(*93*)	Yes
F8	V125	TM III	(*77*)	Yes
	F218	TM V		Yes
	W326	TM VI		
	I329			Yes
	F330			
	Y360	TM VII		Yes
				Yes
				Yes
Amidated C-terminus	N333	TM VI	(*93*)	Yes
Primarily based on multi-dimensional NMR				
Y2	W39	N-terminus	(*97-99*)	Interaction with N-terminus of ligand confirmed
M3	W39	N-terminus		Interaction with N-terminus of ligand confirmed
W5	A334	TM VI		No interaction between the ligand and A334 of the receptor confirmed
M6	N333	TM VI		Interaction with C-terminus of ligand confirmed
D7	Y338	ECL III		No interaction between the ligand and Y338 of the receptor confirmed
Primarily based on photoaffinity labeling				
Y2	R197	ECL II	(*8*)	Yes
M3	L199	ECL II	(*64*)	No interaction between the ligand and L199 of the receptor confirmed
G4	R346	ECL III	(*105*)	No interaction between the ligand and R346 of the receptor confirmed
W5	L99	ECL I	(*63*)	No interaction between the ligand and L99 of the receptor confirmed
	K150	ICL II		
M6	F107	ECL I	(*64*)	No interaction between the ligand and F107of the receptor confirmed
F8	W39	N-terminus	(*104, 136*)	No interaction between F8 of the ligand and W39 of the receptor confirmed

1.6 Antagonists and agonists for the CCK receptor

An overview of diseases which possibly might be treated using CCK1R/CCK2R a(nta)gonists is shown in Figure 1.3. Over the years, various CCK receptor antagonists have been developed and are used in research (*212*), although the only drug of this class that has been widely marketed to date is the anti-ulcer drug proglumide (*180*). Newer drugs have since been developed which are selective for one or other of the CCK receptors; good examples are loxiglumide and dexloxiglumide. Although several CCK receptor antagonists have reached different clinical test phases, the complex and versatile physiological actions of CCK have slowed down their clinical development (*119*). CCK antagonists might be used against ulcers, pancreatitis, pancreatic disorders pancreatic cancer, irritable bowel syndrome, gastric secretion disorders, gastric motility disorders, gastro-oesophagal reflux, anxiety disorders, pain, sleeping disorders and eating disorders. Hence, CCK receptor antagonists have a very big potency for the treatment of several diseases, but more research is necessary to screen for highly selective antagonists with lesser side effects and high bioavailability (*119, 212*). Lorglumide and devazepide are CCK1R antagonists that are often used in *in vitro* research (*26*).

Considering agonists, the first selective molecules were peptides (i.e. A-71378, A7-71623 and AR-15849), but recently also 1,5-benzodiazepine analogues (i.e. Gl18177X and GW5823) and a thiazole derivative (SR14643) have been developed as selective agonists (*20*). Radiolabeled CCK2R agonists can be used to localize CCK2R expressing tumors (*101, 174*). Another CCK-peptide analogue is JMV-180. It acts as a partial agonist, but depending on the situation, e.g. tissue expression and species involved, it can also behave as a full agonist or dual agonist/antagonist (*87, 216, 303*). To date it can be used to study signaling pathways and activation mechanisms of the CCK1R (*5, 253*). CCK1R agonists can possibly be used in the treatment of eating disorders and obesity (*269*). Thusfar, no CCK1R agonists have been found which can effectively reduce body weight in humans. Monotherapy alone with CCKR agonists might not be an effective strategy to treat obesity (*138*). Also for other diseases, as panic disorders and cancer, the same conclusion was drawn that monotherapy with CCK antagonists is not an effective treatment (*21, 43*). Pentagastrin, a CCK2R agonist, can be used to evoke panic attacks to study their treatment (*33*). However, long term administration with various CCK2R antagonists (CI-998 and L-356,260) did not lead to the prevention of panic disorders (*1, 208, 257*). CCK2Rs are expressed or overexpressed in many human tumors, and it seems that CCK-related peptides can cause tumor growth (*15, 230*). To suppress tumor growth, Chau et al. (*43*) suggested that a combination of different agents might be a more effective strategy to treat cancer, and possibly this hypothesis is also valid for the treatment of obesity and panic disorders. However, more animal and controlled human intervention studies are necessary to elucidate the putative

therapeutic potential of CCK receptor ligands (*20, 119*) and this also in combination with other agents as described above. Hence, molecular models can help to reveal these complex ligand-receptor interactions and to develop new lead structures for CCK antagonists and agonists, whereupon, Allen and Roth (*2*) demonstrated the importance of drugs active at GPCRs.

1.7 Bioactive peptides, protein hydrolysates and proteins affecting CCK-induced satiety: mechanisms and active components

In this section, protein-derived food substances that contain the potential to influence satiety through the CCK-signaling pathway, will be described. CCK administration to man significantly reduces food intake in the short term (*144*). As depicted in Figure 1.6, the CCK signaling can be influenced by affecting CCK release (interaction point 1a and 1b) or by interacting with the CCK1R (interaction point 2).

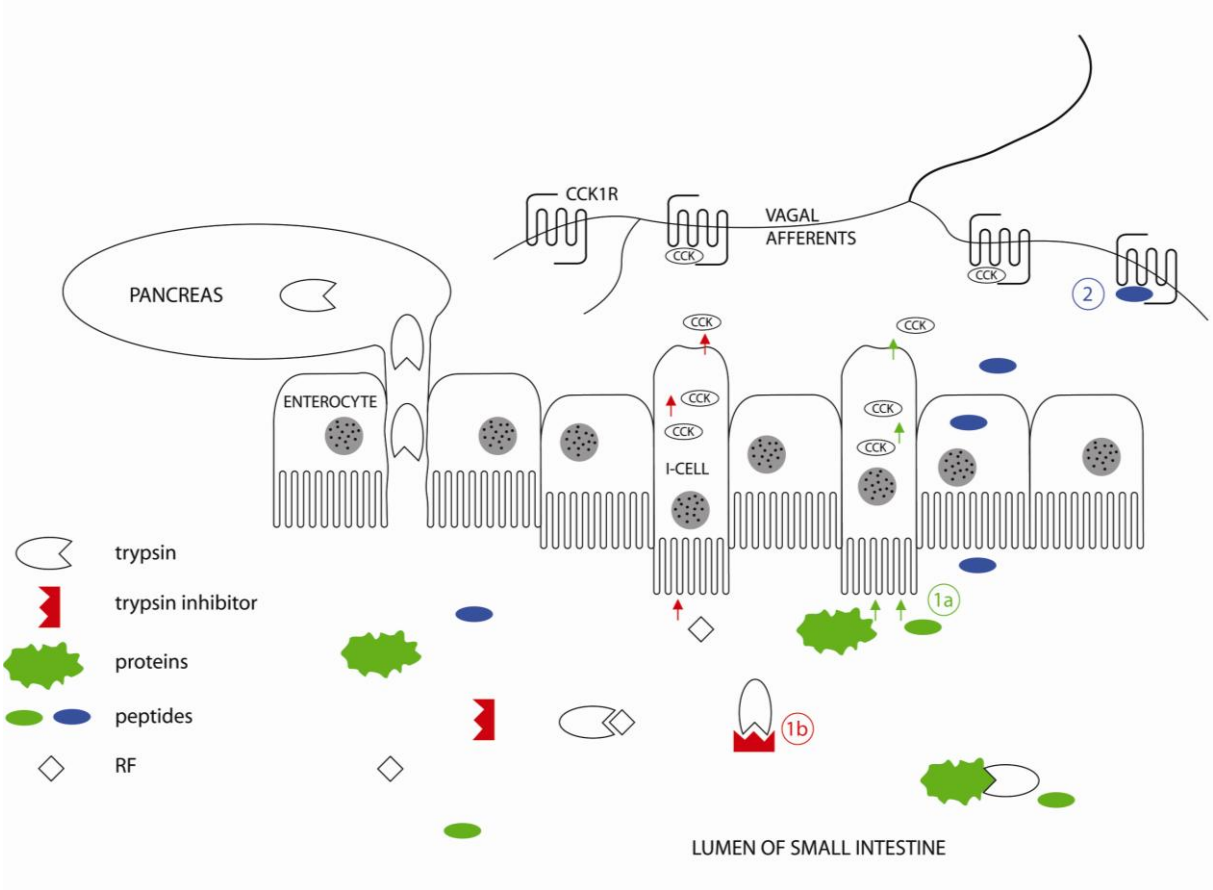


Figure 1.6. Schematic representation of different interaction points to influence the CCK-signaling mechanism. (1a) Stimulation of CCK release by enteroendocrine I-cells with nutrients; mechanism is represented in green. (1b) Inhibition of luminal trypsin activity in favor of trypsin-sensitive CCK-releasing factors (RF); mechanism is represented in red. (2) Direct CCK1R interaction with bioactive peptides; mechanism is represented in blue.

The level of CCK release can be increased via two major interaction points: stimulation of enteroendocrine CCK-releasing I-cells with nutrients (interaction point 1a) and inhibition of luminal trypsin in favour of CCK-releasing factors (interaction point 1b). The different interaction points, the methods for detection of food components that have potential to influence one of these interaction points and the associated proteins and protein hydrolysates will be described in this section.

1.7.1 CCK release

1.7.1.1 Mechanism

CCK is secreted by enteroendocrine I-cells in the proximal small intestine. The apical membrane of these cells contains microvilli which are able to sense nutrients and releasing factors in the lumen of the intestine (Figure 1.6). When the microvilli are stimulated, CCK is released from the basolateral site of the cells into the interstitial space where it can bind to CCK receptors located in the gastrointestinal tract (*162*). CCK release can be enhanced by optimizing the nutrients that stimulate the enteroendocrine I-cells to produce CCK (Figure 1.6, interaction point 1a). Furthermore, CCK release by enteroendocrine I-cells is also induced by endogenously produced releasing factors, including luminal CCK-releasing factor (*162*), diazepam binding inhibitor (*161*) and monitor peptide (*186*). These releasing factors are deactivated by trypsin, but this can be avoided by nutrients competing for trypsin or trypsin inhibitors (*162*). Therefore, nutrients enriched in trypsin inhibitors form a second opportunity to stimulate CCK release (Figure 1.6, interaction point 1b).

1.7.1.2 Methods for screening and active food components

Stimulating CCK release by enteroendocrine I-cells

The ability of food components to stimulate CCK release can be tested *in vitro* with STC-1 cells (a mice intestinal tumor cell line) (*233*) where the amount of CCK produced by the cells is measured after addition of the product (*50*). This can also be tested *in vivo*, where upon ingestion of the product the concentration of CCK in the blood is determined (*202*). An overview of the food compounds with an effect on CCK release is given in Table 1.3. Many proteins and protein hydrolysates from plant or animal origin showed potential to increase CCK release from enteroendocrine I-cells. Intact proteins as well as protein hydrolysates seem to be able to stimulate CCK release (*202*). As the list of proteins and protein hydrolysates having an effect on CCK release is rather long (*51, 82, 90, 262, 264*), this might point to a non-specific nutrient sensing mechanism on the CCK-producing cells (*82*). Still, most studies show a difference in the potential of different proteins or protein hydrolysates to stimulate CCK release (*51, 90, 262, 264*) and Nishi et al. (2003)

reported that the peptide fragment VRIRLLQRFNKRS in soybean β-conglycinin induces a specific CCK-releasing and appetite-suppressing effect (*201*). More research is needed to reveal the exact nutrient-sensing mechanisms that induce CCK secretion by enteroendocrine I-cells to be able to develop targeted CCK-releasing food compounds.

Table 1.3 Food components possibly affecting CCK release

Stimulation of enteroendocrine cells
Wheat protein (*90*)
Pea protein (*90*)
Pea protein hydrolysate (*82, 90*)
(enzymes used: subtilisin + proline-specific endoprotease)
Dolicholin peptone (51 kDa protein from country bean) (*263*)
Soy protein hydrolysate(*82*)
Soybean β-conglycinin peptone (*202*)
Soybean β-conglycinin β 51-63 fragment (*201*)*
Soybean β-conglycinin bromelain (*264*)
Potein® (potato extract) (*192*)
Potein® hydrolysate (*192*)
(enzymes used: pepsin and/or pancreatin)
Potato protein hydrolysate (*82*)
Casein (*90*)
Casein protein hydrolysate (*82*)
Whey protein hydrolysate (*82*)
Egg hydrolysate (*90*)
Blue whiting muscle hydrolysate (*51*)
(most active molecular weight range: 1000 -1500 Da)
Brown shrimp head hydrolysate (*51*)
(most active molecular weight range: 1000 – 1500 Da)
Pork thigh meat peptone (*262*)
Chicken breast meat peptone (*262*)
Inhibition of luminal trypsin
Potein® (*192*)
Potato proteinase inhibitors concentrate (*149*)

Inhibition of luminal trypsin activity to protect releasing factors

Trypsin inhibitors are especially known as an anti-nutritional factor in legume seeds (*241*). The trypsin inhibitory activity can be measured with a colorimetric reaction in which benzoyl-L-arginine-p-nitroanilide is used as a substrate (*139*). An overview of food protein hydrolysates containing trypsin inhibitors of which the positive effect on the luminal CCK-releasing factor has been demonstrated, is given in Table 1.3. Slendesta® is a patented food ingredient from Kemin Industries Inc. (Des Moines, IA) that contains potato proteinase inhibitors claiming satiety induction via this mechanism (patent: US6414124). Soy and potato seem to be especially rich in these trypsin inhibitors (*34, 149*).

1.7.2 CCK1R activation

1.7.2.1 Mechanism

The CCK1R present on vagal afferents, is especially involved in inducing satiety. Vagal afferents are located in the gastrointestinal muscle layers and within the mucosa in the lamina propria in the proximity of the basolateral membrane of the enteroendocrine cells (*225*). Food-derived peptides might be able to reach this receptor and mimic to a certain extent the effect of CCK, therefore being called CCK1R agonists, as depicted by interaction point 2 in Figure 1.6.

1.7.2.2 Methods for screening and active food components

CCK1R agonists screened with cell-based bioassay

CCK1R activation can be measured using CHO cells transfected with the CCK1R. This method is extensively described in Chapter 2. An overview of protein hydrolysates that activate the CCK1R is given in Table 1.4. It should be remarked that these protein hydrolysates require a rather high concentration (0.01 – 1 g/l) (*82*) to show some CCK1R activation compared to CCK itself, which shows full CCK1R activation at a concentration of 1 nM (± 1 µg/l). Another difficulty is the fact that the bioactive peptides need to cross the epithelium intactly to be able to reach the CCK1R in the lamina propria. Oligopeptides containing more than 4 amino acids can be taken up by paracellullar and transcellular routes. In a Caco-2 intestinal transport model, transepithelial transport of peptides with up to 9 residues has been observed (*246*), this issue will be further discussed in paragraph 6.3.3.1.

Table 1.4 Peptides possibly activating CCK1R

CCK1R agonists (based on cell-based bioassay)
Soy hydrolysate (*82*)
Potato hydrolysate (*82*)
CCK-like peptides (based on radiomimmunoassay)
Prolastin (fish protein hydrolysate) (*214*)
Atlantic cod backbones hydrolysate (*250*)
(enzyme used: protamex)
Cod extracts (*223*)
Shrimp extracts (*223*)

CCK/gastrin-like peptides screened with radioimmunoassay

Radioimmunoassay to screen for CCK/gastrin-like peptides was performed using synthetic iodine 125-radiolabeled gastrin and synthetic gastrin as a standard based on the binding competition for antibodies (*214*). Protein hydrolysates from fish or shrimp were screened and proved to contain CCK-like peptides as can be seen in Table 1.4. In contrast, muscle protein hydrolysate from smooth hound did not contain any CCK-like peptides (*31*). It should be taken into account that although some peptides show CCK-like radioimmunoactivity, this is no proof but only an indication for being CCK1R agonist as this needs to be confirmed in a CCK1R activation test (as described in the previous paragraph).

An extensive amount of proteins and protein hydrolysates increasing the release of CCK has been found. This is in contrast to the few protein hydrolysates that are able to activate the CCK1R, which seems to be a more specific process. Thus far, mostly protein hydrolysates have been evaluated for their potential to induce satiety via a CCK-involved mechanism, but also non-protein food components as shown for thylakoids (*147*) and for example phenolic compounds might be promising compounds in this context (*235*). Also certain free fatty acids play an important role (*109*). Next to the 3 interaction points for influencing CCK-induced satiety (Figure 1.6), other interaction points might exist. For example, it also might be possible that some nutrients increase the production of CCK-releasing factors. Next to the screening of CCK1R activating bioactive peptides in this work, further research should focus on food ingredients combining an effect on different CCK interaction points and even on other weight-regulating mechanisms because an integrated approach might form the key to successfully combating obesity.

Chapter 2

Time-resolved quantitative analysis of CCK1 receptor-induced intracellular calcium increase

This chapter has been published in Staljanssens, D.; De Vos, W. H.; Willems, P.; Van Camp, J.; Smagghe, G., Time-resolved quantitative analysis of CCK1 receptor-induced intracellular calcium increase. Peptides 2012, 34, 219-225

Chapter 2 Time-resolved quantitative analysis of CCK1 receptor-induced intracellular calcium increase

2.1 Introduction

As mentioned in paragraph 1.4.3, the CCK1R is a G-protein-coupled receptor, which upon activation, elicits an inositol trisphosphate (IP_3)-induced calcium release from the endoplasmic reticulum (*69, 203*). This intracellular Ca^{2+}-flux is a measure for the activation of the receptor and can be visualized with fluorescent sensor dyes (*182*). Different cell systems functionally expressing one of the CCKR subtypes exist and can be used to screen for ligands with agonistic or antagonistic CCKR binding activity (*25, 71, 252, 254*). However, the use of diverse cell types, fluorescent dyes and measuring techniques makes it difficult to compare the results from different studies on the effectiveness of several ligands. Here, we established a fully controlled, standardized and sensitive cell-based bioassay in 96-well plates to screen and characterize components with CCK1R activity. For validation, the changes in fluorescence intensity observed with a population average technique using a fluorescence plate reader were compared with a single-cell approach using confocal microscopy. Cross-validation of both measuring techniques resulted in a sensitive and specific assay, which can be used for high throughput screening of molecules and protein hydrolysates that interact with the CCK1R.

2.2 Materials and Methods

2.2.1 Cell lines and products

CHO (Chinese Hamster Ovary) cells functionally expressing the rat CCK1R (CHO-CCK1R) were established by Prof. Peter Willems (*254*) and native CHO-K1 cells were obtained from Prof. Georges Leclercq (Ghent University Hospital, Department of Clinic Biology, Microbiology and Immunology, Ghent Belgium). Advanced Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM-F12), fetal bovine serum (FBS), geneticin (G-418 antibiotic), Fluo-4AM, Pluronic F-127 and Hank's buffered salt solution (HBSS) were purchased from Life Technologies (Paisley, UK), probenecid, lorglumide ((±)-4-[(3,4-dichlorobenzoyl)amino]-5-(dipentylamino)-5-oxopentanoic acid sodium salt; CR-1409), bovine serum albumin (BSA) and HEPES from Sigma-Aldrich (St.-Louis, MO), sulfated cholecystokinin octapeptide (CCK-8S) and thrombin receptor activating protein (TRAP-7) from Bachem (Weil am Rhein, Germany), and JMV-180 (Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-2-phenylethylester) from Research Inc. (Barnegat, NJ). Clear black bottom 96-well plates were purchased from Greiner (Frickenhausen, Germany).

2.2.2 Cell culture

CHO-CCK1R and CHO-K1 cells were grown at 37 °C and 5% CO_2 in advanced DMEM-F12 supplemented with 1% streptomycin and penicillin, 1% L-glutamine and 10% FBS. The medium of CHO-CCK1R cells was supplemented with 10 µl/ml geneticin (50 mg/ml) to maintain a stable transfected culture.

2.2.3 Cell-based bioassay to screen for CCK1R activity

The determination of the intracellular free Ca^{2+} concentration was adapted from a method previously reported by Foltz et al. [1]. The fluorescent probe that was used in this assay is the hydrophobic Fluo-4AM, a cell-permeant acetoxymethyl (AM) ester, which is hydrolyzed by cellular esterases and becomes fluorescent upon Ca^{2+}-binding. One half of a 96-well plate is seeded with CHO-CCK1R cells and the other half with CHO-K1 cells, in both at 40,000 cells per well. Cells were incubated at 37 °C and 5% CO_2 for 20-24 h to allow attachment. Next, the medium was removed and 50 µl of DMEM-F12 supplemented with 4 µM Fluo-4AM, 0.02% (w/v) of the surfactant pluronic, 4.55 mg/ml BSA, and 1.6 mM of the anion transport inhibitor probenecid was added to the wells for 1 h at 19 °C, as was determined as the ideal dye loading temperature in preliminary experiments. Subsequently, the wells were washed twice with 150 µl of HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid and 10 mg/ml BSA and finally 100 µl of modified HBSS was added to the wells. Lorglumide was added at a final concentration of 0–40 µM, 30 min before the start of the experiment. Receptor activation leads to a rapid increase in intracellular calcium concentration (within 0–30 s). Hence, fluorescence intensity was measured kinetically. Two setups were used: a fluorescence plate reader and a confocal microscope. In the first setup, an Infinite pro 200 (Tecan, Männedorf, Switzerland) multimode plate reader with automated injection system was handled using i-control™ software. Excitation and emission wavelengths were set to 480 nm and 520 nm respectively, using Quad4 monochromators™ technology. In the second setup, a Nikon A1r confocal laser scanning microscopy system (Nikon Instruments Inc., Melville, NY) was used, mounted on a Nikon Ti-E inverted epifluorescence microscope and equipped with a microscope incubator, Perfect Focus System and resonant scanner. Multiwell dishes were screened, with a Plan Fluor 40 x/0.75 dry objective at full field of view (636 µm x 636 µm), resulting in a pixel size of 1.24 µm x 1.24 µm. Fluo-4AM was excited using a 488 nm multi-line Ar laser and fluorescence was detected through a 525/50 nm bandpass filter.

On both platforms a similar measurement protocol was applied. Measurement started 30 min after washing to allow complete intracellular cleavage of the Fluo-4AM ester. After washing, the plates were immediately placed in the plate reader or microscope incubator to equilibrate at the

measurement temperature of 31 °C, as determined in preliminary experiments. Each well was measured separately. Fluorescence was acquired at 2.5 fps with the plate reader and 3 fps with the confocal microscope. Ligands were added *in fluxo*, i.e. during acquisition: the basal fluorescence of a well was measured for 6 s after which 100 µl of the sample (diluted in modified HBSS) was added instantaneously while the measurement continued for another 34 s. In the plate reader, sample addition was performed automatically, while in the confocal microscopy the sample was added using an electronic repeating pipette (Handystep, BrandTech Scientific Inc, Essex, CT). Each sample concentration was measured in 5 wells (technical replicates) for both cell types and every experiment was repeated 3-4 times (biological replicates).

2.2.4 Image analysis

Confocal images were analyzed with ImageJ freeware (National Institute of Health, Bethesda, MD). For population average measurements, the average fluorescence intensity (per pixel) over the entire image was measured in unprocessed recordings. For single-cell analysis, the following workflow was designed: first, images were aligned by means of a rigid registration (translation and rotation) to remove superimposed motion *e.g.* due to thermal drift. Next, an average projection was made of the complete time stack to provide a well-contrasted image of all the cells by averaging out the noise. This approach of temporal averaging works efficiently even in the absence of an overt calcium flux (e.g. in CHO-K1 cells, cfr. Figure 2.1). An extra smoothing step, by means of Gaussian filtering (sigma=1) removed the remaining noise and allowed for segmenting the cells in a subsequent thresholding step (Huang autothreshold). Touching cells were separated by local maxima finding and conditional region growing, resulting in a complete set of regions of interest (ROI), corresponding to the cell boundaries. All ROI were inspected and manually corrected, where required. Finally, a particle analysis was performed to measure the average pixel intensity per cell ROI (i.e. integrated intensity in a cellular ROI, divided by the ROI area in pixels) through time. This metric was selected to avoid cell-size dependent variations in signal intensity. Single cell results were processed and summarized using Matlab 7.10.0 (R2010a, The Mathworks Inc., Natick, MA).

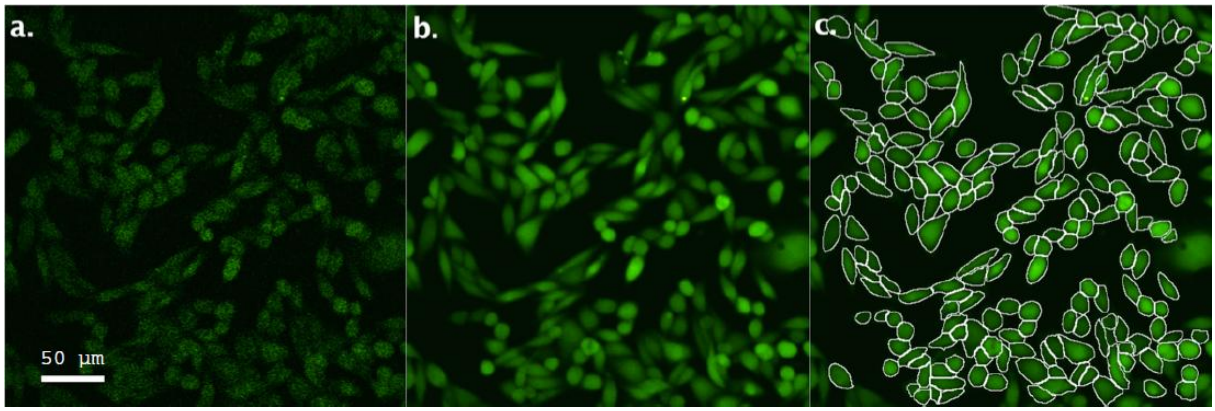


Figure 2.1. Illustration of temporal averaging for image segmentation. (a) original raw image of one single time point of a CHO-K1 cell labeled with Fluo-4AM acquired for 40 s at 3fps. (b) average intensity projection of the complete time stack (40 s, 150 frames) (c) result of segmentation, displayed as white outlines superimposed on the average projection image. Scale bar represents 100 µm.

2.2.5 Data analysis and statistics

For calculation of dose-response curves the following procedure was applied. Per time point i, fluorescence measurements (F_i) were normalized to the average fluorescence before sample addition, which corresponds to the average fluorescence in the first 6 s of the recording (F_0), thereby correcting for differences in the amount of basal fluorescence, due to variations in cell density, dye concentration and/or free calcium concentration. Average normalized fluorescence values of 5 technical replicates were corrected for non-specific responses and background fluorescence by subtracting the average normalized fluorescence values obtained for CHO-K1 cells per time point and per condition. Hence, the relative fluorescence (RF) was calculated using the following formula:

$$RF_i = \frac{F_i(CHO - CCK1R)}{F_0(CHO - CCK1R)} - \frac{F_i(CHO - K1)}{F_0(CHO - K1)}$$

and plotted as a function of time. Subsequently, a single metric was derived from the RF curves: the net response was calculated as the sum of relative fluorescence values, from the moment of sample addition until the end of the acquisition (net response = $\sum_{i=6\,s}^{40\,s} RF_i$). All net responses were expressed as a percentage of the maximal net response of CCK-8S (induced by a concentration of 1 nM). The inhibiting effect of the antagonist was calculated as 100% minus the net response. From these results, sigmoid dose-response curves for the percentage of the maximum response/percentage of inhibition versus sample concentration were derived with Prism v4 software (GraphPad Prism, La Jolla, CA) and median response concentrations, *i.e.* effective EC_{50} and/or inhibitory IC_{50} values, were calculated for the agonists and antagonists, respectively (*286*). The EC_{50}

value represents the agonist concentration at which 50% of its maximum response is reached. The IC_{50} value is the antagonist concentration, which inhibits 50% of the maximum response of CCK-8S (1 nM). The median response concentrations of agonists and antagonists are the mean of at least three independently repeated dose-response curves (biological replicates), which are based on 5 repeated measurements (technical replicates) for each concentration.

Median effect concentrations were statistically compared in S-plus (TIBCO Software Inc., Palo Alto, CA) by means of a One-way ANOVA analysis and the significance of individual differences were calculated using Tukey post hoc tests.

2.3 Results

2.3.1 Optimization

First, we optimized and benchmarked our assay in terms of sensitivity, stability and specificity. When using fluorescent dyes, there is a risk of active dye uptake (*e.g.* by pinocytosis) leading to compartmentalization of the dye in vesicles (*170*). Indeed, using epifluorescence microscopy, we found that the fluorescent dye showed significant compartmentalization when the dye was loaded at 37 °C. By lowering the loading temperature to 19 °C, we observed a homogeneous cellular distribution of the dye indicating passive diffusion into the cell.

Given that a complete experiment, corresponding to one 96-well plate, takes about one hour to screen, it is imperative that the cellular response in different wells remains stable through time. When performing pilot experiments using the plate reader, we observed at 37 °C a gradual decrease in cell response to a single concentration of CCK-8S (0.1 and 1 nM) from well to well. Conversely, at lower temperatures (28 °C) a gradual increase in response was observed. The most stable allover response through time was obtained at a measurement temperature of 31 °C (Figure 2.2).

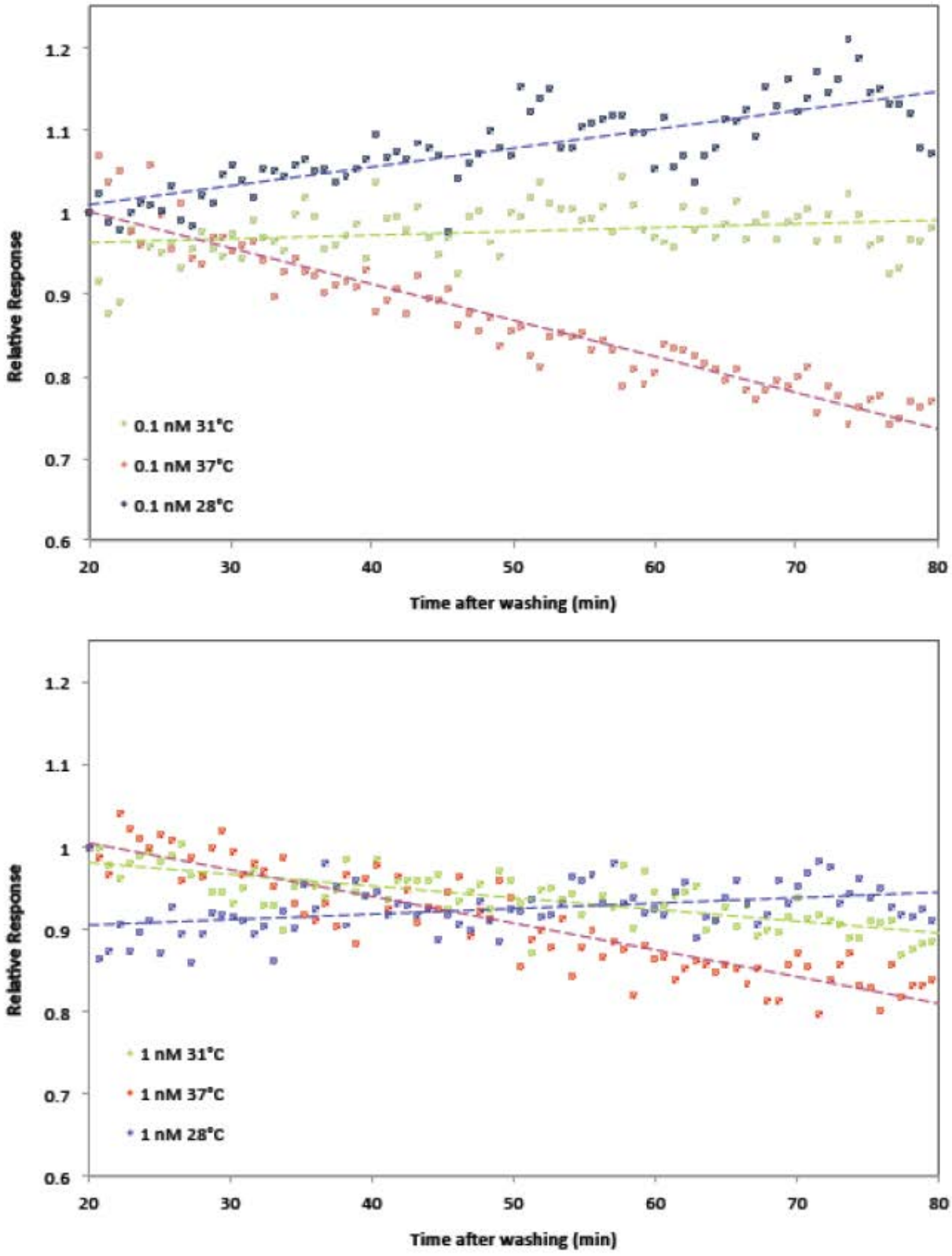


Figure 2.2. Temperature and concentration dependence of cell response from well to well. Fluo-4AM CHO-CCK1R cells in 96-well plates were exposed to 0.1 nM (a) or 1 nM CCK-8S (b) at different measurement temperatures. Every well, represented by a single data point in the plot, was treated (and measured) sequentially. Trend lines from a linear regression were plotted for demonstrational purposes. The response has been expressed relative to that of the first well.

To ensure ligand specificity, native CHO-K1 cells were included in the assay as negative controls. When stimulated with CCK-8S, CHO-K1 cells did not show a significantly different response from cells treated with buffer. However, both CHO-K1 as CHO-CCK1R showed a similar response when stimulated with TRAP-7, an agonist for the thrombin receptor (Figure 2.3). This confirmed that CHO-K1 cells could be used to correct for non-specific responses.

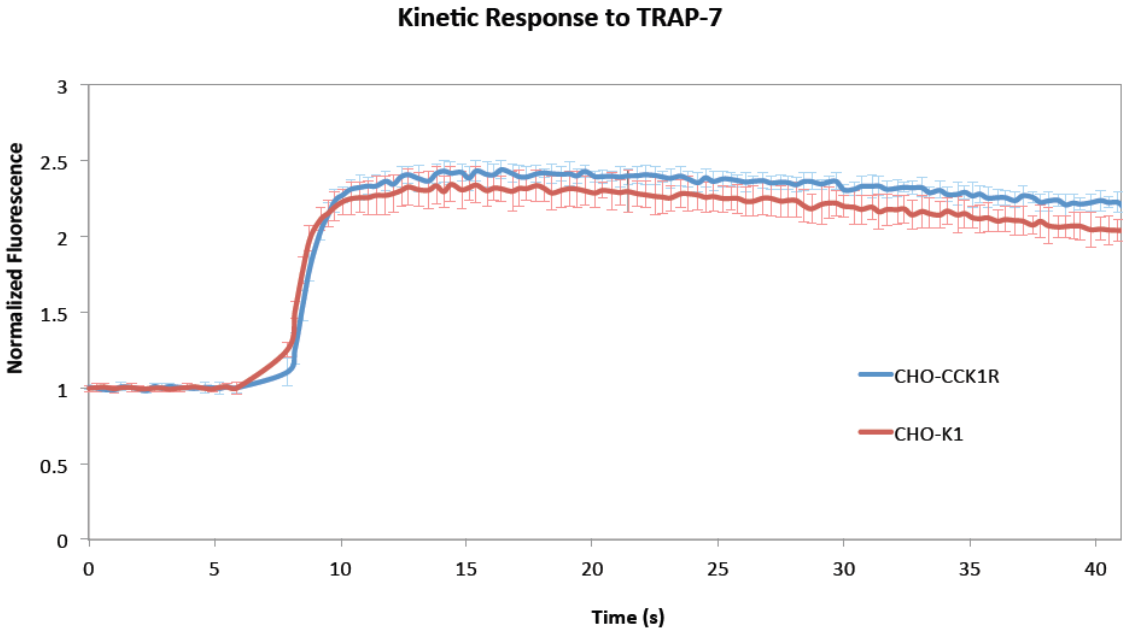


Figure 2.3. Kinetics of normalized fluorescence (F_i/F_0) ± SD in Fluo-4AM labeled CHO-CCK1R and CHO-K1 to 250 µM of TRAP-7. Experiments were carried out in the presence of 50 µM lorglumide to inhibit effects via the CCK1R (n=6).

2.3.2 The plate reader assay allows for sensitive and accurate measurement of agonist and antagonist effects on the cell population level

First, we measured the cellular response to the natural ligand, CCK-8S. The change in fluorescence was monitored in time for increasing concentrations of CCK-8S (0.001 nM–1 nM) (Figure 2.4). Typically, RF curves (Figure 2.4a) showed strong dose-dependent kinetics in terms of the time point and height of the maximum RF, but also in terms of peak persistence.

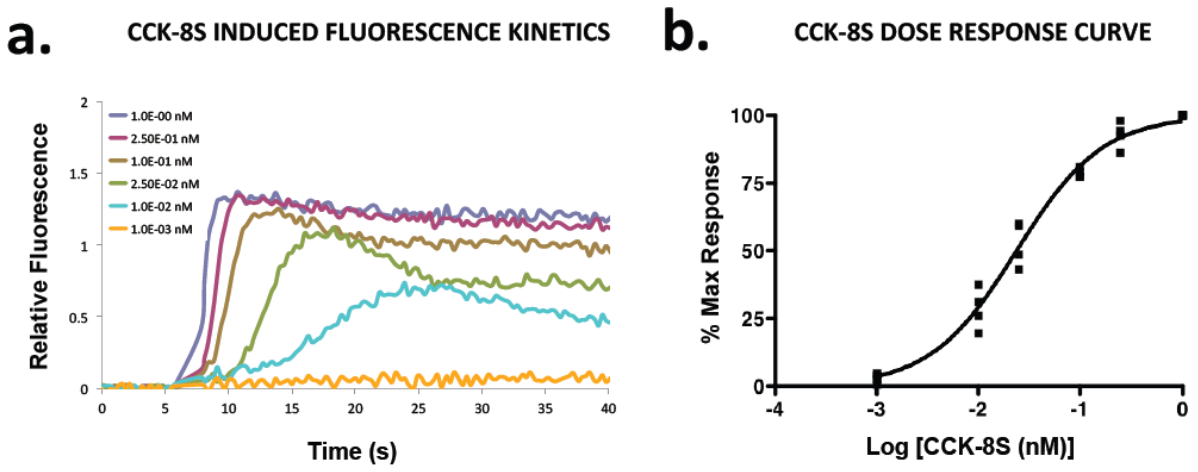


Figure 2.4. Dose-dependent CCK1R-mediated calcium fluxes in cell populations monitored with a plate reader. (a) Kinetics of relative fluorescence (RF) of Fluo-4AM labeled CHO-CCK1R cells to increasing concentrations of CCK-8S (0.001–1 nM). The curves represent the mean of 5 technical replicates (wells). (b) Representative dose-response curve for CCK based on 4 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, *i.e.* the net response induced by 1 nM CCK.

The integrated area below the curve incorporates these different parameters and was therefore used in the calculations. A significant increase in signal could be detected down to a concentration of 0.01 nM CCK-8S. Dose-response curves were established from calculating the net response per condition (Figure 2.4b) and were used to derive the EC_{50} values (Table 2.1).

Table 2.1. Median effect concentrations (EC50 and IC50) for the natural ligand CCK-8S, the partial agonist JMV-180 and the antagonist lorglumide, measured with the fluorescence plate reader (population average) and the confocal microscope (population average and single-cell). Median effect concentrations for a ligand do not significantly differ between measuring techniques, calculated with ANOVA followed by a post hoc Tukey test (df = 2; CCK-8S: F = 3.05, p = 0.11; JMV-180: F = 0.48 p = 0.64; lorglumide: F = 0.72, p = 0.52).

Ligand	Median effect concentration (EC_{50} and/or IC_{50} ± SE)		
	Fluorescence plate reader	Confocal microscope	
	Population average	Population average	Single-cell average
CCK-8S	24 ± 4 pM	50 ± 13 pM	47 ± 13 pM
JMV-180	20 ± 4 nM	31 ± 12 nM	31 ± 12 nM
Lorglumide	3 ± 1 µM	4 ± 1 µM	5 ± 2 µM

Next, the effect of a partial agonist, JMV-180, was determined. The EC_{50} of JMV-180 was a 1,000-fold higher with respect to the natural ligand, pointing at a much lower affinity towards the receptor (Table 2.1). Moreover, the maximum response that could be evoked by JMV-180 compared to 1 nM CCK-8S was more than halved (mean ± SEM; 38 ± 8%), illustrating the lower potency of this partial agonist to activate the CCK1R (Figure 2.5a).

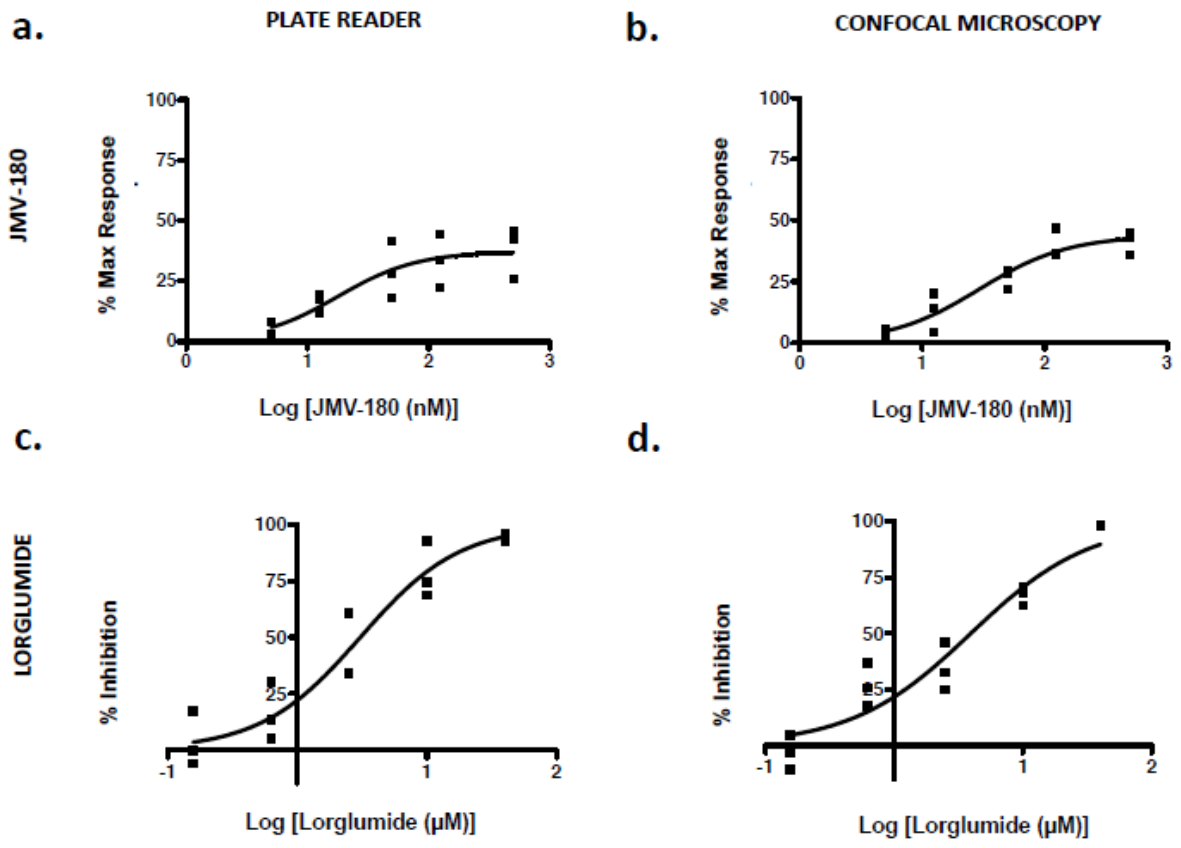


Figure 2.5. Representative dose-response curves for JMV-180 (a,b) and lorglumide (c,d) based on 3 experiments in which the measurements for each concentration are repeated 5 times, expressed as a percentage of the maximum response and the percentage of inhibition from the maximum response, respectively. Graphs on the left are the results measured with the fluorescence plate reader (a,c) and on the right with the confocal microscope (b,d).

In addition to the aforementioned agonists, the full antagonist lorglumide was tested for its potential to inhibit a CCK-8S-induced response. Increasing concentrations of lorglumide clearly demonstrated a dose-dependent inhibition of the response to 1 nM CCK-8S. The IC_{50} value was 3 ± 1 µM (Figure 2.5c, Table 2.1). Full inhibition of 1 nM CCK-8S was obtained at 40 µM lorglumide.

2.3.3 Confocal microscopy provides both population as well as single-cell information

Using confocal microscopy, the same kinetic experiments were performed as with the plate reader. First, the population-average response was determined on whole images. Per well, one single region was acquired at full field of view (636 µm x 636 µm), corresponding to 100-150 cells, and the average pixel intensity was measured over the entire image through time (Figure 2.6a, See supplementary Movies 1 and 2 in the online publication (*259*)). This assay was performed for CCK-8S with and without lorglumide and for JMV-180 and resulted in highly similar RF curves, dose-response curves and median effective concentration values (EC_{50} and IC_{50}) as those obtained for the plate reader (Figure 2.6b,c; Table 2.1; Figure 2.5b,d). To determine the actual single-cell response, we measured fluorescence kinetics in individual cells by means of automated image analysis (Figure 2.7a, Figure 2.1).

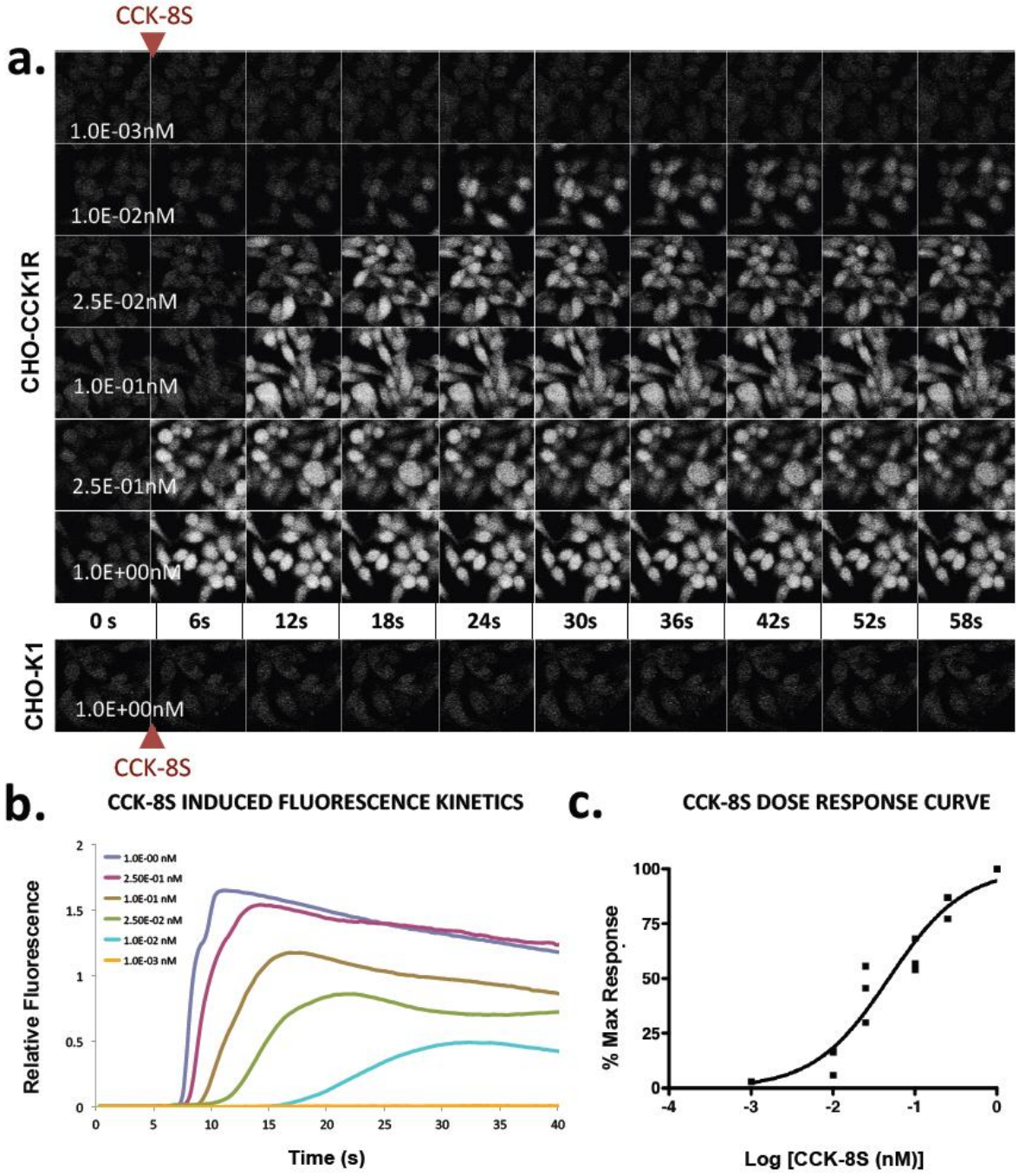
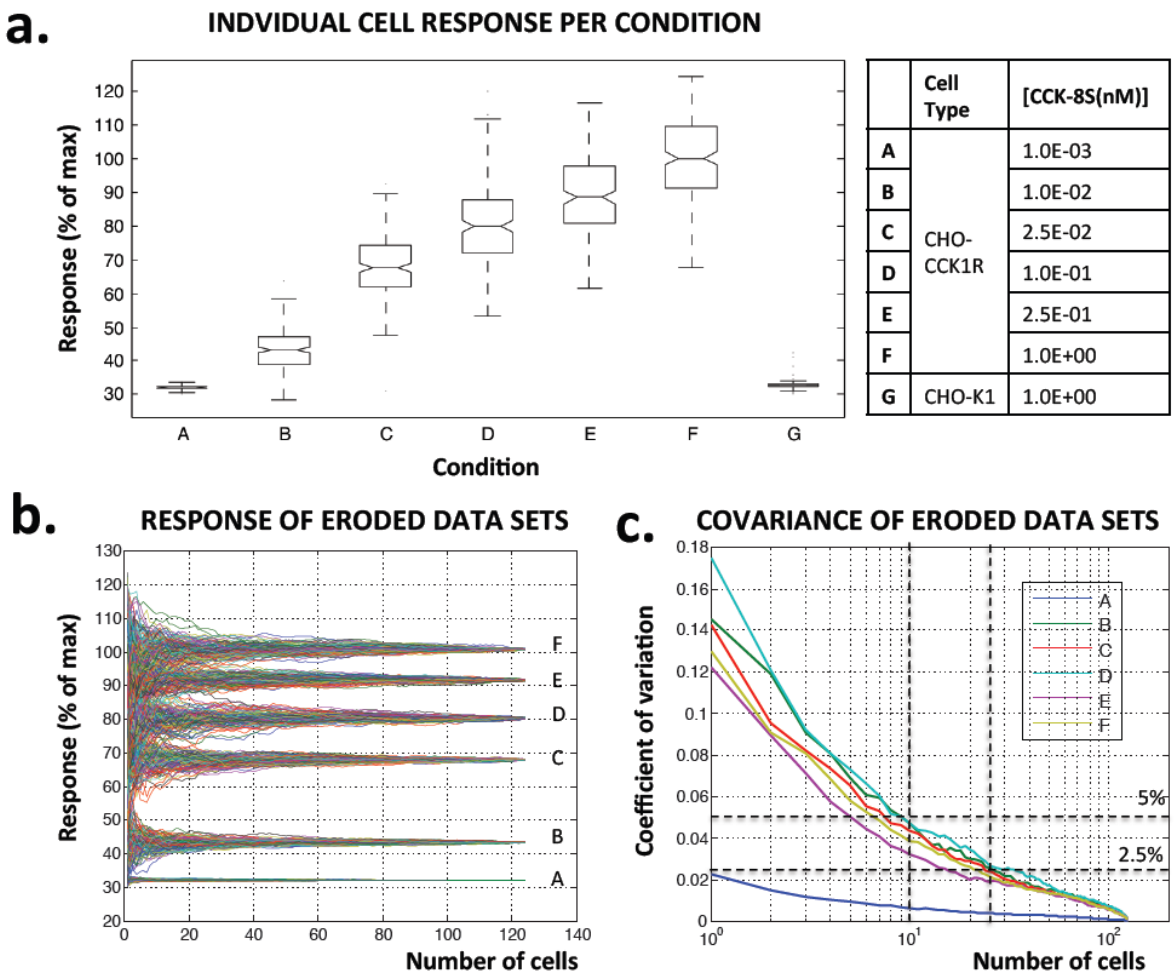


Figure 2.6. Dose-dependent CCK1R-mediated calcium fluxes in cell populations monitored with a resonant scanning confocal microscope. (a) Montage from *in fluxo* confocal microscopy, combined from equally cropped regions selected from raw time-lapse series, acquired as described in the Materials and Methods section. (b) Kinetics of relative fluorescence (RF) of Fluo-4AM labeled CHO-CCK1R cells to increasing concentrations of CCK-8S (0.001–1 nM). The curves represent the mean of 5 technical replicates (wells). (c) Representative dose-response curve for CCK based on 3 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, *i.e.* the net response induced by 1 nM CCK.



	Cell Type	[CCK-8S(nM)]
A	CHO-CCK1R	1.0E-03
B		1.0E-02
C		2.5E-02
D		1.0E-01
E		2.5E-01
F		1.0E+00
G	CHO-K1	1.0E+00

Figure 2.7. Dose-dependent CCK1R-mediated calcium fluxes in individual cells monitored with a resonant scanning confocal microscope (a) Boxplot representing the single-cell response of CHO-CCK1R cells to increasing concentrations of CCK-8S (0.001–1 nM), measured as the average intensity of individual cells and expressed as a percentage of the maximum response, *i.e.* the average response induced by 1 nM CCK. (b) *In silico* erosion. Per condition, the response was calculated for all cells within one representative image per CCK-8S concentration. Next, cells were progressively removed from permutated data sets, one by one, down to the single cell and per step the average net response was calculated. This was repeated 100 times per condition. Every line represents one complete erosion cycle. (c) From the eroded data sets the covariance (CoV) was calculated and plotted as a function of the number of cells and represented on a logarithmic scale for visualization purposes. Ten cells reduce the CoV to 5% and 25 cells reduce the CoV to 2.5% (dotted black lines).

The individual fluorescence kinetics in time varied from cell to cell, not only in magnitude and time point of the maximum fluorescence intensity but also in fluorescence fluctuation behavior (Figure 2.8).

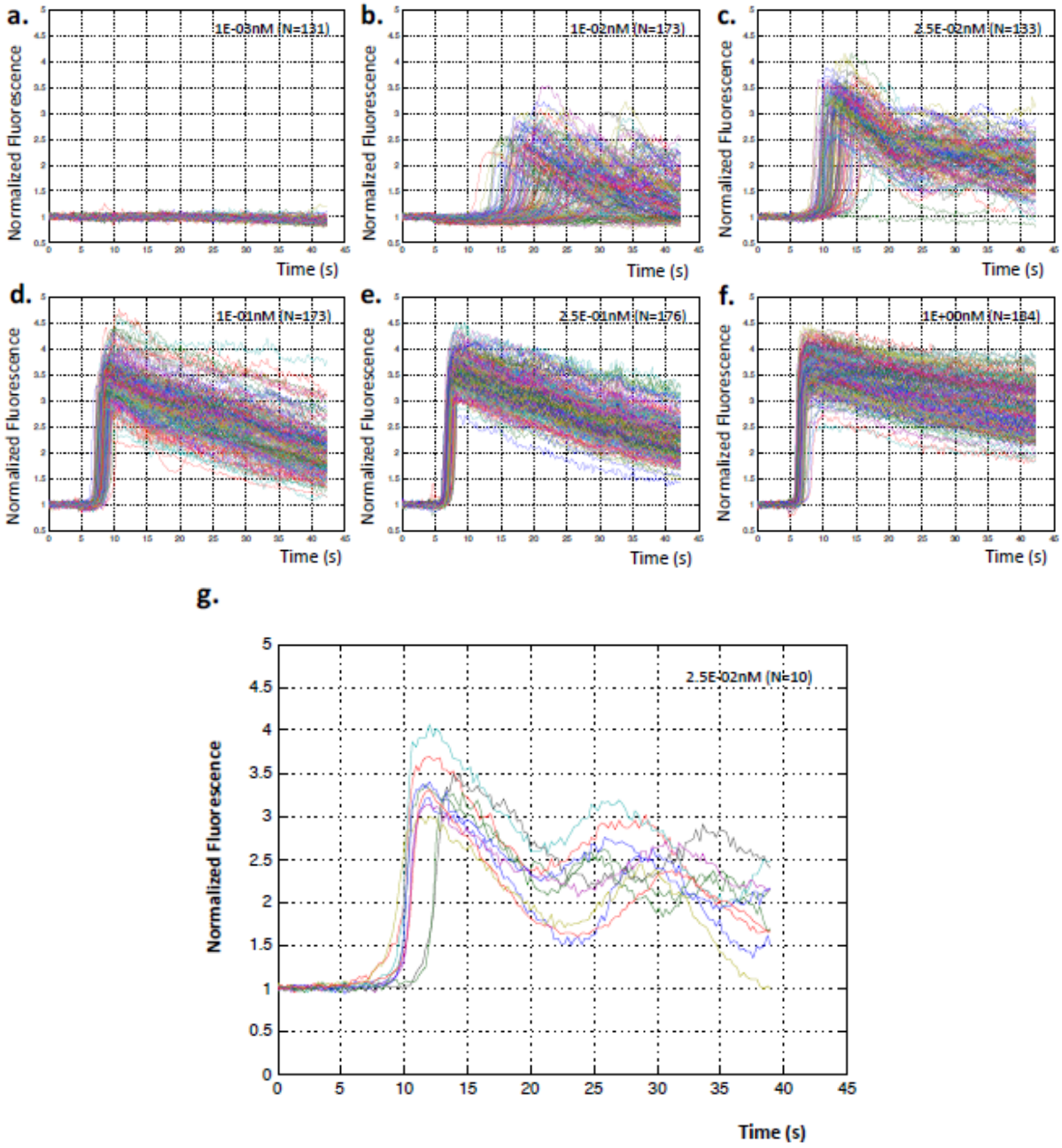


Figure 2.8. Inter-individual and temporal variation in normalized fluorescence kinetics (F_i/F_0) in response to CCK-8S, monitored in Fluo-4AM labeled CHO-CCK1R cells with a resonant scanning confocal microscope, decreases with increasing concentration: (a) 1E-03 nM, (b) 1E-02 nM, (c) 2.5E-02 nM, (d) 1E-01 nM, (e) 2.5E-01 nM, and (f) 1n M. (g) 10 randomly selected curves from cells treated with 2.5E-02 nM CCK-8S demonstrate the fluorescence fluctuations in individual cells at low concentrations.

The time lag between the moment of sample addition and the moment of maximum fluorescence intensity became progressively shorter with increasing agonist concentration. In parallel, the synchrony with which the fluorescence of individual cells changed increased as well. This is reflected in the magnitude of the standard deviation of the time point of maximum fluorescence (Figure 2.9).

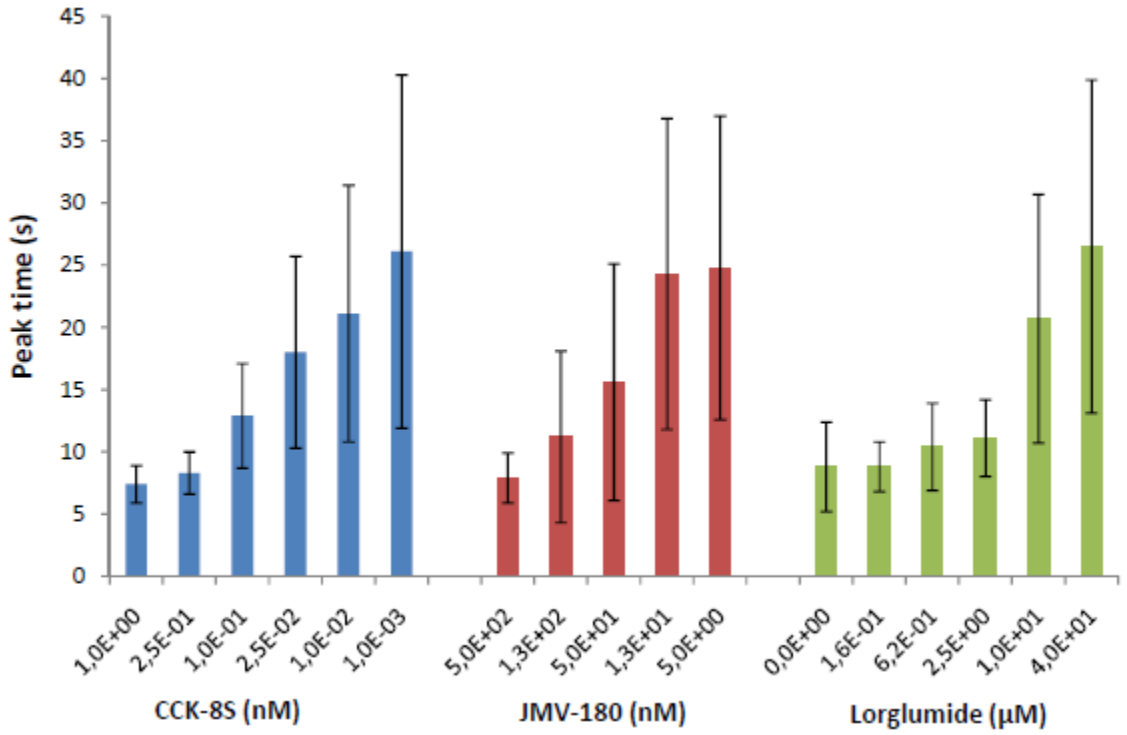


Figure 2.9. Histogram comparing the average peak time (maximum fluorescence) of individual cells of all experiments, for increasing concentrations of CCK-8S, JMV-180 and lorglumide. Error bars represent standard deviations.

Especially at low doses a cellular behavior became evident that was not observed in population averages: calcium fluxes show several less intense oscillations after the first primary fluorescence peak (Figure 2.8g). These fluctuations were no longer observed from a dose of 0.1 nM CCK or higher. At high concentrations, the oscillatory mode changed into one single increase after which no oscillations are seen anymore. In brief, fluorescence kinetics In CCK-8S stimulated cells became more uniform with increasing concentrations, which is in accordance with earlier observations (*295*). In comparison, fluorescence oscillations were more pronounced in cells treated with JMV-180 and also persisted at higher doses (Figure 2.10).

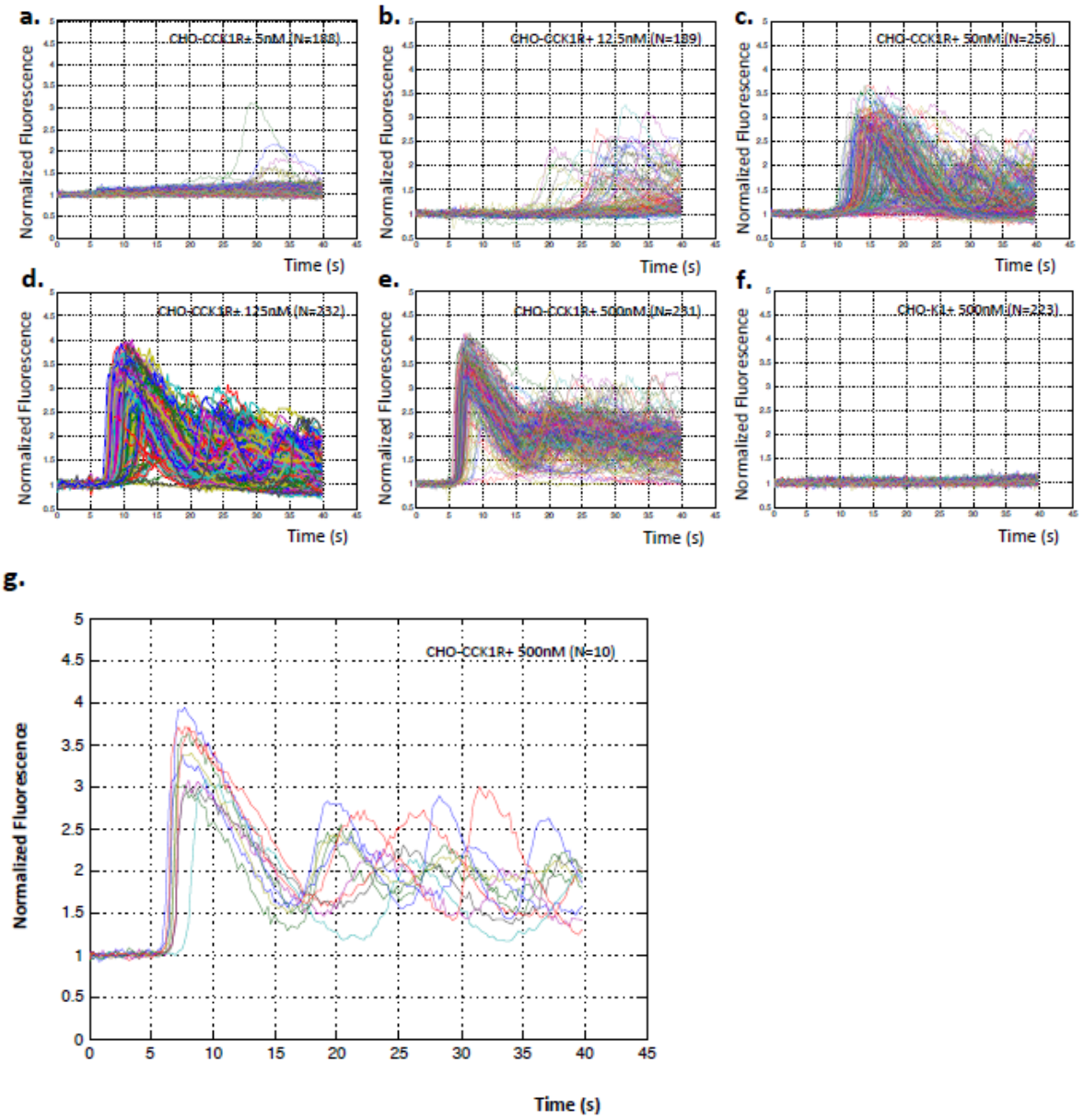


Figure 2.10. Inter-individual and temporal variation in normalized fluorescence kinetics (F_i/F_0) in response to JMV-180, monitored in Fluo-4AM labeled CHO-CCK1R cells with a resonant scanning confocal microscope, is more pronounced than after treatment with CCK-8S and persists with increasing concentration: (a) 5.0 nM, (b) 12.5 nM, (c) 50 nM, (d) 125 nM, and (e) 500 nM. For comparison, (f) shows the profiles of Fluo-4AM labeled CHO-K1 cells treated with 500 nM JMV-180. (g) 10 randomly selected curves from cells treated with 500 nM JMV-180 illustrate the persistence of fluorescence fluctuations in individual cells at high concentrations.

Despite the inherent variability, all doses were significantly different from each other (Figure 2.7a). The median effective concentrations (EC_{50} and/or IC_{50}) based on single-cell analysis were not significantly different from those based on the average image intensity nor from those derived from the plate reader (Table 2.1).

To determine the robustness of the single-cell analysis, we calculated the number of cells required to discriminate dose-dependent responses using an *in silico* approach. To this end, image data sets containing all individual cell responses were progressively eroded by omitting one single, randomly selected cell at a time (on permutated data sets), down to one single cell, and by calculating the average response with each step. This was repeated 100 times to obtain a distribution that represents the variability within the data set (Figure 2.7b). The covariance (std/mean) plots of these distributions demonstrated that as little as 10 cells are sufficient to determine the population mean with 95% accuracy, or 25 cells are sufficient to determine the population mean with 97.5% accuracy (Figure 2.7c). *In silico* erosion was performed for all experiments and confirmed that 10-20 cells are sufficient to determine the population mean with 95% accuracy (Figure 2.11).

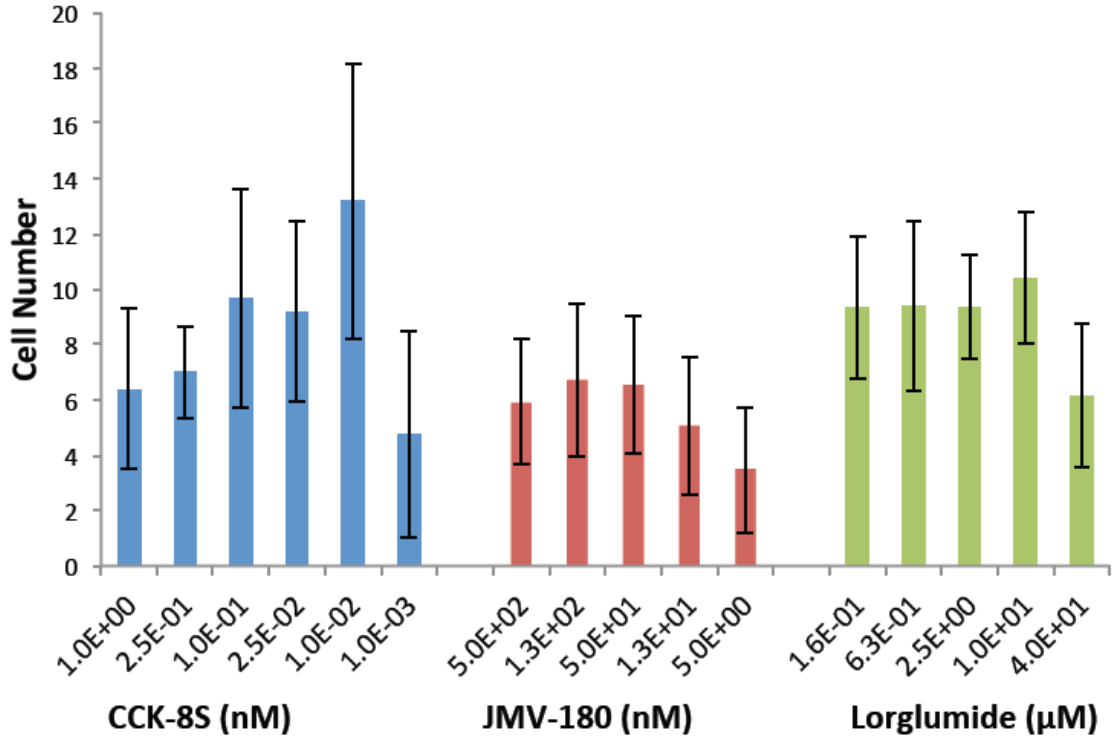


Figure 2.11. Histogram comparing for all experiments the number of cells required to obtain a coefficient of variation of the cell response equal to or smaller than 5%, as calculated from *in silico* erosion data sets. Error bars represent standard deviations.

2.4 Discussion

We have established a robust cell-based bioassay to screen for compounds which activate CCK1R or inhibit its binding to the natural ligand, and which works on two different platforms. This technology was first developed by Foltz et al. (*82*) for a fluorescence plate reader. We have now optimized this technology by making it more sensitive, specific and more robust and by cross-validating this assay

on a different platform, namely the confocal microscope. During optimization, we discovered that lowering the loading and measurement temperature had an important influence on the sensitivity and temporal stability of the cell response. It is conceivable that the degree of cell compartmentalization and the decay of the fluorescence through time, which were observed at higher temperatures, are due to a higher cell metabolism. At higher temperatures, transport mechanisms like endocytosis/pinocytosis (leading to compartmentalization) and exocytosis (leading to loss of signal) become more prominent (*170*) and active breakdown of the fluorescent dye could be promoted (*62*).

The EC_{50} measured for CCK-8S in this project with the fluorescence plate reader and the confocal microscope were not significantly different, although purely based on the SEM one could say a higher accuracy was obtained with the plate reader. This could be due to a different demand for manual interaction between both approaches: the measurements with the fluorescence plate reader were almost fully automated, whereas the measurements with the confocal microscope were carried out manually, thereby introducing pipetting inaccuracies and causing complete experiments to last longer and have more fluorescent probe decay. Also, sample mixing in the medium may have occurred faster in the plate reader causing cells to react more synchronously. On the other hand, a bigger SEM in confocal measurements may be the reflection of the high heterogeneity between individual cells, as such providing more accurate information of the true cellular response. In addition, the visualization of cells allows immediate verification of cellular health and density. Confocal microscopy also allows discrimination of off-target effects like bad solubility and background fluorescence of the sample (*e.g*. when protein hydrolysates are tested). With the fluorescence plate reader, background fluorescence of the sample causes a major concern as the fluorescence of the entire well is measured, which may obscure more subtle fluorescence increases at the level of the cells. In contrast, the confocal microscope only detects the fluorescence in a selected focal plane on the bottom of the well, which is predominantly within the cells. Hence, confocal microscopy allows measuring a more specific response and testing higher sample concentrations.

Furthermore, experiments carried out with cells in suspension under continuous stirring revealed 10 to 50-fold higher EC_{50} values (around 0.19-1.08 nM) for CCK-8S compared to our experiments (Table 1) and were less sensitive (*25, 71, 252*). These differences could not be ascribed to large differences in medium composition (e.g. BSA concentration) or probenecid concentration, indicating that attached cells are a better means to measure Ca^{2+}-fluxes. However, the EC_{50} of the partial agonist JMV-180 measured with cells in suspension (25 nM) was comparable to the EC_{50} measured in this

project (*252*). Receptor activation can also be measured by inositol trisphosphate (IP_3) production. Cawston et al. (*42*) reported an EC_{50} value for IP_3 production of JMV-180 of 18 nM, which is similar to the EC_{50} value of JMV-180 we found for Ca^{2+} increase (Table 2.1), and at the maximum concentration only induce 9% of the IP_3 production compared to that of CCK-8S could be induced. The lower potency of JMV-180 compared to the natural ligand can be explained by the differential positioning of the C-terminal end within the binding site of CCK1R (*5*). A partial agonist only exerts a subset of the biological functions of the natural ligand and therefore has a reduced tendency to evoke side effects and cause receptor intolerance. This makes a partial agonist more suitable as a therapeutic than the natural ligand (*42*). In this context, it is also noteworthy that JMV-180 elicited highly different fluorescence kinetics compared to CCK-8S. In accordance with previous observations (*254, 273*), more pronounced and more frequent oscillations were observed, even at high doses, indicating an altered cellular response. These differences could be due to differential coupling of low- and high affinity CCK1R binding sites to second-messenger systems and Ca^{2+} signal transduction pathways, e.g. differences in activation of Protein Kinase C and IP_3 production (*279, 304*). Lorglumide is a full antagonist of the CCK1R and it is 2,300 times more selective for CCK1R than for CCK2R. It is a potent inhibitor in comparison to other glutaramic acid analogues (*20*); an IC_{50} value of 0.13 µM was reported by Makovec et al. (*169*) for this antagonist. The latter IC_{50} value is 15 times lower compared to the IC_{50} measured in this project, which is probably due to the use of a different experimental design. Hence it can be concluded that comparison between the median effect concentrations (EC_{50} and IC_{50}) of different CCK receptor agonists and antagonists between studies should be made with caution, as different cell types, measuring techniques and controls are used. Therefore, we propose this standardized procedure to enable comparison of the CCK1R activity of different ligands. Moreover, with little adaptations, this assay could be converted into a screening system for ligands for the CCK2R, the sulfakinin receptor and other Ca^{2+}-influencing GPCRs.

In conclusion, the proposed cell-based bioassay can be used to screen for protein hydrolysates and molecules with CCK1R activity in a standardized manner. Measurement can be accomplished on two platforms. The fluorescence plate reader is more suitable to perform a primary screen, but the confocal microscope can be used to validate the activity of the components on the single-cell level, especially in a context of strong autofluorescence background.

Chapter 3

Screening of soy and milk protein hydrolysates for their ability to activate the CCK1 receptor

This chapter has been published in Staljanssens, D.; Van Camp, J.; Billiet, A.; De Meyer, T.; Al Shukor, N.; De Vos, W. H.; Smagghe, G., Screening of soy and milk protein hydrolysates for their ability to activate the CCK1 receptor. *Peptides* **2012,** 34, 226-231

Chapter 3 Screening of soy and milk protein hydrolysates for their ability to activate the CCK1 receptor

3.1 Introduction

In this chapter, the performance of the cell-based bioassay as described in Chapter 2 is evaluated to screen more complex formulations such as protein hydrolysates for their ability to activate the CCK1R. The potential of the fluorescence plate reader for the screening of such complex matrices was investigated and compared with the confocal microscope. We focused on soy and milk proteins, well-known and convenient food proteins of which beneficial health effects have been proven (*92, 145, 151, 190*), but which differ significantly in source and structure.

3.2 Materials and Methods

3.2.1 Products

Hexane, sodium bisulfite, α-lactalbumin, κ-casein, pepsin, trypsin and chymotrypsin were purchased from Sigma-Aldrich (Bornem, Belgium/St.-Louis, MO). Soybeans were purchased in the local grocery store (Heuschen & Schrouff OFT B.V., Canada). Enzymatic soy hydrolysates E110, AM41, A2SC and A3SC were obtained from Organotechnie (La Courneuve, France). Dialysis membranes were purchased from Spectra Laboratories Inc. (Rancho Dominguez, CA).

3.2.2 Preparation of 7S fraction from soy protein

The 7S fraction from soybean protein (β-conglycinin) was extracted following a method based on pH precipitation described by Liu et al. (*164*). In brief, soybean seeds were ground with a coffee mill and defatted with hexane to obtain defatted soybean flour. The soybean flour was extracted twice with 0.03 M Tris-HCl (pH 8.5) for 1 h at 45 °C in a ratio of 15% (w/v). Subsequently, sodium bisulfite was added to the extraction product to a concentration of 0.01 M. The solution was kept overnight at 4 °C and centrifuged. Next, NaCl was added to a concentration of 0.25 M to the supernatant and the solution was centrifuged again. The pH of the obtained supernatant was adjusted to 4.8, which caused the 7S protein fraction to precipitate. The precipitate was dialyzed over pure water and subsequently freeze-dried.

3.2.3 Hydrolysis simulating gastrointestinal peptic digestion

An in vitro gastrointestinal digestion was performed on α-lactalbumin, κ-casein and the 7S soy proteins as described before (*260*). Briefly, the lyophilized proteins were dissolved in distilled water

(4% w/v), pH was lowered to 2 and pepsin was added in a ratio of 0.4% (w/w) to the sample. The solution was kept at 37 °C and shaken for 2 h. Next, the pH was set to 6.5 and trypsin and chymotrypsin were added also in a ratio of 0.4% (w/w), and the solution was shaken for 2.5 h at 37 °C. The reaction was terminated by heating the samples for 15 min to 80 °C. The 7S soy hydrolysate was designated 7SH.

3.2.4 Cell-based bioassay to screen for CCK1R activity

The determination of the intracellular free Ca^{2+} concentration was performed as described in chapter 1. Samples were added *in fluxo*: after 6 s of acquisition, the protein hydrolysate sample or CCK-8S was added instantaneously while the acquisition continued for another 34 s. For each sample concentration, measurements were repeated in five wells for both cell types (technical replicates) and every experiment was repeated 2-4 times (biological replicates). For inhibition experiments, lorglumide was added at a final concentration of 50 µM, 30 min prior to the start of the experiment.

3.2.5 Data analysis and statistics

Statistical analyses were performed using one-sample Student's *t*-tests, two-sample Student's *t*-test or non-parametric Wilcoxon rank sum tests. Differences between net responses were considered significant for p-values smaller than 0.05. Sigmoid dose-response curves were generated for the net response versus sample concentration relationships and the EC_{50} value (the sample concentration at which 50% of the maximum response was reached) was calculated using Prism v4 software (GraphPad Prism, La Jolla, CA) (*286*).

Specifically for confocal microscopy, the robustness of the single cell analysis was determined using an *in silico* approach, which calculates the minimal numbers of cells to determine the net response accurately [20]. To this end, selected image data sets containing all individual cell responses were progressively eroded by omitting one single, randomly selected cell at a time, down to one single cell, and by calculating the average response with each step. This was repeated 100 times on permutated data sets to obtain a distribution that represents the variability within the data set. From these distributions, the coefficient of variation (COV= standard deviation/mean) was derived and plotted against the number of cells.

3.3 Results

3.3.1 The fluorescence plate reader fails to give accurate results for complex matrices of protein hydrolysates

First, four commercial crude soy hydrolysates E110, AM41, A2SC and A3SC were tested for their potential to induce a CCK1R-mediated calcium flux. Measurements were performed with the fluorescence plate reader since this setup proved to be a fast and reliable tool for pure compounds, as discussed in the previous chapter. A net response was measured for these hydrolysates between 90 and 300%. However, there was a high variability (25 to 40%) and the fluorescence profiles showed atypical kinetics. Upon addition of the protein hydrolysates an immediate (within 0.3 s) increase in fluorescence was observed. The increase was 5 to 7 times higher than that observed for CCK-8S and this was the case both for CHO-CCK1R and CHO-K1 cells (Figure 3.1a). Moreover the fluorescence level remained stable throughout the acquisition period, irrespective of the administered dose (data not shown). For comparison, addition of CCK-8S resulted in an exponential increase of the fluorescence signal in CHO-CCK1R cells with a delay of minimum 3 s after sample addition and no significant signal increase in CHO-K1 cells. This suggested that the crude protein hydrolysates induced a non-specific rise in fluorescence, possibly due to the presence of an autofluorescent component.

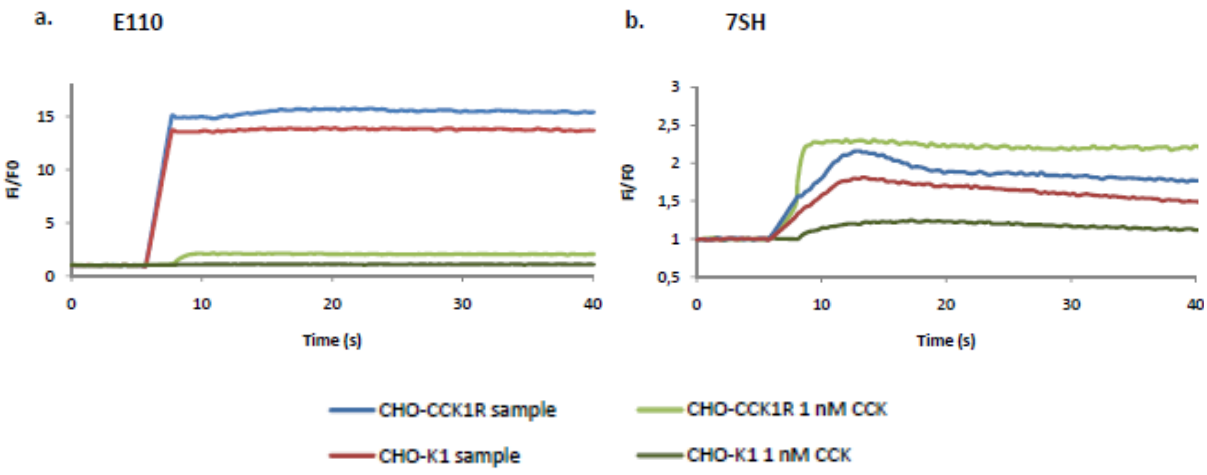


Figure 3.1. Normalized fluorescence kinetics (F_i/F_0) for CHO-CCK1R and CHO-K1 in cell populations monitored with a plate reader. Results for sample as well as for 1 nM are shown. The curves represent the mean of 5 technical replicates (wells). (a) Representative curve for soy hydrolysate E110 (3 g/l). (b) Representative curve for 7SH (1 g/l).

Since it was difficult to pinpoint the exact origin of the strong (auto-)fluorescence in the complex hydrolysates, we decided to measure the fluorescence kinetics in hydrolysates of purified soy and milk proteins. The gastrointestinal digested 7S fraction from soy protein (1 g/l), gastrointestinal

digested κ-casein from milk (3 g/l) and gastrointestinal digested α-lactalbumin (3 g/l) from milk were evaluated. The fluorescence profiles of these hydrolysates demonstrated more reliable kinetics than those of the crude hydrolysates: the fluorescence increase was lower than that induced by 1 nM CCK8S, reached a maximum response after several seconds and gradually faded (Figure 3.1b). In addition, the fluorescence increase of CHO-CCK1R was significantly higher than that of CHO-K1 cells. For the 7SH, a net response of 13.4±3.0% (n=5, p=0.01) was measured, which was significantly different from zero. Net responses were obtained of 7.7±1.4% (n=2, p=0.11) for the κ-casein hydrolysate and 19.9±4.2% (n=2, p=0.13) for the α-lactalbumin hydrolysate, but these were not significantly different from zero.

3.3.2 Confocal microscopy allows to measure CCK1R activation accurately, excluding false positives

3.3.2.1 Only the 7S soy hydrolysate shows a significant net response

As described in the previous chapter, we noted that it was important to validate the data from the plate reader with a confocal microscope, especially in a context of strong autofluorescence background. In addition, especially for crude hydrolysates, highly variable results were obtained with the plate reader, as reported above. Therefore, the experiments with the soy and milk protein hydrolysates were repeated using confocal microscopy and the population-average response was calculated. To this end, the average pixel intensity of an image comprising 150 to 250 cells was measured per well. Interestingly, the high responses found with the fluorescence plate reader for the crude soy hydrolysates were not reproduced with the confocal microscope. Compared to the CCK8S-induced response, a delayed and limited increase in fluorescence was observed (Figure 3.2a). CHO-CCK1R cells and CHO-K1 cells reacted alike, resulting in a low net response for all hydrolysates. The net responses for soy hydrolysate E110, AM41, A2SC and A3SC were not higher than 7.7% and none were significantly different from zero. Taken together, these results showed that the crude hydrolysates failed to induce a CCK1R-mediated Ca^{2+}-flux.

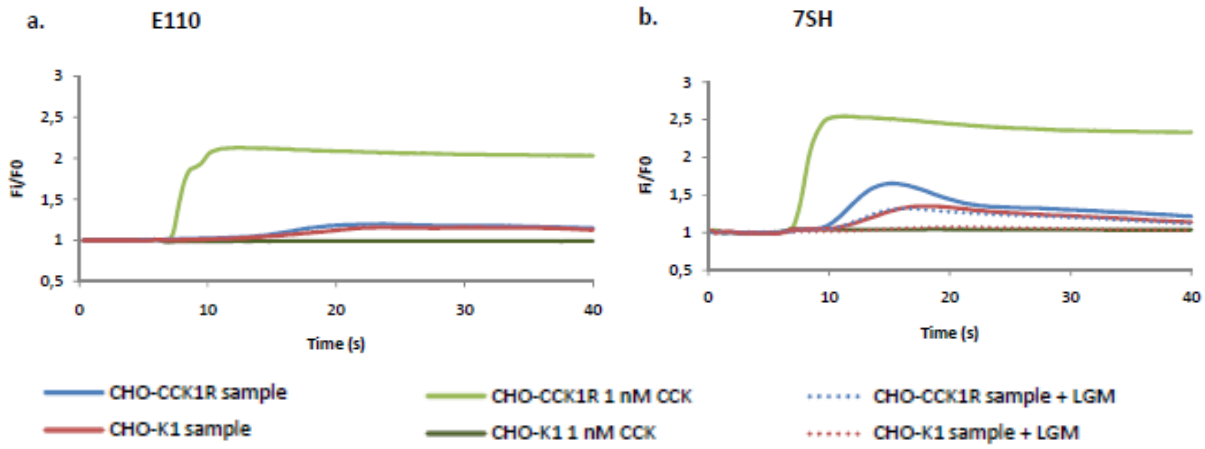


Figure 3.2. Normalized fluorescence kinetics (F_i/F_0) for CHO-CCK1R and CHO-K1 in cell populations monitored with a resonant scanning confocal microscope. Results for sample as well as for 1 nM are shown. The curves represent the mean of 5 technical replicates. (a) Representative curves for soy hydrolysate E110 (3 g/l). (b) Representative curves for 7SH (1 g/l). Dotted lines represent the fluorescence kinetics of the experiments performed in the presence of lorglumide.

As for the hydrolysates from purified proteins, confocal analysis of the 7SH (1 g/l) showed a significant net response of 14.3±1.8% (n=4). Moreover, the fluorescence profiles demonstrated reliable kinetics. An exponential increase was observed in fluorescence signal, which was significantly higher for CHO-CCK1R than for CHO-K1 cells; it reached a maximum and subsequently decreased (Figure 3.2b). For this hydrolysate, a dose-response curve was established (Figure 3.3), from which an EC_{50} value was calculated of 0.069 mg/l (95% confidence interval: 0.028-0.170 mg/l; R^2=0.79).

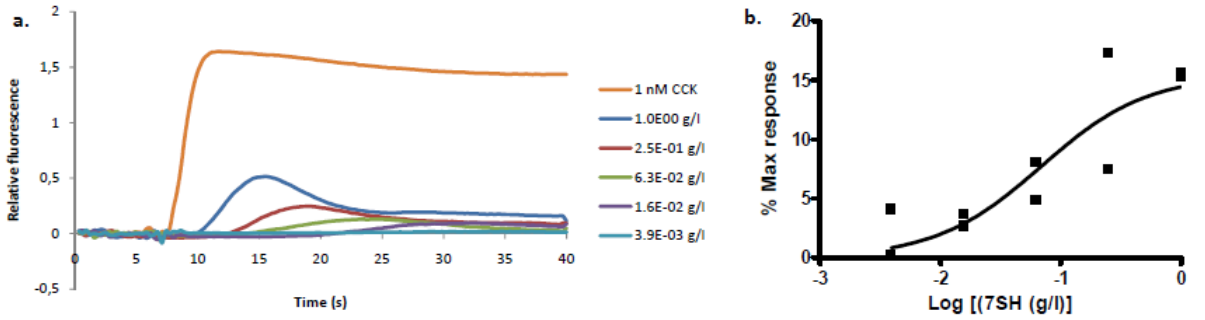


Figure 3.3. Dose-dependent CCK1R-mediated calcium fluxes in cell populations monitored with a resonant scanning confocal microscope. (a) Representative relative fluorescence kinetics (RF) of increasing concentrations of 7SH (0.0039-1 g/l). The curves represent the mean of 5 technical replicates. (b) Dose-response curve for 7SH based on 2 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, i.e. the net response induced by 1 nM CCK8S.

For the milk κ-casein hydrolysate, a net response of 10.6±5.1% (n=2) was measured, but it was not significantly different from zero (p=0.28). The net response of the milk α-lactalbumin hydrolysate was negligible.

3.3.2.2 Single-cell variations for the 7S soy hydrolysate

To complement the aforementioned population-average approach and obtain a better insight in the actual single-cell response, we measured fluorescence kinetics in individual cells by means of automated image analysis. As documented before [20], CHO-CCK1R cells treated with 1 nM CCK-8S showed a strong, highly synchronous fluorescence profile. This profile was characterized by a steep increase in fluorescence signal, reaching a maximum at 3 s after the sample addition and a subsequent gradual decrease. In contrast, CHO-K1 cells showed no significant response to the CCK8S stimulation, except for a sporadic non-specific fluctuation in calcium flux. When exposed to varying concentrations of the 7SH, CHO-CCK1R cells demonstrated intensity fluctuations with a strong intercellular variability, both in magnitude and time point of the maximum fluorescence intensity. The time lag between the moment of sample addition and the moment of maximum fluorescence intensity became progressively shorter with increasing hydrolysate concentration. In parallel, the response of individual cells became more synchronous at higher concentrations. Notably, CHO-K1 cells also showed pronounced individual cell responses, which were significantly different from CCK8S-treated CHO-K1 cells for concentrations of 7SH higher than 6.3E-02 g/l. These fluctuations were lower and less abundant than those observed in CHO-CCK1R cells, but much more pronounced than basal calcium fluctuations (Figure 3.4).

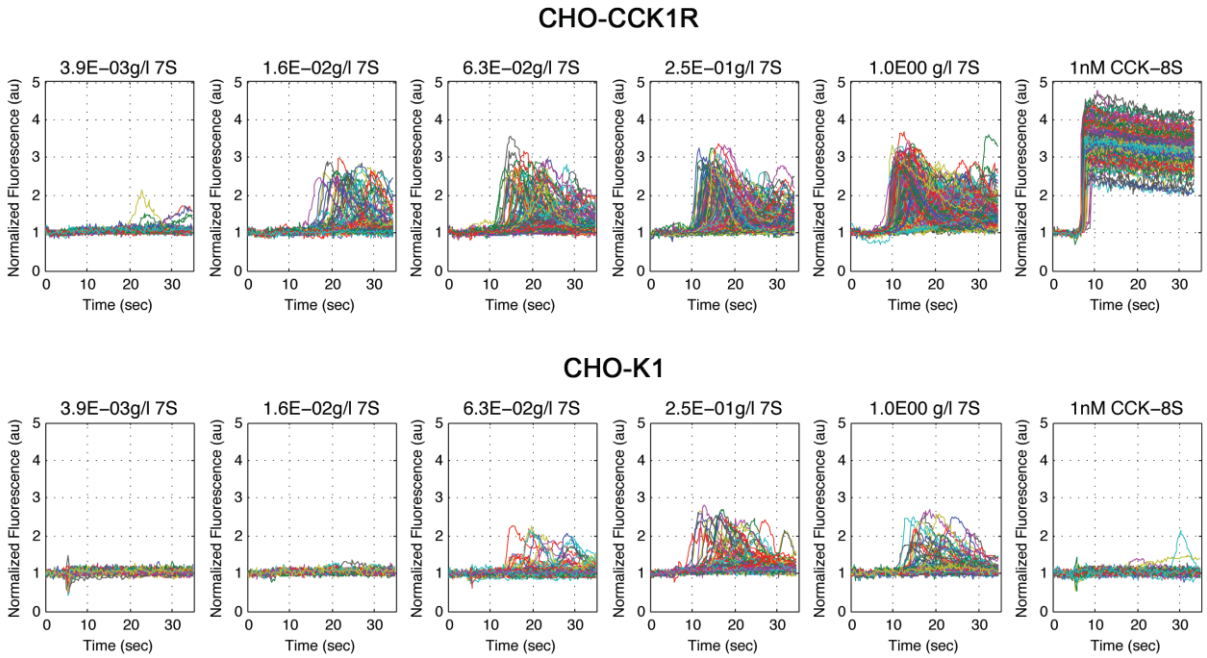


Figure 3.4. Inter-individual and temporal variation of normalized fluorescence kinetics (F_i/F_0) in response to varying doses of the 7SH and 1 nM CCK8S, monitored in CHO-CCK1R and CHO-K1 cells with a resonant scanning confocal microscope. Every plot displays the kinetic fluorescence profiles of all cells of one arbitrarily selected recording.

Despite the inherent variability and fluctuations in both cell types, all doses, except for 3.9E-03 g/l, induced a response in CHO-CCK1R cells, which was significantly different from the dose-matched CHO-K1 controls (Figure 3.5a). As a measure for the robustness of the single cell analysis, we determined the numbers of cells required to obtain an accurate estimate of the average net response on representative datasets of the 7SH (Figure 3.5b). When the coefficient of variation of the net response was plotted against the numbers of cells that were included (as described in the material and methods), a strong exponential decrease was observed for increasing numbers of cells (Figure 3.5c). On average, across the entire dataset, 15±2 cells were required to determine the net response (±5%) with 95% confidence.

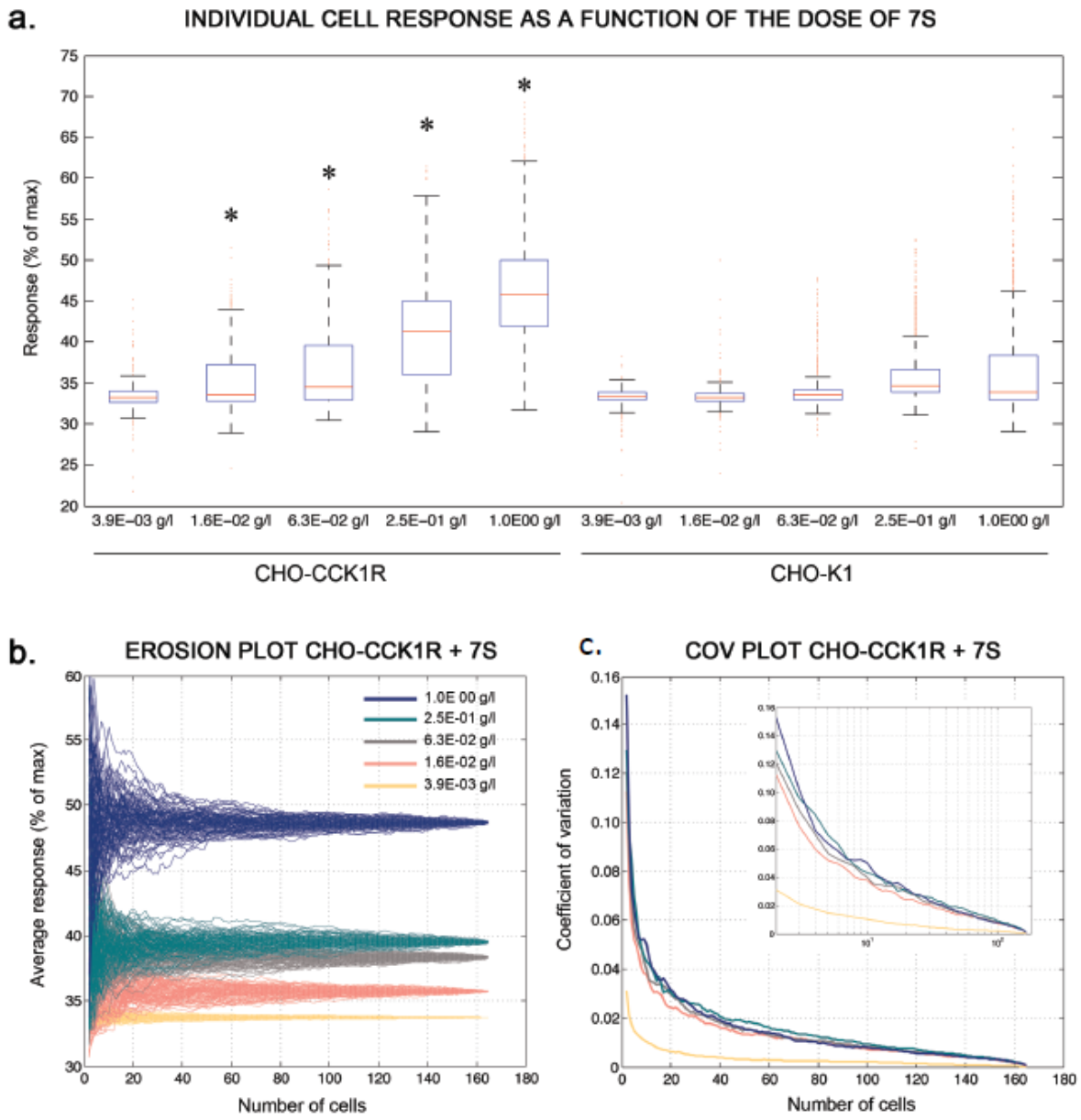


Figure 3.5. Dose-dependent calcium-mediated fluorescence fluxes in individual cells monitored with a resonant scanning confocal microscope. (a) Boxplot representing the single-cell response of CHO-CCK1R and CHO-K1 cells to increasing concentrations of the 7SH (0.0039-1 g/l), measured as the average intensity of individual cells and expressed as a percentage of the maximum response, i.e., the net response induced by 1 nM CCK8S. The boxplots are based on the results of five technical replicates. Stars indicate significant differences from the dose-matched CHO-K1 control (3.9E-03 g/l: p= 9.1E-01, 1.6E-02 g/l: p=1.8E-02, 6.3E-02 g/l: p=1.0E-02, 2.5E-01 g/l p=3.8E-02, 1.0 g/l p=4.8E-05). (b) *In silico* erosion. Per condition, the response was calculated for all cells within one representative image per concentration of 7SH. Next, cells were progressively removed from permutated data sets, one by one, down to the single cell and per step the average net response was calculated. This was repeated 100 times per condition. Every line represents one complete erosion cycle. (c) From the eroded data sets the coefficient of variation (COV) was calculated and plotted as a function of the number of cells. The inset shows the same plot represented on a logarithmic scale to facilitate discrimination of the exponential part of the plot (cell numbers <20).

Finally, single cell analysis on image datasets of the soy hydrolysate E110 showed significant intensity fluctuations, which were equally abundant in CHO-CCK1R and CHO-K1 cells. Comparison with the fluorescence profiles of background regions (without cells) confirmed that the fluorescence kinetics as measured by the population-average method, were predominantly caused by non-specific calcium fluxes (Figure 3.6).

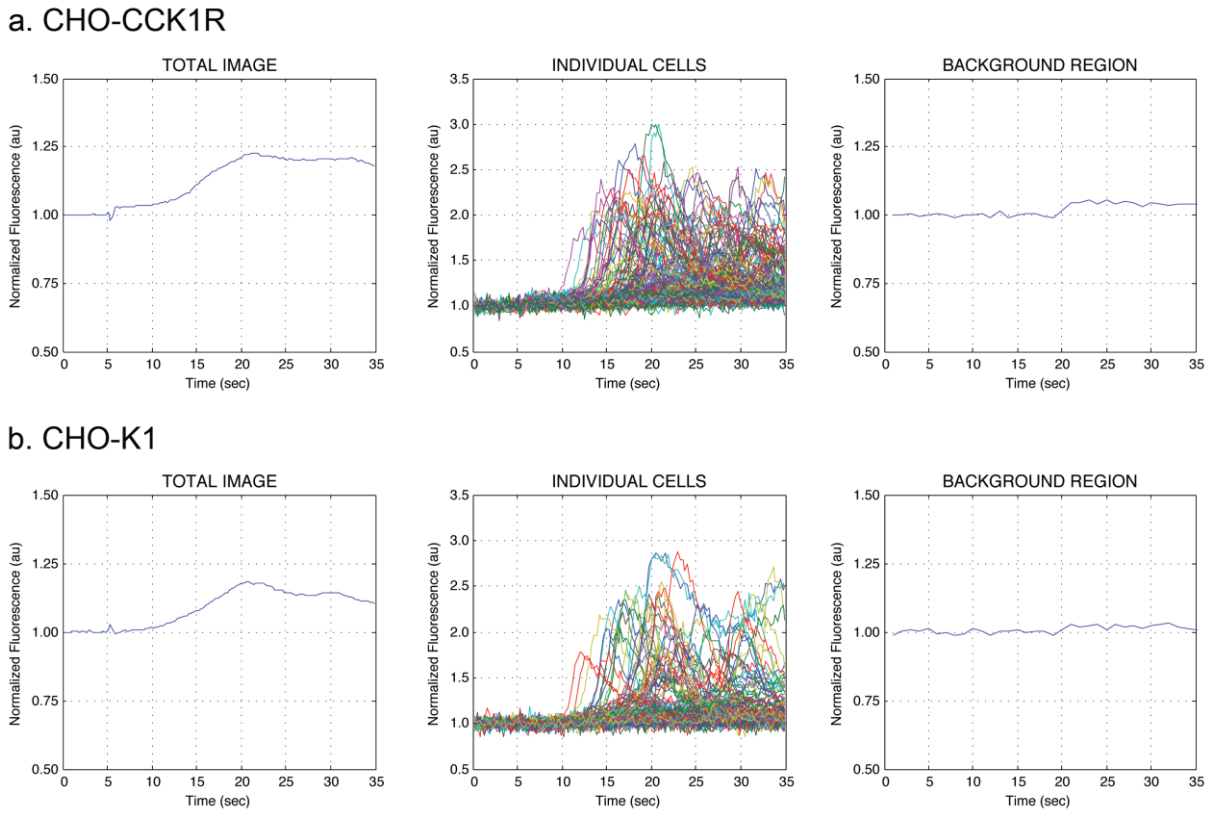


Figure 3.6. Normalized fluorescence kinetics (F_i/F_0) in response to 3 g/l of the soy hydrolysate E110, monitored in CHO-CCK1R (a) and CHO-K1 (b) cells with a resonant scanning confocal microscope. Kinetic curves are displayed for one representative recording of each cell type. The left plot shows the average signal of the whole image, the middle plot shows the kinetics of the average signal per cell for all cells and the right plot displays the average signal of an image region where no cells are present (background). The relative stability of the background signal and the pronounced intensity fluctuations of individual cells confirm that the measured response across the whole image can mostly be attributed to a cellular component (calcium fluxes).

3.3.2.3 The effect of lorglumide on the 7S soy hydrolysate

To test the specificity of the CCK1R response elicited by the 7SH (1 g/l), confocal microscopy experiments were repeated in the presence of 50 µM lorglumide, a known antagonist of CCK1R. The fluorescence profiles of lorglumide-treated CHO-CCK1R cells showed a significant decrease compared to the profiles of cells without lorglumide pretreatment. But surprisingly, this decrease was also

observed with the CHO-CCK1R cells (Figure 3.3b), and as a result, the net response showed no significant change between lorglumide-treated and non-treated cells.

3.4 Discussion

In this study, we have screened raw soy hydrolysates and purified soy and milk proteins for their potential to activate the CCK1R. First, crude protein hydrolysates were tested, which were commercially available. A primary screen was performed with a fluorescence plate reader. However, this device produced dubious results. Very high fluorescence responses were measured for the complex matrices, which mainly seemed to be attributed to background autofluorescence within the sample rather than to a CCK1R-induced Ca^{2+}-increase. Why CHO-CCK1R cells showed a higher non-specific fluorescence response than CHO-K1, when measured with the plate reader is still unclear. Theoretically, the subtraction of the relative fluorescence of the CHO-K1 cells from that of the CHO-CCK1R cells should correct for non-CCK1R-induced Ca^{2+} increases and sample autofluorescence. One possibility is the presence of technical inconsistencies such as non-linearity effects in the detection of strong fluorescence (saturation). Visual inspection of the complex hydrolysates with a widefield microscope confirmed that the formulations showed strong autofluorescence (data not shown). However, in confocal microscopy the excessive signal increases, caused by the autofluorescent components in the sample, were not detected. This is presumably a result of its optical sectioning capacity. By reducing the field depth to the bottom of the well, registration becomes mostly restricted to signals stemming from the cells. However, in the fluorescence plate reader, fluorescence is measured across the entire well, which may obscure more subtle fluorescence increases at the level of the cells due to autofluorescence of the sample. Indeed, single cell analysis of the crude E110 protein hydrolysate showed that there were cellular responses, even though they were non-specific for the CCK1R as they occurred both in CHO-CCK1R and CHO-K1 cells. Hence, confocal microscopy allows for measuring a more specific response and testing higher sample concentrations when working with autofluorescent samples.

As for the hydrolysates from purified proteins, the 7SH yielded a significant response with a fluorescence profile pointing to a real CCK1R activation. Single cell fluctuations showed resemblance to those observed after administration of low concentrations of CCK8S or partial agonists such as JMV-180 (Chapter 2), suggesting partial activation of the CCK1R. The EC_{50} value amounted to 66 mg/l which is in the same range as the EC_{50} value of a commercial soy protein hydrolysate (Quest International, Naarden, the Netherlands) described by Foltz et al. (*82*). Unfortunately there were some confounding factors, which complicate the interpretation of the data. First, the 7SH was not

completely specific since it induced a less pronounced but significant response in CHO-K1 cells. Activation of CHO-K1 cells was also observed for other hydrolysates, albeit to a variable extent, e.g. for soy hydrolysate E110. This is in contrast to the results of Foltz et al. who reported no activation of CHO-K1 cells upon hydrolysate treatment (*82*). Our results suggest that not only CCK1R but also other receptors might become activated, which induce an intracellular calcium flux. In fact, many receptors (other GPCRs, receptor tyrosine kinase-type receptors…) use a similar signaling machinery involving Ca^{2+} as a second messenger (*107, 143, 215, 226*). Activation of these receptors may complicate downstream effects and obscure the envisioned cellular outcome. A second complication was discovered after lorglumide treatment. While a decrease was observed in the fluorescence kinetics of CHO-CCK1R cells, pointing to a specific CCK1R activation, we also found an unexpected decrease in CHO-K1 cells. This indicates that lorglumide might also inhibit other receptors, as was previously suggested by Gaudreau et al. for opioid receptors (*89*). Interestingly, it has been reported that hydrolysates and peptides of the 7S fraction of soy protein are also capable of releasing CCK from STC-1 cells and reducing appetite in rats (*202, 262, 264*). Therefore it might be possible that the appetite-suppressing effect of the 7S soy hydrolysates described in the latter studies have a double mode of action, i.e. stimulation of the release of CCK on the one hand and direct activation of the CCK1R on the other hand.

In conclusion, the fluorescence plate reader seems less suited to measure complex formulations and therefore can only be used to perform a rough primary screen. Confocal microscopy is crucial to exclude false positive and to distinguish specific from non-specific effects. Moreover, an equally high accuracy was obtained with pure compounds (Chapter 2), indicating that the confocal microscope is as reliable for measuring complex formulations as it is for pure compounds. Using confocal microscopy, we discovered that the gastrointestinal digested 7S fraction from soy protein contains CCK1R activity. We also have indications that CCK1R-activating bioactive peptides might be released from κ-casein in milk, since substantial activity was seen, although not significant at the tested concentrations in the current experiments. Further research is needed to increase the *in vitro* net responses of the protein hydrolysates, which includes optimization of hydrolysis and purification of the active fractions and peptides. Finally, validation of the effect of the active components in an *in vivo* model is imperative. To our knowledge, this study is the first in which the effect of non-pharmaceutical CCK1R agonists, i.e. food protein hydrolysates, is analyzed in detail at the cellular level. This knowledge may facilitate the screening and discovery of novel products with CCK1R activity, thereby contributing to the battle against obesity.

Chapter 4

Evaluation of the CCK1R activity of bioactive peptides from 7S and 11S soy protein hydrolysate fractions

---

This chapter is submitted as Staljanssens, D., Smagghe, G., Huybregts, L., De Vos, W., Zotti, M.J., Gevaert, K., Boon, P., Raedt, R. & Van Camp, J. Bioactive peptides from purified soy protein hydrolysate fractions activate the cholecystokinin receptor type 1. *Regulatory Peptides*.

.

Chapter 4 Evaluation of the CCK1R activity of bioactive peptides from 7S and 11S soy protein hydrolysate fractions

4.1 Introduction

As the gastrointestinal digest of the 7S fraction of soy protein showed good CCK1R activity, also the CCK1R activity of the gastrointestinal digest of the other major storage proteins in soybean, namely the 11S fraction (glycinin) was characterized. Next, the protein hydrolysates of 7S and 11S were separated in different molecular weight fractions to determine which fractions induce the strongest activation/response. The potential of the different molecular weight fractions to activate the CCK1R was reevaluated and peptides present in the low molecular weight fractions were identified with liquid chromatography – mass spectrometry (LC-MS). Working with purified proteins allowed us to compare the obtained results with *in silico* data. Since the amino acid sequence of the proteins present in the purified 7S and 11S fractions is known, this enables prediction of the amino acid sequence of the active peptide(s). Furthermore, the structure of one predicted active peptide was analyzed by superimposition with the natural ligand CCK and subsequently its potency to activate the CCK1R was measured. Finally, the effect on food intake by soybean fractions with CCK1R activities, was evaluated *in vivo* using an experiment with rats.

4.2 Materials and methods

4.2.1 Animals and products

Cytochrome C, substance P, Val-Tyr and Bradford reagens were purchased from Sigma-Aldrich (Bornem, Belgium/St.-Louis, MO). Soybeans were purchased in the local grocery store (Heuschen & Schrouff OFT B.V., Canada). A HiLoad 16/60 Superdex 30 prep grade, a Superdex peptide 10/300 GL column and PD MidiTrap G-10 columns were purchased from GE Healthcare (Freiburg, Germany). Sprague-Dawley rats were purchased from Janvier (Le Genest Saint Isle, France) and standard laboratory rat food from Carfil Quality (Oud-Turnhout, Belgium). SR146131 was obtained from Sanofi-Aventis (Paris, France) (Figure 4.7).

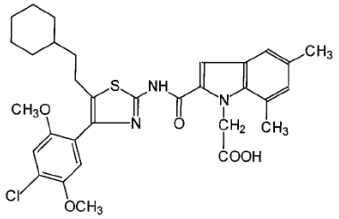


Figure 4.1. Molecular structure of SR146131 (*25*)

4.2.2 Preparation of 11S protein fraction from soybean

The 11S fraction was extracted as described in paragraph 3.2.2 for 7S, by adapting the pH to 4.8, following the method of Liu et al (*164*). The precipitates were dissolved in water, pH was set to 7.4 and dialyzed against water for 24 h using a 12-14 kDa molecular weight cut-off (MWCO) membrane. The purity of the isolated proteins was evaluated with SDS-PAGE and results were comparable with those of Liu et al (*164*).

4.2.3 Enzymatic hydrolysis

A gastrointestinal peptic digestion was performed on the 11S and 7S protein fraction as described in paragraph 4.2.3 directly after dialysis (without an additional lyophilisation step). The concentration of the 7S and 11S proteins after purification was determined with the Bradford method (*32*). The solution was centrifuged for 15 min at 10,000 g to remove insoluble parts. The 11S hydrolysate will accordingly be called 11SH.

4.2.4 Size exclusion chromatography

The peptides present in the hydrolyzed 7S and 11S soy protein fractions were separated using size exclusion chromatography. The peptides were applied to a HiLoad Superdex 30 pg column in a concentration of 50 mg/ml with an injection loop of 5 ml using an Aktä Purifier (GE Healthcare, Freiburg, Germany). A flow rate of 1.6 ml/min was applied and fractions of 10 ml were collected from an elution volume of 30 to 120 ml. The absorbance was measured at 214 nm. The column was calibrated using cytochrome C (12327 Da), aprotinin (6511 Da), substance P (1348 Da) and Val-Tyr (96 Da). Fractions with a molecular weight higher than 1500 Da (fractions A-E) were dialyzed against water and fractions with a molecular weight between 700 Da and 1500 Da (fraction F) were desalted with water on PD MidiTrap G-10 columns using gravity flow. Upon completion, the different peptide fractions were freeze-dried.

4.2.5 Peptide identification with LC-MS

These experiments have been performed in cooperation with Prof. Kris Gevaert (Department of Biochemistry, Ghent University). Peptide fractions were dissolved to a concentration of 1 µg/µl. The peptides were analyzed on an Ultimate 3000 HPLC system (Dionex, Amsterdam, The Netherlands) with a 30 min gradient of water/acetonitrile on an in-house packed 15 cm column (Reprosil-Pur Basic C18-HD 3 µm, Dr. Maisch, Germany), in-line connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany). Per LC-MS/MS analysis, 1 µl of the peptide mixture was

consumed. The mass spectrometer was operated in positive mode with data-dependent acquisition, automatically switching between MS and MS/MS acquisition for the six most abundant ion peaks per MS spectrum. Full scan MS spectra were acquired at a target value of 1E6 with a resolution of 60,000. The six most intense ions were then isolated for fragmentation in the linear ion trap. In the LTQ, MS/MS scans were recorded in profile mode at a target value of 5,000. Peptides were fragmented after filling the ion trap with a maximum ion time of 300 ms and a maximum of 1E4 ion counts. Instrument settings for LC-MS/MS analysis and the generation of MS/MS peak lists were as previously described (*91*). MS/MS peak lists were then searched with Mascot using the Mascot Daemon interface (version 2.3.0, Matrix Science). The spectra from the soy samples were searched in the *Glycine max* database, downloaded from UniProt on January 10th, 2013. Acetylation on the N-terminus, pyroglutamate formation of N-terminal glutamine and methionine oxidation to methionine sulfoxide were set as variable modifications. The protease was set to none to allow cleavage after every amino acid. The mass tolerance on the precursor ion was set to ±10 ppm and on fragment ions to ±0.5 Da. In addition, Mascot's C13 setting was set to 1. Only peptides that were ranked one and had an ion score at least equal to the corresponding identity threshold at 99% confidence were withheld and further data handling was done in the ms_lims database (*118*). Alignments between the proteins and the CCK-8 sequence (DYMGWMDF) were made using T-coffee Multiple Sequence Alignment Tools freeware (Bioinformatics and Genomics Programme Center for Genomic Regulation, Barcelona, Spain, (*204*)).

4.2.6 Superimposition and electrostatic structure calculations of CCK and PALSWLR

These experiments were performed in cooperation with Moises João Zotti (Department of Crop Protection, Ghent University). The human CCK ligand (CCK-8S, DY(SO_3H)MGWMDF-$CONH_2$) and PALSWLR were optimized at physiological pH using the modeling tool of Discovery Studio Visualizer (Accelrys, San Diego, CA). All structural-electrostatic calculations were performed on a Red Hat Linux workstation using the programs DelPhi v. 4 (*237*), CHARMM (Chemistry at HARvard Macromolecular Mechanics) (*36*), MODELLER release 9v4 (*238*) and Discovery Studio Visualizer (Accelrys, San Diego, CA). Starting from the initial peptides a minimization run was performed using Smart Minimizer algorithm and CHARMM as force field. These peptides were subjected to 1,000 minimization steps with solvent dielectric constant setup to 80. Thereafter, an alignment was created with Align123 algorithm, a progressive pairwise alignment algorithm modified from the CLUSTAL W program (*272*) and then the structures were superimposed. The electrostatic potential calculations were performed with DelPhi, a program that solves the Poisson-Boltzmann equation on a cubical lattice using the finite-difference technique.

4.2.7 *In vivo:* Food intake study with rats

These experiments have been performed under supervision of Prof. Robrecht Raedt (Department of Internal Medicine, Faculty of Medicine and Health Sciences, Ghent University). The *in vivo* experiments with rats were performed as previously described for the CCK1R agonist SR146131 (Figure 4.1) by Bignon et al. with slight modifications (*24*). The experimental set-up was validated using this agonist and similar results as in the study from Bignon et al. were obtained (*24*). Male Sprague-Dawley rats of 8-12 weeks old and a weight of 225-250 g were housed in separate steel cages in an acclimatized room kept at 24 °C with a 12 h dark-light cycle (Figure 4.2).

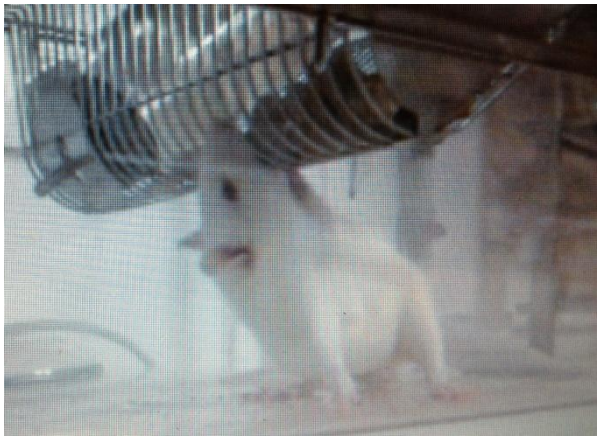


Figure 4.2. Eating Sprague-Dawley rat given weighed amount of food. No bedding is present in the cage during the experiment to be able to measure the spilled food.

The rats were fed a standard laboratory diet (3.5% fat, 16.5% protein, 39% sugars and starch, vitamins, minerals, amino acids and fatty acids) and tap water was freely available. One week before the start of the experiment, access to food was only allowed between 10 a.m. and 4 p.m. to get them acquainted with the experimental protocol. Before an experiment, rats were fasted between 4 p.m. and 10 a.m. The lyophilized peptide fractions D to F of 7SH and 11SH as described above were pooled and dissolved in about 0.5 ml of tap water in such way that each rat received an oral dose of 200 mg sample per kg body weight (BW) using a metal gavage. Control rats were administered 0.5 ml volume of tap water. Half an hour after oral administration of the sample with use of a gavage, the rats were given access to a weighed amount of food and food intake was measured by weighing the remaining food 1, 3, 6 and 24 h after food access taking spillage into account. The applied study design was a balanced crossover design, i.e. the rats were divided in four batches of 3 rats and each batch of rats received each intervention (7SH fractions, 11SH fractions or the control diet) once (four biological repeats). The fourth batch of rats was not considered here, as they were used for exploratory research (results not shown). Between experiments, there was minimally 1 wash-out day. The

cumulative food intake per intervention group was expressed as mean ± SEM based on 4 measurement days with 3 rats each resulting in 12 measurements per intervention group. The experiments were approved by the ethics committee for animal experiments of the Faculty of Medicine and Health Sciences at Ghent University. Differences between intervention and the controls were tested using one-way ANOVA (S-Plus, TIBCO software Inc., Palo Alto, CA). Statistical significance was set at $P<0.05$ and all tests were two-sided.

4.3 Results

4.3.1 *In vitro* CCK1R activity of 7SH and 11SH

In the previous Chapter, it was reported that the gastrointestinal digest of the 7S protein from soybean (7SH) contains significant CCK1R activity. We analyzed the activity of the peptic gastrointestinal digest of the other important storage protein in soybean (11SH) in a similar way and measured a significant net response of 10.5 ± 0.8% (n=3, p=0.038) for a concentration of 1 g/l. The fluorescence kinetics for each cell type are shown in Figure 4.3.

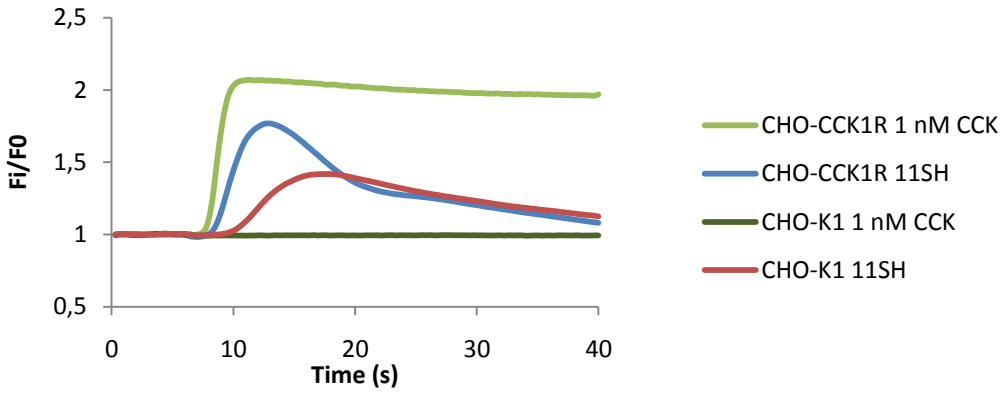


Figure 4.3. Representative normalized fluorescence kinetics (Fi/F0) of 11SH for CHO-CCK1R and CHO-K1 in cell populations monitored with a resonant scanning confocal microscope. Results for sample as well as for 1 nM CCK are shown.

Upon stimulation with 11SH, CHO-CCK1R cells showed a fluorescent response that was lower and not as stable as when stimulated with CCK, but this fluorescence increase was higher and faster as compared to the response of CHO-K1 cells being stimulated with 11SH. These results showed that 11SH contains considerable CCK1R activity and should thus be further pursued. Although the difference in response to the hydrolysates between CHO-CCK1R cells and CHO-K1 cells was significant for 7SH (Chapter 2) as well as for 11SH, the CHO-K1 cells also showed a response to the hydrolysates which was not negligible. This points to non-specific activation of other membrane receptors that induce a cytoplasmic calcium release in CHO cells, plausibly due to the presence of peptide

components or amino acid motifs, different from those activating the CCK1R. As this possibly might lead to *in vivo* side-effects, it is necessary to remove these non-specific responses by further purification of the sample.

4.3.2 Gel filtration chromatography

To obtain specific CCK1R responses, without activation of CHO-K1 cells, and to identify the active peptides responsible for the CCK1R response, the peptides in the hydrolysates were separated in different molecular weight fractions using gel filtration chromatography. The peptide profile for 7SH obtained with the Superdex 30 column and the collected fractions can be seen in Figure 4.4a. The obtained fractions were applied to a Superdex peptide column to evaluate the fractionation. A good separation was obtained between the different molecular weight peptide fractions (Figure 4.4b). Similar results were obtained for 11SH (Figure 4.4c and d). An overview of the molecular weights and the corresponding number of amino acids of the different peptide fractions A-J is shown in Table 4.1. Fully biologically active forms of CCK require a peptide length of at least seven amino acids (*162*), which is why fractions G to J were excluded for further testing as these fractions contain peptides with only five or less amino acids.

Table 4.1 Overview of different molecular weight peptide fractions estimated on gel filtration standards

Fraction	Elution Volume (ml)	MW (Da)	Estimated number of amino acids
A	30 - 40	31718 - 67811	240 - 513
B	40 - 50	14835 - 31718	112 - 240
C	50 - 60	6939 - 14835	52 - 112
D	60 - 70	3246 - 6939	24 -52
E	70 - 80	1518 - 3246	11 - 24
F	80 - 90	710 - 1518	5 - 11
G	90 - 100	332 - 710	2 - 5
H	100 - 110	155 - 332	1 - 2
I	110 - 120	73 - 155	0 - 1
J	120 - 130	34 - 73	-

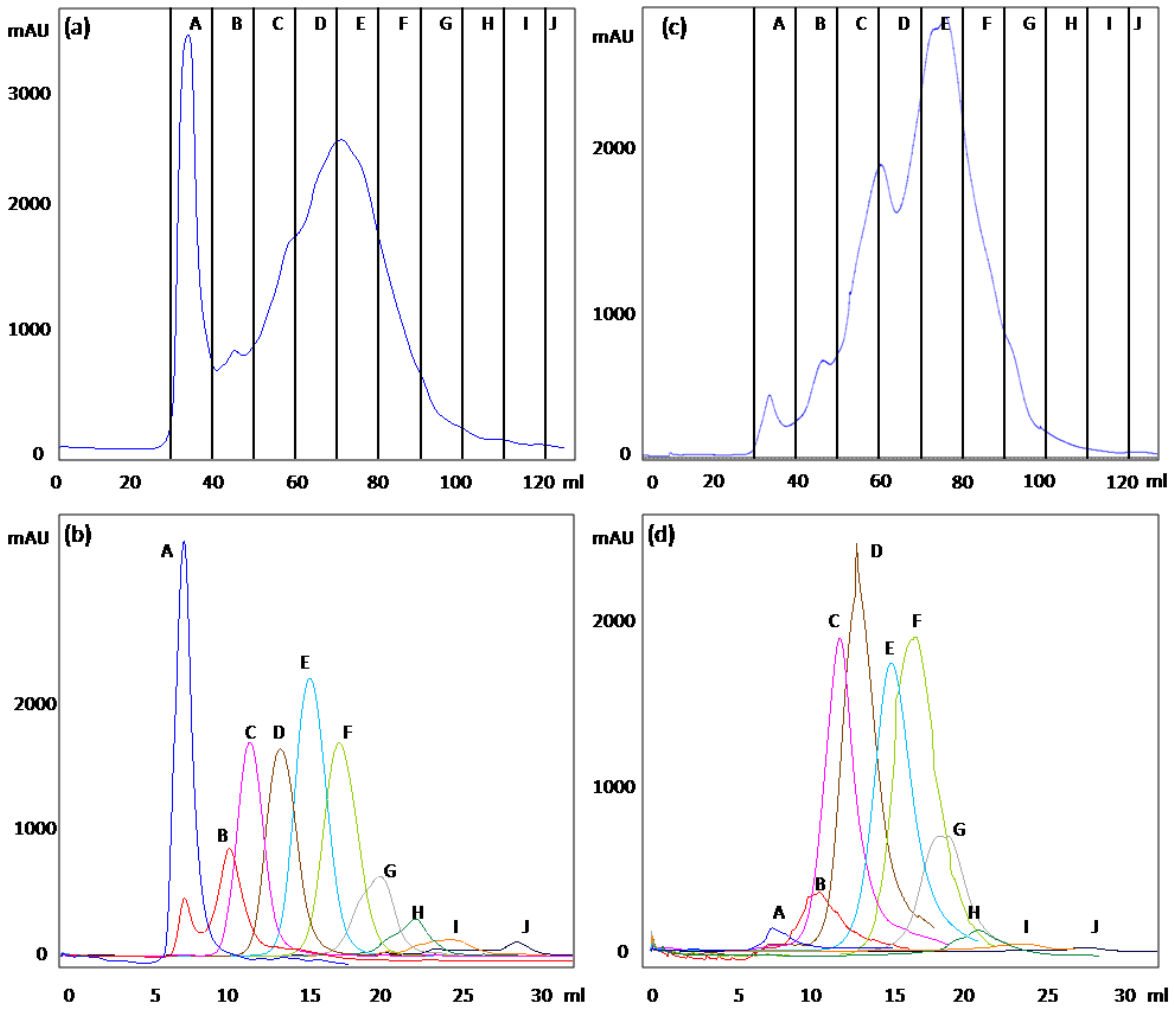


Figure 4.4. (a,c) Representative chromatographic profiles of 7SH and 11SH, respectively, obtained with Superdex 30 column, different peptide fractions which are collected are indicated. (b,d) Representative chromatographic profiles of the collected peptide fractions obtained after reinjection on a Superdex peptide column for 7SH and 11SH, respectively. Absorbance was measured at 214 nm.

4.3.3 *In vitro* CCK1R activity of different molecular weight fractions

The CCK1R activity of the different molecular weight fractions was measured and the net responses for each fraction (A-F) from every hydrolysate are shown in Table 4.2. The relative fluorescence kinetics for each fraction can be found in Figure 4.5a and b, for 7SH and 11SH, respectively. All fractions showed a net response that was significantly different from zero, except fraction F from 7SH due to a high standard error. As the relative fluorescence curve for this fraction is relatively high in comparison to the other fractions, it was decided not to exclude this fraction for further evaluation.

Table 4.2. Net response, expressed as a percentage of the maximum response induced by 1 nM CCK, by 1 g/l of the different molecular weight fractions from 7SH and 11SH ± SEM (n=2-4), * indicates that the value is significantly different from 0 (p<0.05)

Net response ± SEM (%)	7SH	11SH
Frac A	26.2±2.8*	15.6±3.6*
Frac B	12.5±2.6*	12.9±1.5*
Frac C	9.7±1.3*	11.6±0.9*
Frac D	11.8±2.1*	10.2±2.0*
Frac E	10.8±0.7*	7.96±2.9*
Frac F	19.6±10.4	5.2±1.1*

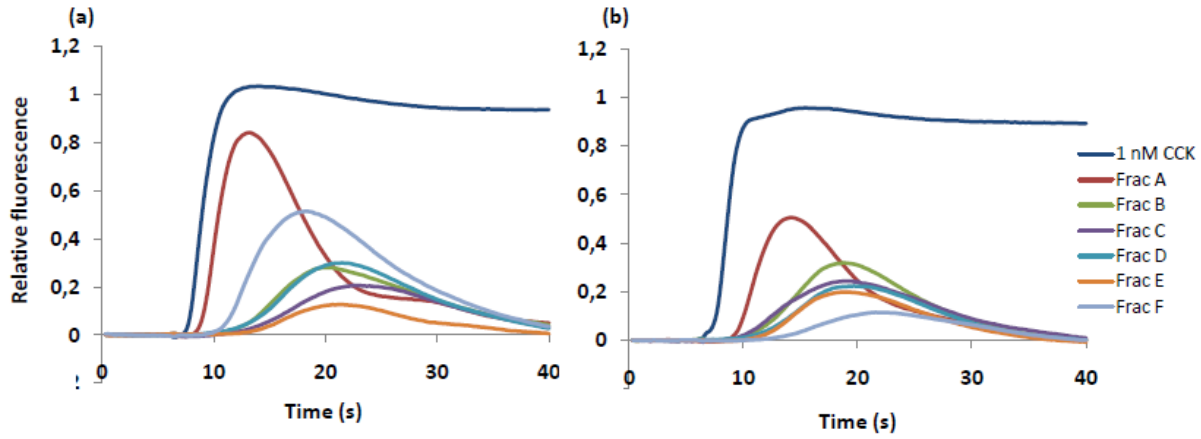


Figure 4.5. Representative relative fluorescence kinetics (RF=Fi(CHO-CCK1R)/F0(CHO-CCK1R)-Fi(CHO-K1)/F0(CHO-K1)) for 1 g/l of different molecular weight peptide fractions. (a) Peptide fractions from 7SH. (b) Peptide fractions from 11SH.

Fraction A, containing the polypeptides with the highest molecular weight, gave the strongest and fastest responses for both hydrolysates. Notably, activation of CHO-K1 cells was seen for fraction A only (see Figure 4.6 and Figure 4.7 for 7SH and 11SH, respectively), indicating that this fraction contains the peptide residues responsible for the non-specific responses that appeared when the CCK1R activity of the complete hydrolysate was measured. So despite the high net response, this fraction was judged to be less suited for further *in vivo* evaluation due to the non-specific responses seen for CHO-K1 cells. Conversely, fractions B to F for both hydrolysates did not activate the CHO-K1 cells but did significantly activate the CHO-CCK1R cells, which means that these fractions show specific and reliable CCK1R activity and are suitable for *in vivo* evaluation (see Figure 4.6 and Figure 4.7 for 7SH and 11SH, respectively).

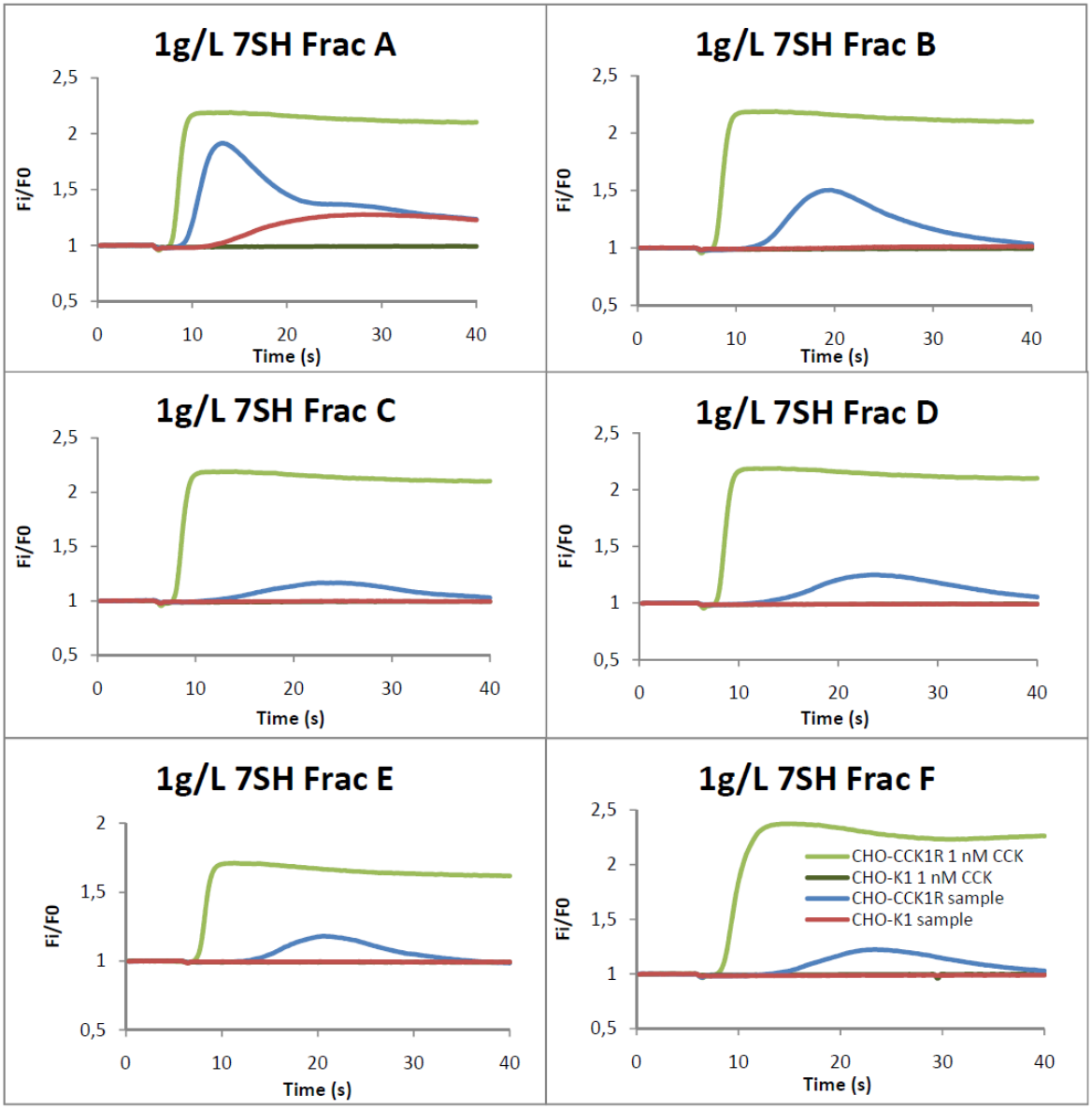


Figure 4.6: Representative normalized fluorescence kinetics (Fi/F0) of different molecular weight peptide fractions for 7SH for CHO-CCK1R and CHO-K1 in cell populations monitored with a resonant scanning confocal microscope. Results for sample as well as for 1 nM CCK are shown.

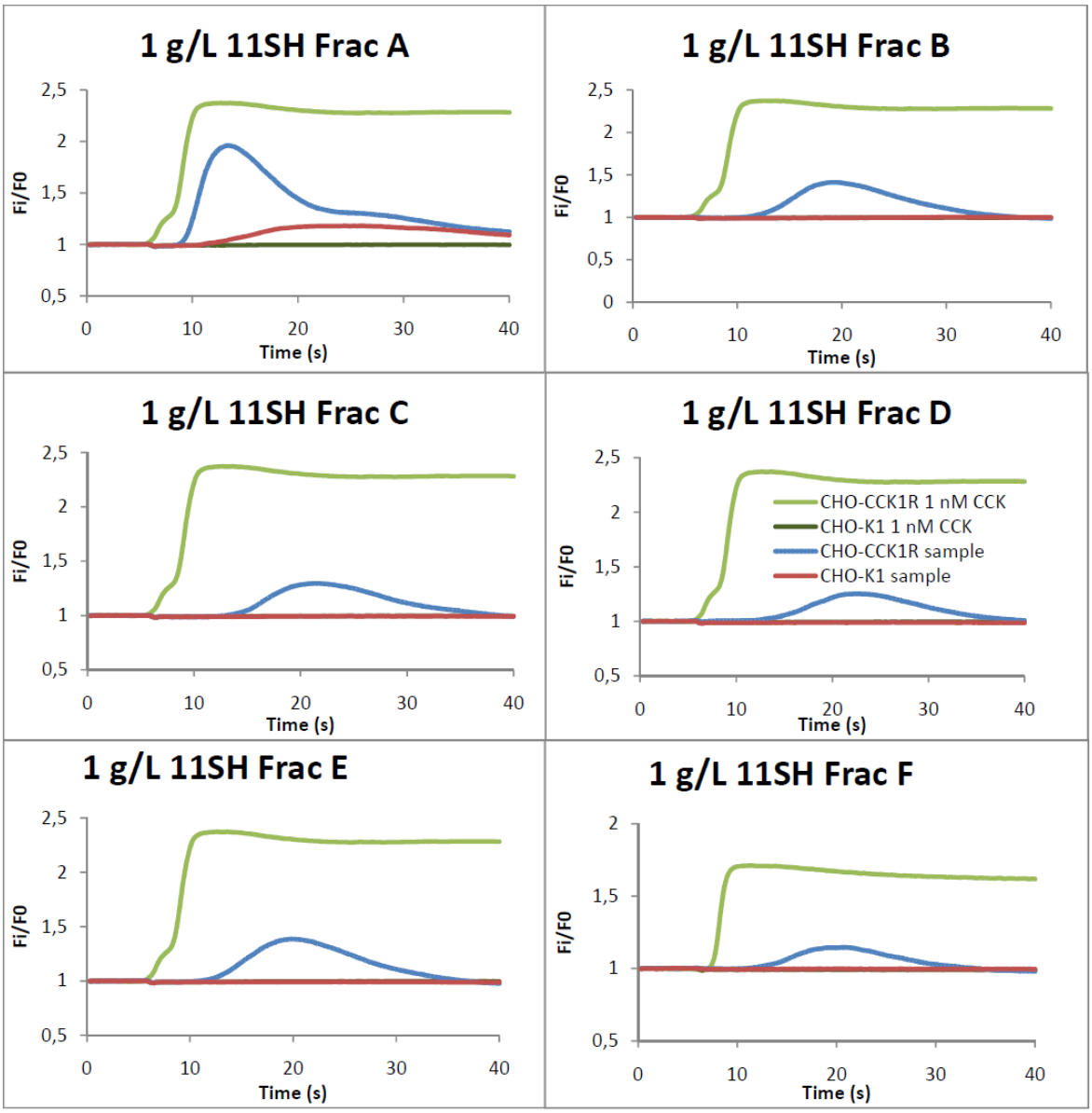


Figure 4.7: Representative normalized fluorescence kinetics (Fi/F0) of different molecular weight peptide fractions for 11SH for CHO-CCK1R and CHO-K1 in cell populations monitored with a resonant scanning confocal microscope. Results for sample as well as for 1 nM CCK are shown.

Considering the *in vivo* evaluation, peptides with a molecular weight as low as or lower than in fraction D (containing 52 amino acids or less, see Table 4.1) were retained for *in vivo* experiments as those peptides might be intactly absorbed through the intestinal wall. Although it is unlikely that peptides with a chain length of up to 30-50 amino acids can penetrate the intestinal wall, there are some examples supporting this hypothesis such as the significant physiological effect of enteral administration of biological active (poly)peptides like insulin (*236, 246*).

4.3.4 Peptide identification

The peptides present in fraction E and F of 7SH and fraction E of 11SH were analyzed by LC-MS/MS. Between 100 and 200 different peptides were found in each fraction, so it is quite impossible to directly pinpoint to the exact peptide(s) responsible for the CCK1R activity measured in the fractions (an overview of all the identified peptides can be found in Supplementary Table 1). Therefore, an attempt was made by screening the proteins in 7S and 11S *in silico* using amino acid aligment with the CCK-8 sequence. In this way, best homology was found for the amino acid motif PALSWLR and this sequence appeared in three peptides: NH_2-TATSLDFPALSWLR-COOH, present in the 7SH fraction E, and NH_2-TSLDFPALSWLR-COOH and NH_2-PALSWLRL-COOH, both present in 7SH fraction F. Therefore, PALSWLR was considered as the best candidate structure to account for CCK1R activity.

4.3.5 Superimposition, electrostatic potential characterization and evaluation of CCK1R activity of PALSWLR

To evaluate the possible activity of PALSWLR, it was superimposed with CCK (Figure 4.8a) and evaluated *in vitro* for CCK1R activity. We focused on electrostatic potential landscapes, which is useful to elucidate the molecular interaction in various chemical and biological systems. We noted that aspartic acid located in position 7 of CCK contains a remarkably negative character (Figure 4.8b, c and f), while a more positive profile is observed for arginine at position 7 in PALSWLR (Figure 4.8d, e and f). This remarkable difference in the potential at this position could hamper the proper electrostatic fit of PALSWLR in the binding region (Figure 4.8e). So, despite the homology between PALSWLR and CCK-8S, this superimposition indicates that this candidate peptide might show no CCK1R activity. At the same time, also the CCK1R activity was tested, and in accordance with the sumperimposition and electrostatic potential data, no activity could be measured at a concentration as high as 1 mM (data not shown). It is well known that sulfatation of tyrosine in position 2 in CCK accounts for a 1,000 fold increase in activity for CCK1R (*69, 79*) compared to the non-sulfated peptide. The tested peptide PALSWLR contains alanine in position 2, and although the electrostatic potential landschape is quite similar at this point, we believe that a sulfate group is obligatory for activity. In addition, we can also not exclude the possibility that the CCK1R activation by the soybean peptide fractions has been induced by a synergistic effect of more than one peptide.

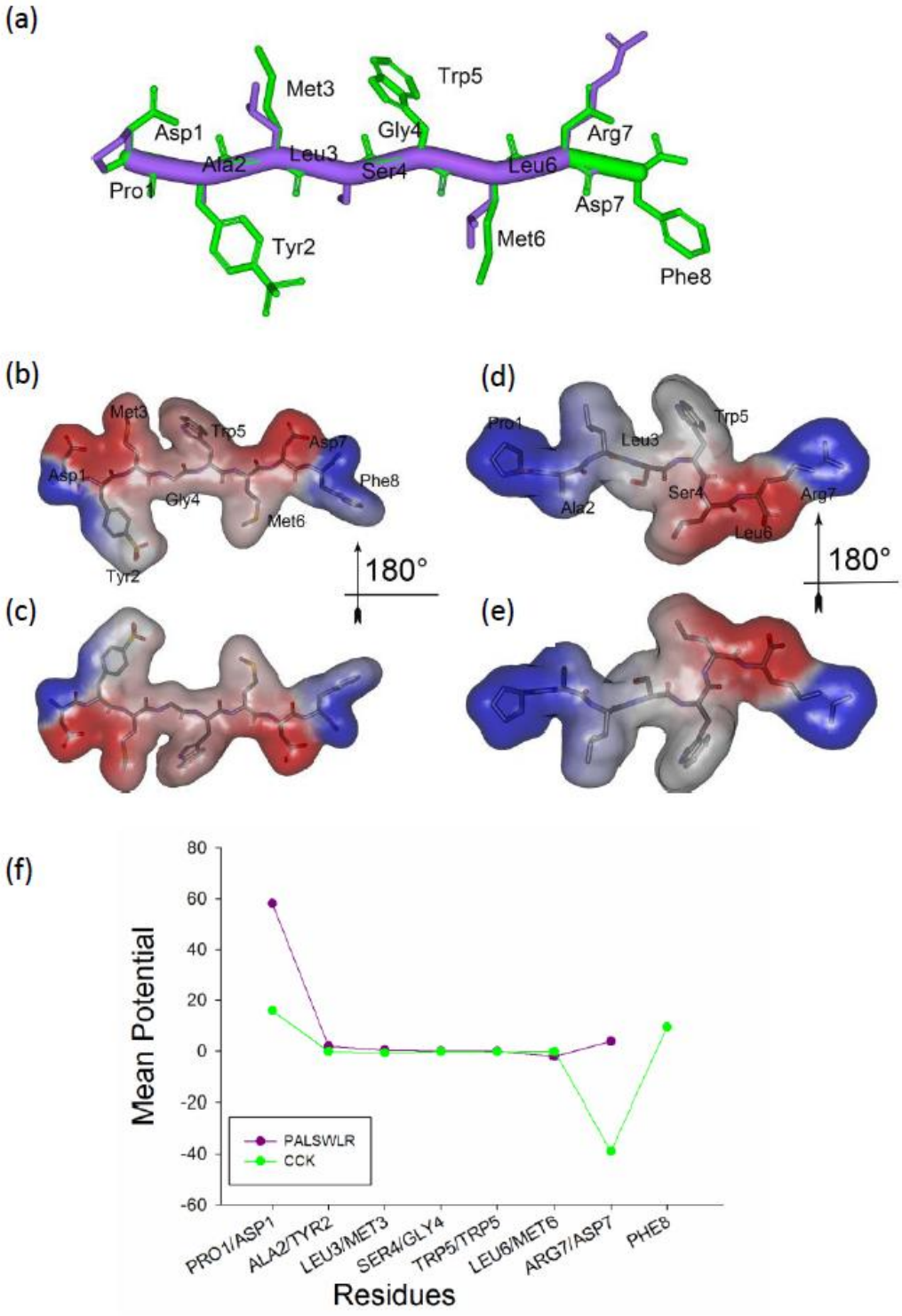


Figure 4.8. Superimposition and electrostatical potential of CCK (DY(SO3H)MGWMDF-CONH2) and PALSWLR. (a) Superimposition of CCK and PALSWLR to compare peptide backbones and side-chains. The peptides were superimposed based on residues alignment (data not shown). CCK is depicted in green and PALSWLR in purple. (b,c) Electrostatic potential of CCK. (d,e) Electrostatic potential of PALSWLR (c,d). The color gradients of the peptide surfaces range from blue (highest hydrophilic area) to red (highest lipophilic area). The arrows indicate the rotation of peptides around their horizontal axis. (f) Mean potential of each residue (values of electrostatic potential averaged on the atoms of each residue) derived from electrostatic calculations.

4.3.6 *In vivo* effect on food intake

Fasted rats were administered the grouped fractions D-F from each hydrolysate as these fractions contain peptides with a chain length short enough to penetrate intactly through the intestinal wall (<50 amino acids) but long enough to activate the CCK1R (>7 amino acids) (*162, 236, 246*). Food intake was measured 1, 3, 6 and 24 h after a weighted amount of food was introduced into the cage. The primary outcome was the food intake at 1 h follow-up, as CCK works at the short term. At 1 h follow-up, no significant difference on food intake could be demonstrated compared to the control rats ($p>0.5$). Neither could significant differences be found for the other time points (Figure 4.9) ($p>0.5$).

4.4 Discussion

In this study, it was shown that the gastrointestinal digested hydrolysate from the 11S soy protein, as shown for the 7S soy protein hydrolysate in Chapter 3, also contains significant CCK1R activity. The 7S and 11S soy protein hydrolysate were separated into different molecular weight fractions and these fractions also showed significant CCK1R activity. The peptides present in some of the fractions were identified, but thus far it remained impossible to determine which peptide(s) exactly were responsible for the CCK1R activity. The peptide, PALSWLR, was selected based on homology to CCK for further evaluation but it showed no CCK1R activity.

To assess whether the peptide fractions retain their activity *in vivo*, the effect on food intake of the peptide fractions with a molecular weight between 700 and 7000 Da were evaluated as these are most likely to penetrate the intestinal wall. However, no effect on food intake was seen. There are several possible explanations for the lack of effect. Firstly, this might be due to application of too low doses. However previous studies did report significant reductions in food intake of rats at comparable or even lower doses using other bioactive protein hydrolysates (*202, 264*). A comparable dose of β-conglycinin bromelain hydrolysate led to a significant reduction (7%) in food intake in rats after 1 h (*264*). Duodenal infusion of a β-conglycinin peptone at a low dose (5 mg), led to a significant food intake reduction of 17% (*202*) after 1 h. However, whilst these studies demonstrated that food intake can be affected via the CCK-signaling system (Figure 1.6), they differ in that the presumed mechanism involved is augmentation of CCK release by stimulation of the CCK-producing enteroendocrine cells (*202, 264*). The novelty of this study is that the peptides are meant to have their effect at the level of the CCK1R located on vagal afferents in the intestinal mucosa (*225*). So as a consequence, these peptides need to cross the intestinal wall and therefore higher or more concentrated doses might be needed. In addition, it should be noted that CCK1Rs on vagal afferents

involved in inducing satiety, would be in the low-affinity state which in turn might even increase the needed dose (*160*). Secondly, it might be possible that the active peptides are not able to cross the intestinal wall, as peptide uptake is dependent on the length and hydrophobicity of the peptide (*246*). Thirdly, another explanation for the absence of effect might be that active peptides are degraded in the gastrointestinal tract and lose their activity. However, this scenario is unlikely as a protein gastrointestinal digestion was already performed on the peptides, which makes these -at least in part- resistant to further cleavage by gastrointestinal proteases. Fourthly, the peptides present in fraction D en E might be too big to be absorbable through the intestinal wall. A study that reports on the absorption of a peptide with a chain length of 50 AA exists (*236*), however this study refers to a very low amount of intestinal absorption of insulin. Generally, a chain length of 8 to 12 AA is a more reasonable cut-off for peptide absorption (*246*).

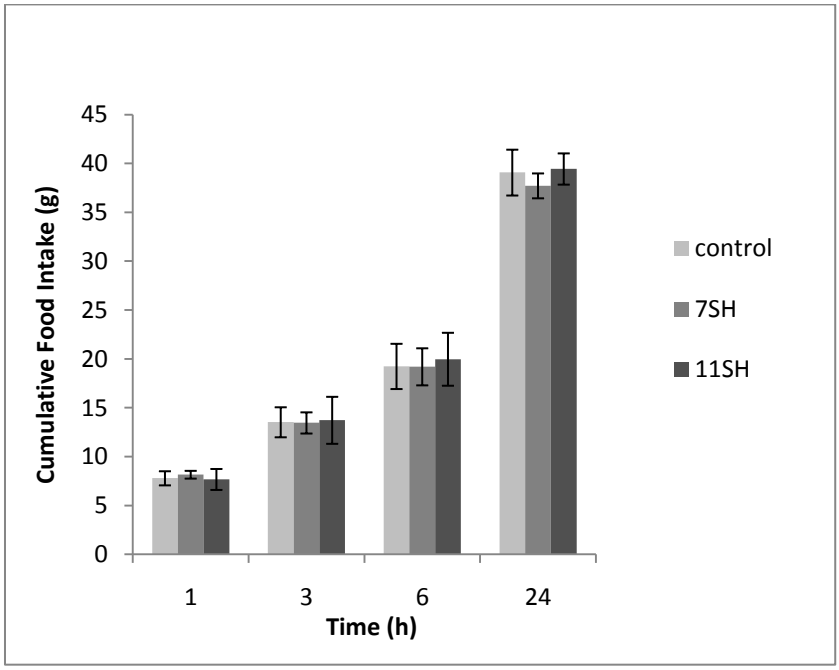


Figure 4.9. Cumulative food intake of fasted rats after administration of 200 mg/kg BW of 7SH Fraction D-F, 11SH Fraction D-F or control (tap water).

In the *in vitro* tests, CCK gave a maximum response at a concentration of 1 nM in the cell culture medium. The concentration of the peptides present in the different molecular weight fractions tested varied between 0.2 and 1 mM (calculated based on the estimated molecular weight), which is up to one million times more than the concentration of CCK in the *in vitro* tests. So based on molarity, the activity of the peptides present in the different molecular weight fractions is not very high. It is plausible that the activity is caused by only a limited number of specific peptides and that the abundance of these peptides is very low. At this point it is difficult to identify the peptides

responsible for the CCK1R activity. Therefore, it would be useful to develop a technique to “fish out” these bioactive peptides from a complex matrix, as is the soybean protein hydrolysate. To this end, a new technique will be worked out in the next chapter.

Chapter 5

Development of CCK1R-nanoscale apolipoprotein bound bilayer particles (NABBs)

This research was performed at the Rockefeller University (New York, NY) in the the Laboratory of Molecular Biology and Biochemistry under supervision of Prof. Thomas Sakmar with the help of Prof. Thomas Huber, Manija Kazmi, Carlos Rico, Tian He, and Minyoung Park.

Chapter 5 Development of CCK1R-nanoscale apolipoprotein bound bilayer particles (NABBs)

5.1 Introduction

Nanoscale apo-lipoprotein bound bilayer particles (NABBs) are a unique native-like bilayer membrane system for incorporation of GPCRs. They have a diameter of about 12 nm and consist of a discoid bilayer of phospholipids, held together by a protein belt structure from engineered zebra fish apo-lipoproteins (zap1) (*16*) (Figure 5.1). The advantage of NABBs over sol-gel entrapped proteins and proteins in immobilized membranes (*23, 251*) is that the GPCR can be incorporated rapidly in the NABBs and that it remains in a more natural conformation (*16*). NABBs containing the CCK1R (CCK1R-NABBs) could be used as a highly-innovative affinity-selection mass spectrometry technique to fish out CCK1R binding peptides.

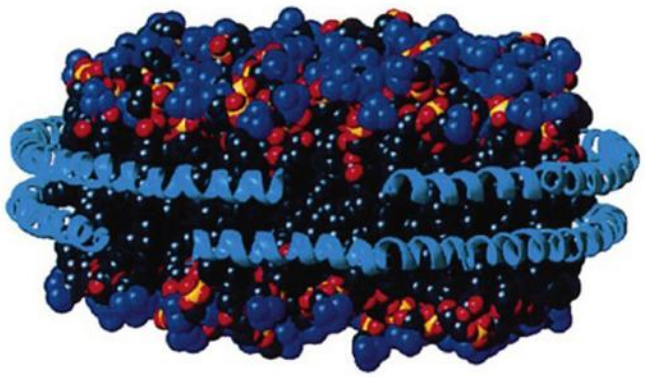


Figure 5.1. Structure of a NABB particle. They consist of a phospholipid bilayer surrounded by 2 zap1 molecules in an anti-parallel helical configuration forming a belt-like structure.

In this chapter, the first step to develop such a technique, i.e. the formation of CCK1R-NABBs, is elaborated. To form CCK1R-NABBs, it was necessary to obtain the CCK1R and the belt protein, zap1, in a purified form. To produce purified CCK1R, it was heterologously expressed in HEK-293T cells, but before purification could start, functional expression of the receptor had to be confirmed. Therefore calcium fluxes upon receptor activation were measured, as was done previously for CHO-CCK1R cells. Besides calcium fluxes, receptor internalization after ligand binding was assessed. Receptor internalization is a cellular mechanism to desensitize the cell for circulating hormones that induce a biological response in the cell by reducing the number of cell surface receptors (*299*). In addition, during these experiments, the functionality of a fluorescent labeled CCK analogue (FCCK) was evaluated. The CCK1R was purified using immunoprecipitation from the lysed HEK-293T cells. Purification of CCK1R was performed both with and without the presence of devazepide, a small

molecule CCK1R antagonist, as it has been reported that this stabilizes the receptor (*49*). Also a small amount of cholesterol detergent was added to facilitate CCK1R function (*3, 217*). Zap1 protein was heterologously expressed in *E. coli* and purified by affinity and subsequent size exclusion chromatography. In the final step, these proteins were combined with lipids and detergents in the presence of devazepide, which then self-assembled into NABBs. This is the first report on the incorporation of the CCK1R into NABBs.

5.2 Materials and methods

5.2.1 Cells, plasmids and reagents

HEK-293T cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown at 37 °C and 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose and 2 mM glutamine) , obtained from Life Technologies (Paisley, UK) and supplemented with 10% fetal bovine serum (FBS), obtained from Atlanta Biologicals (Lawrenceville, GA). pcDNA3.1(+), Lipofectamine PLUS, PLUS reagent, Lipofectamine 2000, Optimem medium, gels for SDS-PAGE and Dynabeads Protein G were also obtained from Life Technologies. CCK-8S was purchased from Bachem (Bubendorf, Switzerland). Black with clear bottom 384-well plates were purchased from Corning Life Sciences (Tewksbury, MA). 35 mm² glass dishes were purchased from IBIDI (µ Dish high, Planegg, Germany). A FLIPR calcium 4 assay kit was purchased from Molecular Devices (St. Grégoire cédex, France). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), were obtained from Avanti Polar Lipids (Alabaster, AL). Dodecyl β-D-maltoside (DM), cholecesteryl-hemasuccinate (CHS) and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Anatrace, Inc. (Maumee, OH). Sepharose 2B, poly-D-lysine-hydro-bromide (PDL), phenylmethanesulfonyl fluoride solution (PMSF) and devazepide (DVZ) were obtained from Sigma-Aldrich. Detergents were obtained from Cisbio (Bedford, MA). A Micro-Spin column was obtained from Pierce (Rockford, IL). An Amicon Ultra 10 kDa cutoff centrifugal filter device and Immobilon, a polyvinylidene difluoride (PVDF) membrane, were obtained from Millipore (Billerica, MA). 1D4-sepharose 2B resin was prepared by the Sakmar lab. 1D5-peptide (TETSQVAPA) was purchased from Biobasic Canada Inc. (Markham, ON, Canada). Primary antibodies against the engineered 1D4 epitope were obtained from the National Cell Culture Center. Horseradish peroxidase (HRP)-conjugated α-mouse antibody, an α-His_6 tag monoclonal antibody conjugated with HRP-α-mouse, reagents for enhanced chemiluminescence, Terrific Broth medium and Sephadex G-50 Superfine were purchased from GE Healthcare (Amersham, Freiburg, Germany). A HyBlot CL autoradiography film was purchased from Denville Scientific, Inc. (South Plainfield, NJ). His60 Ni resin was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Pierce detergent removal resin and BupH

phosphate buffered saline packs were purchased from Thermo Scientific (Rockford, IL). A fluorescent labeled CCK analog (FCCK, Figure 5.2), designed after the analog described by Harikumar et al. (*115*) with slight modifications, was purchased from Bachem.

ATTO647N - AEEAc - Gly - Asp - Tyr(SO_3H) - Nle - Gly - Trp - Nle - Asp - Phe - amide

Figure 5.2: Fluorescent labeled CCK analogue (FCCK). ATTO647N: fluorescent dye, AEEAc: Amino-Ethoxy-Ethoxy-Acetic acid

5.2.2 Construction of CCK1R cDNA

The rat CCK1R cDNA (acc. nr. M88096), with a C-terminal 1D4 epitope of 18 amino acids (DEASTTVSKTETSQVAPA) and N-terminal serotonin 5HT3 membrane import sequence was constructed by Genewiz. The CCK1R and serotonin 5HT3 membrane import sequence were N-terminally preceded by a Kozak sequence (CACC) for better expression. A DYV linker was C-terminally incorporated after the 5HT3 membrane import sequence. Restriction enzymes used were C-terminal EcoRI, BamHI at the C-terminal of the rat CCK1R sequence and N-terminal NotI. The rat CCK1R sequence was codon-optimized for expression in mammalian cells by Genewiz. Figure 5.3 gives an overview of the construct. The construct was subcloned into pcDNA3.1(+) using either EcoRI and NotI (further referred to as E/N) or BamHI and NotI (further referred to as B/N). In the former case, the serotonin 5HT3 membrane import sequence was included in the construct, in the latter it was not. The serotonin 5HT3 membrane import sequence is used to increase the probability of incorporation of the GPCR into the membrane (*156*).

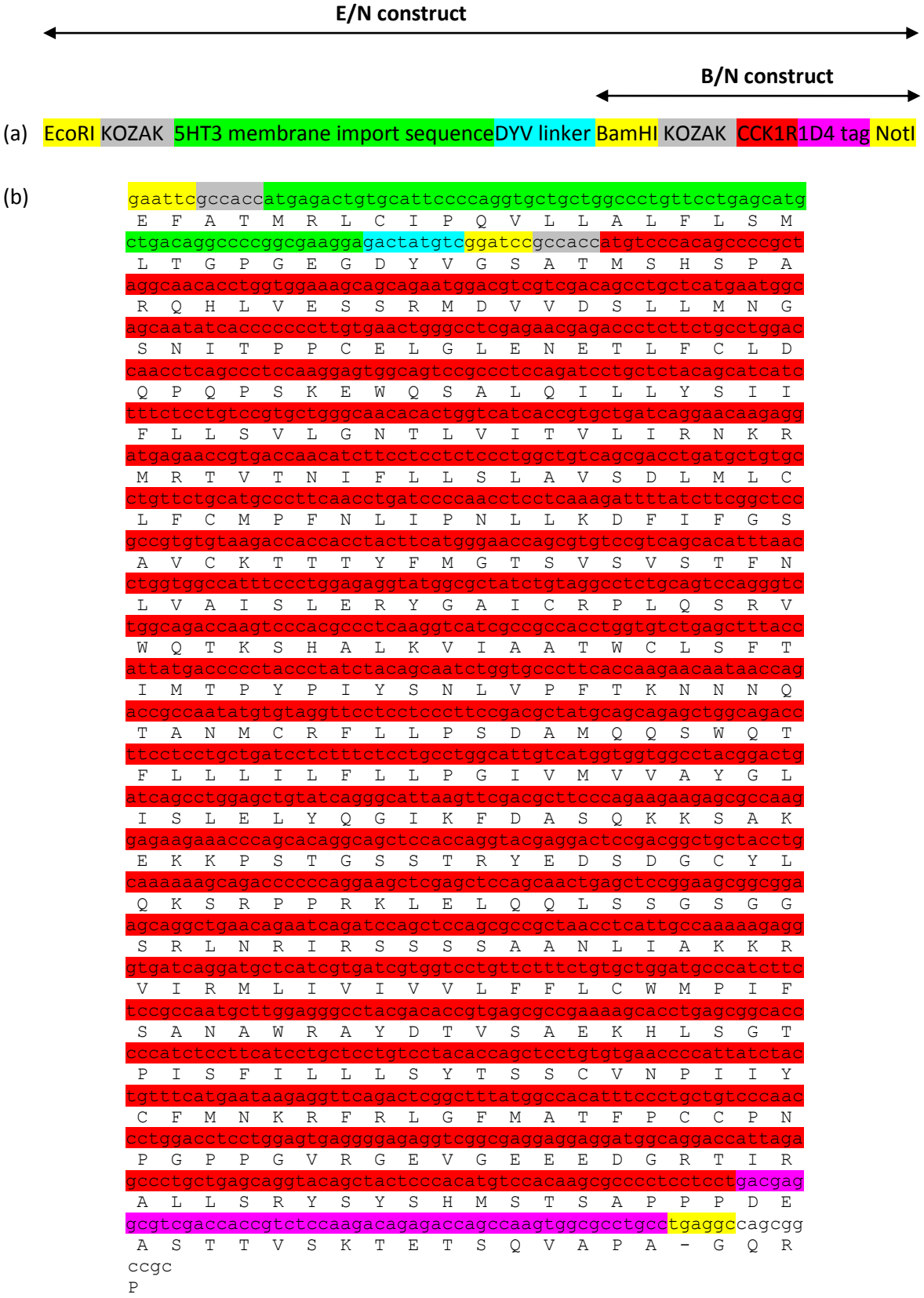


Figure 5.3. (a) Schematic overview of CCK1R cDNA construct with tag sequence and with (E/N construct) and without (B/N construct) the import sequence. (b) DNA sequence (lowercase) and resulting amino acid sequence (upper case). Colors of the DNSA sequence correspond to the different parts in the construct shown in (a).

5.2.3 Transfection of HEK-293T Cells.

5.2.3.1 For receptor purification

HEK-293T cells were transfected with this plasmid DNA using Lipofectamine PLUS as previously described for earlier experiments with rhodopsin receptor (*300*). In brief, 3.5 µg of plasmid DNA and 10 µl of PLUS reagent were dissolved in 0.75 ml DMEM and incubated for 15 min at room temperature. Next, this mixture was added to 0.5 ml DMEM containing 17 µl of Lipofectamine PLUS and incubated for another 15 min after which the total volume was brought to 4 ml with DMEM. Subsequently, this solution was added to a 10 cm diameter cell culture dish containing HEK-293T cells at 70-80% confluence. After 4 h, 4 ml of DMEM with 20% FBS was added to the cells. The transfected cells were incubated at 37 °C with 5% CO_2 and harvested after 48h with PBS containing 1% PMSF. The cell pellets were stored at -20 °C until further use.

5.2.3.2 For fluorescent measurements

For fluorescent measurements using a plate reader, HEK-293T were transfected while replating in 384-well plates (black with clear bottom) with either the E/N or B/N construct. The plates were pretreated with 10 µl of 0.1 mg/mL of PDL per well, washed thrice with double distilled water and air dried. An amount of 10 ng DNA was mixed with 0.075 µl of Lipofectamine 2000 and 10 µl DMEM per well and incubated at room temperature for 20 min. This mixture was added to 10 µl per well of DMEM supplemented with 20% FBS, containing 800,000 cells/ml and subsequently plated in the 384-well plates. Control cells were transfected with the same amount of empty vector DNA (pcDNA3.1(+)). Cells were incubated for 48 h at 37 °C with a 5% CO_2 atmosphere.

For fluorescent measurements using fluorescence microscopy, HEK-293T cells were cotransfected with CCK1R B/N and green fluorescent protein (GFP, pEGFP-C1, Genbank Accession #: U55763) while replating in 35 mm² glass dishes. The glass dishes were pretreated with 700 µl of 50 ug/mL PDL in 2mM HEPES for 1h and then washed thrice with nuclease free water and dried overnight. For 1 dish, 0.8 µg of CCK1R B/N and 0.8 µg of GFP were mixed in 100 µl of Optimem medium and 4 µl of Lipofectamine was added to 100 µl of Optimem medium in a separate tube. These two mixtures were incubated for 5 min. at room temperature and then combined and further incubated for an additional 20 min. After that, 400 µl of DMEM with 10% FBS was added to the DNA-Lipofectamine mixture. Meanwhile, HEK-293T cells were diluted to 3.33E5 cells/ml in DMEM with 10% FBS. Subsequently, 600 µl of the DMEM containing the DNA-Lipofectamine mixture was mixed with 600 µl

of the diluted cell suspension and seeded in a glass dish. For control cells, empty vector DNA instead of CCK1R B/N was used. Cells were incubated for 48h at 37 °C with 5% CO_2 .

5.2.4 Calcium-flux measurement in HEK-293T cells expressing the CCK1R construct

Calcium-flux in HEK-293T cells transfected with the CCK1R B/N construct or the empty vector after addition of CCK-8S or FCCK-8S was measured in a 384-well plate using a FlexStation II 384 Plate Reader (Molecular Devices). Two days after transfection, cells were incubated for 1 h at 37 °C with 20 µl dye (FLIPR Calcium 4 Assay Kit) per well. The dye was solved in HBSS-H (Hank's Balanced Salt Solution, with 20mM HEPES buffer, pH 7.4), supplemented with 0.4% BSA (according to the manufacturer). Subsequently the plate was allowed to stand for 15 min before the start of the measurement in the FlexStation preheated to 37°. The ligands were diluted in HBSS-H supplemented with 0.4% BSA. The fluorescence was monitored during 90 s, with a 2.5 s interval and 10 µl of the ligands were added *in fluxo* 20 s after the start of the measurement. The excitation and emission wavelength were respectively set to 488 nm and 530 nm. Each sample concentration was measured in 3 wells (technical replicates) for both transfections and every experiment was repeated 3-4 times (biological replicates). The difference between the maximum fluorescence signal and the baseline (Δmax-min) was calculated for each cell type and subsequently that of the control cells was subtracted from that of the cells transfected with CCK1R to correct for background fluorescence of the fluorescent labeled ligand. From these results, sigmoid dose-response curves for $(\Delta\text{max-min})_{CCK1R}$ - $(\Delta\text{max-min})_{control}$ versus sample concentration were derived with GraphPad Prism and EC_{50} values were calculated. The EC_{50} values are the mean of at least three independently repeated dose-response curves (biological replicates), which are based on triplicates (technical replicates) for each concentration.

5.2.5 Receptor internalization

These experiments were performed in collaboration with Prof. Thomas Huber. Visualization of receptor internalization was performed on HEK-293T cells in glass dishes cotransfected with GFP and the CCK1R B/N construct or the empty vector using a Zeiss AxioVert 200M inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Two days after transfection, the medium in the glass dishes was exchanged with the assay medium (DMEM-F12 containing 15 mM HEPES and supplemented with 10% FBS and 1% L-glutamine). The dishes were kept at room temperature. Subsequently, half of dishes were incubated for 15 min in assay medium supplemented with 1 µM of devazepide (DVZ), a CCK1R antagonist. The ligands were dissolved in DMSO and diluted in the assay buffer in such a way that the concentration of DMSO in the assay medium was 1% (v/v). The medium

was removed from the dishes and replaced with 1 ml of assay medium containing FCCK for 30 s. In the case of pretreatment with devazepide, assay medium containing FCCK and 1 µM devazepide was used. Then the medium was removed and after 45 s, the dishes were washed with 1 ml of assay medium. The washing step was repeated two more times. The dishes were kept at 4 °C and re-equilibrated to room temperature for 20 min before imaging. Per dish, 7x7 regions were imaged using a motorized x,y-stage with CRISP aligned to the glass/media interface using an X-Cite 120 lamp (Lumen Dynamics, Mississauga, Canada) with an intensity of 4 to 5. Three types of images were acquired for each position: 500 ms of brightfield exposure, 5 ms of epifluorescence with a 18 nm bandpass centered at 482 nm to visualize the GFP, and 2500 ms of epifluorescence with a 14 nm bandpass centered at 640 nm to visualize FCCK, using 20 x gain on a electron multiplying-charge coupled device (EM-CCD), eVolve 512 (Photometrics, Tucson, AZ). The emission filter is a quadruple bandpass (centered at 446, 523, 600, and 677 nm, Semrock, Rochester, NY).

5.2.6 Solubilization and purification of heterologously expressed CCK1R

The cell pellets of the transfected HEK-293T cells were lysed with 1 ml of lysis buffer (DPBS containing 19.6 mM DM, 1.96 mM CHS-Tris), 50 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1 mM $CaCl_2$ and protease inhibitors) per 10 cm dish for 1 h on a nutator and subsequently centrifuged at 1000 g for 15 min at 4 °C to remove the cell debris. Next, 100 µl of 1D4-sepharose resin was added to the cleared lysate and incubated on a nutator for 16 h at 4 °C. The resin was spun down at 1,000 g for 10 min and the supernatant was removed. It was subsequently washed thrice for 15 min with wash buffer (DPBS containing 1.96 mM DM, 0.196 mM CHS-Tris). Then the resin was put into a Micro-Spin column and the receptor was eluted by incubating the beads with 100 µl of 1D5-peptide solution (0.36 mg/ml in wash buffer) per plate on ice for 30 min and subsequent centrifugation for 5 min at 10,000 g. The elution was repeated twice and analyzed with sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot. Experiments were performed both with and without 5 µM devazepide.

5.2.7 Western Blot

Western Blot analysis was performed as described before (*146*). First, samples were resolved by SDS-PAGE (NuPage Novex 4-12% Bis-Tris Gel) and subsequently transferred onto a PVDF membrane using a semi-dry transfer cell (Biorad). To detect the CCK1R, a primary antibody against the 1D4 epitope (1:5000) and HRP (horseradish peroxidase)- conjugated anti-mouse (1:10000) as secondary antibody were used. Zap1 was probed with an α-His_6 tag monoclonal antibody conjugated with HRP-α-mouse.

The membrane was treated with enhanced chemiluminescence reagents and the signal was visualized on a HyBlot CL autoradiography film.

5.2.8 Production and purification of zap1

These experiments have been performed in collaboration with Carlos Rico and other members of the Sakmar lab. Recombinant zebra apo-lipoprotein (zap1) was produced and purified as described previously by Banerjee et al. (*16*) with slight modifications. BL21-DE3 cells, a *rosetta2* strain of *E. coli,* were transformed with pE28-zap1. A starter culture was grown overnight in LB medium supplemented with 50 µg/ml kanamycin and chloramphenicol. The next day, this starter culture was diluted 1:100 in 1 liter of Terrific Broth medium. When the OD_{600} (optical density at 600 nm) reached 0.8, expression of zap1 (see Figure 5.4 for protein sequence) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside.

M G S S **H H H H H H** S S G L E V L F Q G P H M A S Q A D A P T Q L E H Y K A A A L V Y L N Q V K D Q A
E K A L D N L D G T D Y E Q Y K L Q L S E S L T K L Q E Y A Q T T S Q A L T P Y A E T I S T Q L M E N T K Q
L R E R V M T D V E D L R S K L E P H R A E L Y T A L Q K H I D E Y R E K L E P V F Q E Y S A L N R Q N A E
Q L R A K L E P L M D D I R K A F E S N I E E T K S K V V P M V E A V R T K L T E R L E D L R T M A A P Y A
E E Y K E Q L V K A V E E A R E K I A P H T Q D L Q T R M E P Y M E N V R T T F A Q M Y E T I A K A I Q A

Figure 5.4. Protein sequence of recombinant zap1. The sequence contains a hexa-histidine-tag (His_6), indicated in bold.

After 2 h of incubation, the cells were harvested by centrifugation and the cell pellet was resuspended in 50 ml storage buffer (40 mM Tris pH 8, 0.3 M NaCl, 5 mM 2-mercaptoethanol, 1x aprotinin, complete EDTA-free protease inhibitor tablets (1 tablet per 50 ml), 2 mM PMSF) and stored at -80 °C.

The resuspended cells (50 ml) were thawed on ice and 500 µl of 100% Triton and 50 µl of DNAse were added to the cells and incubated on ice for 30 min. Then, 25 mL of the sample was mixed with 10 ml of Tris-buffer (25 mM Tris pH 8, 0.3 M NaCl) and sonicated for 15 min with intervals of 5 s (5 s "on", 5s "off") with an output level of 24 W or greater. Next, the zap1 protein was purified using affinity chromatography. 2 ml of His60 Ni Superflow Resin was equilibrated by adding 20 ml of equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 7.4) on ice. Then, the resin was spun down (1,500 g, 5 min, 4 °C) and the supernatant was removed. Next, 25 ml of the sample was applied to the resin and put on a nutator for 1 h at 4 °C. The resin was spun down (1,500 g, 5 min, 4 °C) again and the supernatant containing the cell debris was removed. Next, 20 ml of equilibration buffer and 20 ml of wash buffer (50 mM sodium phosphate, 300 mM sodium

chloride, 40 mM imidazole, pH 7.4) were applied to the column (resin was spun down and supernatant was removed after each step). Then, 10 ml of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole, pH 7.4) was added to the resin to elute the recombinant zap 1. Again the resin was spun down and the supernatant containing the recombinant zap1 was concentrated using a 10 kDa MWCO centrifugal filter. The buffer was exchanged with Tris-HCl pH 8, 1 mM EDTA buffer to get rid of the imidazole. This sample was applied to a Microcon-MC 0.45 µm microporous membrane spin filter unit to remove protein precipitate. The sample was further purified by SEC using a Superdex 200 10/30 GL column connected to an Äkta purifier at a flow rate of 0.5 ml/min buffer G (10 mM Tris pH 8, 0.15 M NaCl, 0.5 mM TCEP). Fractions of 0.5 ml were collected. The fractions containing the sample were combined and concentrated using a 10 kDa MWCO centrifugal filter. The quality of the purification of each step was analyzed with SDS-PAGE.

5.2.9 Incorporation of CCK1R into NABBs

The method as described by Knepp et al. (*146*) with slight modifications was followed to make NABBs. A ratio of 1/75 belt protein zap1/lipids was used. For NABBs containing the CCK1R (CCK1R-NABBs), 50 µl of the CCK1R elution was combined with 2.5 nmol of zap1, 187.5 nmol of POPC (14.25 µl of 1% (w/v) POPC/CHAPS), 0.33% DM-CHS buffer (196 mM DM, 19.6 mM CHS-Tris), 1.5% sodium cholate and brought to 150 µl with buffer S (20 mM Tris-HCl (pH 7), 100 mM $(NH_4)_2SO_4$ and 10% glycerol) and incubated on ice for 30 min. Subsequently, this mixture was put on 1 ml of Pierce Detergent Removal resin which was pre-equilibrated with 2 column volumes (CV) of buffer S. The elution was done under gravity flow by addition of buffer S. Five fractions of 150 µl were collected and the protein content was determined by monitoring the absorbance at 280 nm. To make empty NABBs (NABBs without receptor), the same recipe was followed but the 50 µl of CCK1R elution was replaced by 50 µl of PBS without Ca^{2+} and Mg^{2+} supplemented with 0.1% of DM-CHS buffer. Experiments were performed with or without 5 µM devazepide.

5.2.10 Binding of FCCK to CCK1R-NABBs

CCK1R-NABBs were further purified using a Sephadex G-50 Superfine SEC column with a column volume (CV) of 2 ml to remove the 1D5-peptide from receptor purification. To prepare the column, the Sephadex G-50 resin was first blocked with 5 CV of 10 mg/ml BSA in BupH (0.1 M Na_3PO_4, 0.15 M NaCl, pH 7.2) and subsequently washed twice with 5 CV of BupH. Then, 300 µl of CCK1R-NABBs from the combined third and fourth elution obtained after detergent removal was applied to the column and 5 fractions of 300 µl were collected on ice by addition of buffer S on the column. In the mean time, Dynabeads labeled with 1D4-antibody were prepared as followed; 25 µl of Dynabeads Protein

G were washed twice in 100 µl of BupH and subsequently incubated with 100 µl BupH supplemented with 1 mg/ml 1D4-antibody for 10 min at room temperature. Next, the labeled beads were washed in 100 µl Buffer S and incubated for 1 h on ice with 200 µl of CCK1R-NABBs from the third fraction resulting from the Sephadex G-50 SEC, to capture the CCK1R-NABBs on the beads via the 1D4-tag in the CCK1R. The unbound fraction was washed away with 100 µl of buffer S.

Then, a solution of 1 nM FCCK and a solution of 1 nM FCCK and 10 µM CCK-8S were prepared in buffer S. Next, 20 µl of each solution together with 2 µl of the CCK1R-NABBs captured on 1D4-beads was added to a 384-well plate and fluorescence was acquired with a Zeiss AxioVert 200M inverted microscope using similar settings as described in 5.2.5.

5.3 Results

In preliminary experiments, the expression of both constructs, i.e. CCK1R E/N and CCK1R B/N, after transfection in HEK-293T cells was compared on western blot. The HEK-293T cells transfected with CCK1R B/N construct yielded higher receptor expression as well as higher calcium flux. Therefore it was decided that all further experiments would be performed using the latter construct.

5.3.1 Functionality of heterologously expressed CCK1R and fluorescently labeled CCK analogue (FCCK)

The functionality of the CCK1R after heterologous expression in HEK-293T cells after transfection with CCK1R cDNA was evaluated with two different assays: 1) measuring the calcium flux after activation of the receptor and 2) assessing receptor internalization after ligand binding. The calcium flux was measured for both the natural ligand CCK-8S and FCCK (as depicted in Figure 5.2).

5.3.1.1 Calcium-flux measurement

The calcium flux induced by FCCK in HEK-293T expressing the CCK1R was compared to that induced by the natural ligand CCK-8S. Ligand concentrations ranging from 0.002 to 1000 nM were tested, and HEK-293T cells transfected with the empty vector were used as a negative control. The control cells exhibited no increase in fluorescence, except for the highest concentrations of FCCK, which is probably due to background fluorescence of the ligand itself. Therefore it was decided to subtract the fluorescence increase of the control cells from that of CCK1R expressing cells. Sigmoid dose-response curves were generated for the corrected calcium flux $(\Delta\text{max-min})_{CCK1R}$ - $(\Delta\text{max-min})$) versus sample concentration and EC_{50} values were calculated (Figure 5.5). They amounted to 7.0 ± 4.5 nM (SEM, n=3) and 69 ± 41 pM (SEM, n=3) for FCCK and CCK-8S respectively. The EC_{50} value of FCCK is a

100-fold compared to that of CCK-8S, pointing at lower affinity of FCCK towards the CCK1R which can arise from steric hindrance of the dye. However, the full agonism of FCCK seems to be retained as the highest calcium flux that can be obtained is similar for both ligands. Furthermore, the EC_{50} value found for CCK-8S in this experiment for HEK-293T cells is not significantly different from the results obtained for CHO-CCK1R cells (24 ± 4 pM) as described in Chapter 2.

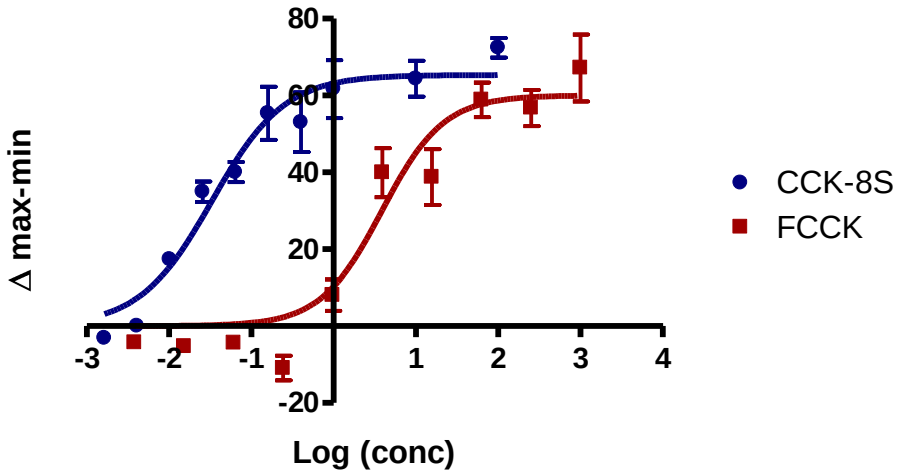


Figure 5.5. Representative dose-dependent CCK1R-mediated calcium fluxes in HEK-293T cells for increasing concentrations of CCK -8S and FCCK (0.002 – 1000 nM) based on 3 experiments (biological replicates) in which the measurements for each concentration were repeated 3 times (technical replicates).

5.3.1.2 Receptor internalization

HEK-293T cells were cotransfected with GFP and the CCK1R cDNA at a ratio of 1:1. The cells were incubated for 30 s with FCCK, with or without preincubation with devazepide. Subsequently, receptor internalization was visualized by monitoring the fluorescence of FCCK. Three different concentrations, 10 nM, 100 nM and 1000 nM of FCCK were tested, but only the highest concentration enabled visualization of the internalization of FCCK-bound CCK1R (Figure 5.6). To confirm that FCCK binds specifically to the CCK1R and that the observed internalization of FCCK is not due to non-specific endocytosis, the experiment was repeated in the presence of 1 µM devazepide, an antagonist. When treated with devazepide, no more fluorescence from FCCK could be seen. Also the control cells that were transfected with GFP and the empty vector only showed fluorescence for GFP and no fluorescence for FCCK. These experiments demonstrate specific binding of FCCK to the CCK1R. From the calcium flux assay and the receptor internalization experiment, it can be concluded that the CCK1R is functionally expressed in the CCK1R and that FCCK is functional as a full agonist, based on the equal amount of calcium that can be released by CCK-8S and FCCK, for this receptor.

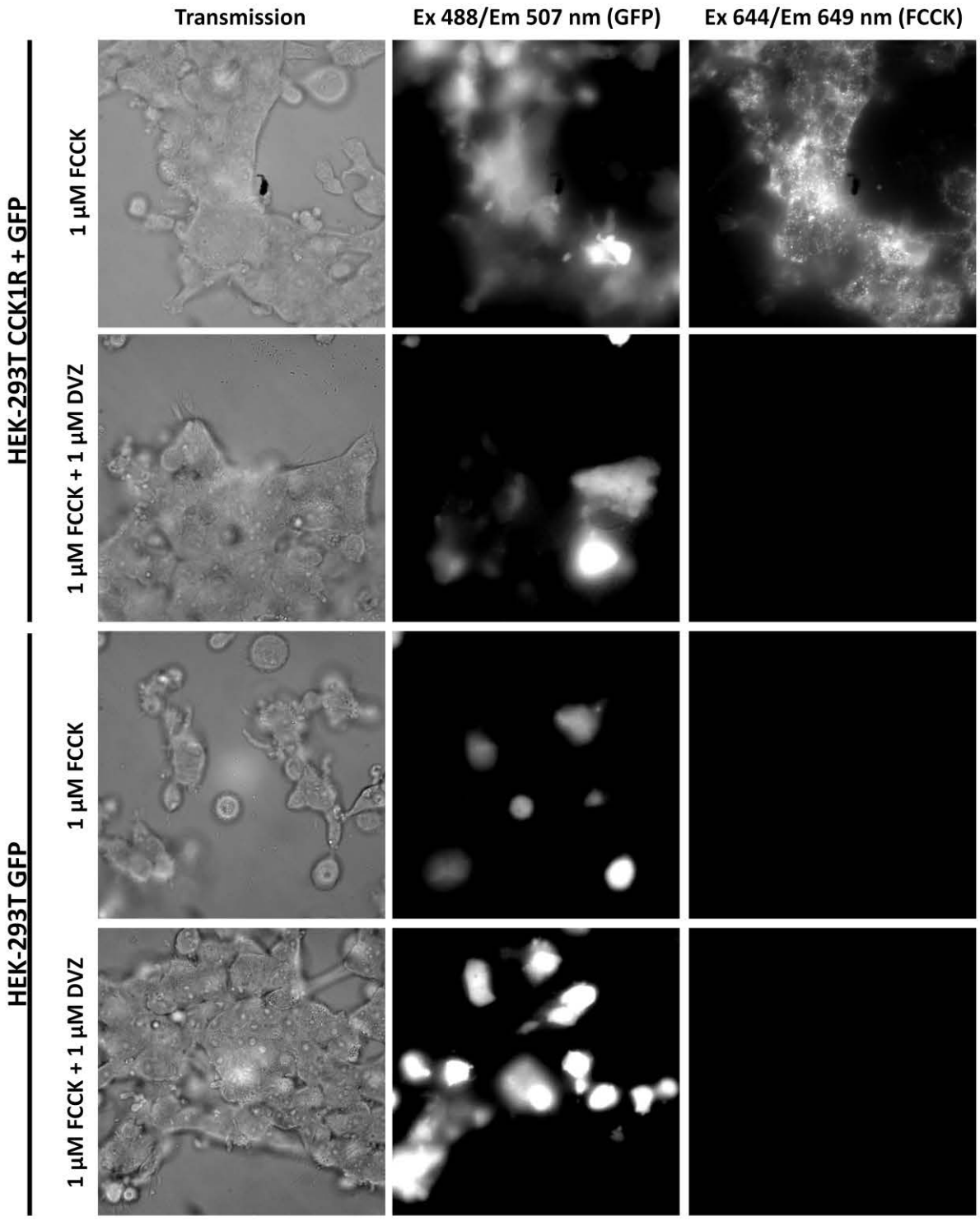


Figure 5.6. Image of HEK-293T cells transfected with either GFP and CCK1R or GFP and the empty vector after incubation with 1 µM FCCK, with or without treatment with 1 µM devazepide (transmission). For each image (82 µm squares) the fluorescence of GFP (excitation/emission wavelength 488/507 nm) and of FCCK (excitation/emission wavelength 488/507 nm) are shown.

5.3.1 Solubilization and purification of the recombinant CCK1R

HEK-293T cells heterologously expressing the CCK1R were lysed and the CCK1R was captured via its 1D4-tag on 1D4-sepharose resin. It was subsequently eluted using 1D5-peptide. On the coomassie stained gel after SDS-PAGE it can be seen that the cell lysate contains a wide range of proteins which can no more be detected in the elutions containing the purified receptor (Figure 5.7). The CCK1R including the 1D4-tag has a MW of 51.3 kDa. On the western blot (Figure 5.7), the CCK1R shows up as a band around 60 kDa, which is higher than the calculated MW, but this is probably due to glycolysation of the receptor. Treatment of the sample with PNGaseF to remove the N-glycans resulted in a decrease in MW (own research data). The band around 60 kDa is clearly enriched for the CCK1R elutions in comparison to the cell lysate, showing that the purification of the CCK1R was successful. The band around 20 kDa seen in the CCK1R elution lane can be 1D4-antibody that came off the 1D4-beads. For the cell lysate, also a band around 150 kDa and 37 kDa appears, the former can be dimerized receptor and the latter is probably truncated receptor, which are removed during purification as they stick to the 1D4-beads. As a reference, 10 ng of purified rod-outer-segment (ROS) rhodopsin was loaded on the gel. So approximately, 50 ng of CCK1R is present in the 6.5 µl of CCK1R elution loaded on the gel. This means that about 3 µg of purified CCK1R can be obtained per 10 cm diameter culture dish of HEK-293T cells transfected with 3.5 µg of pcDNA.

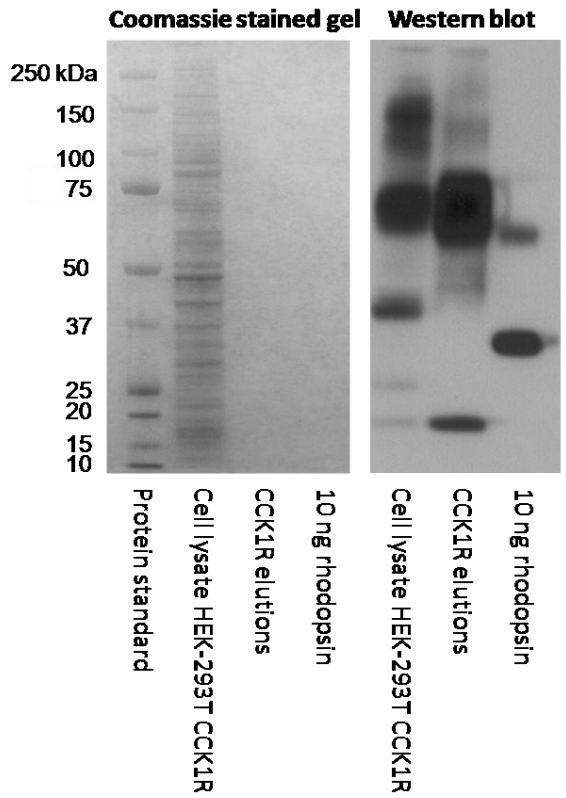


Figure 5.7. Coomassie stained gel and western blot against 1D4 for recombinant CCK1R purification. 6.5 µl of cell lysate and combined CCK1R elutions were loaded on the gel.

5.3.2 Production and purification of recombinant zap1

The zap1 protein was expressed in BL21-DE3 and purified using affinity chromatography (AC) and subsequent SEC (Figure 5.8). From the coomassie stained gel obtained with SDS-PAGE in Figure 5.8a it can be seen that after elution of the His60 Ni Superflow Resin for AC, the band of zap1 (MW 30 kDa) is enriched but that impurities are still present. Therefore this sample was applied on a Superdex 200 10/30 column for further purification using SEC (Figure 5.8b).

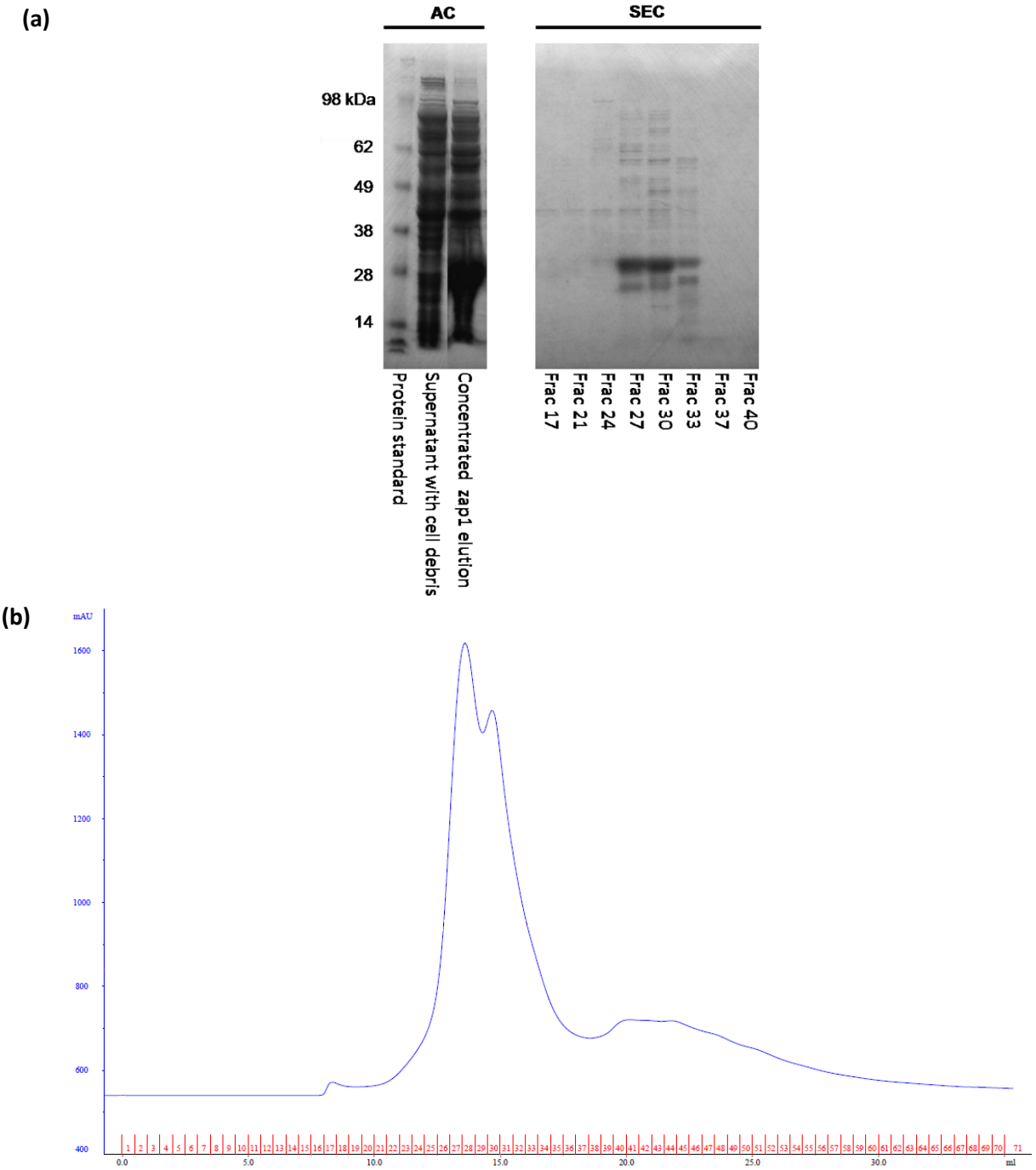


Figure 5.8. Purification of zap1. (a) Coomassie stained gels obtained with SDS-PAGE after AC and SEC. 6.5 µl of sample was loaded in each lane. (b) Size exclusion chromatographic profile using Superdex 200 column of sample after AC (absorbance at 280 nm).

Fractions of 0.5 ml were collected and the purity was assessed with SDS-PAGE (Figure 5.8a), a clear band around 30 kDa can be seen with less impurities compared to before SEC. Fractions 22 to 36 were combined and concentrated using a 10 kDa MWCO centrifugal filter. In total, 800 µl of purified zap 1 having a concentration of 9.48 g/l, determined with UV-VIS, was obtained. This means that the yield for this purification method was 18 mg recombinant zap1 for 1 liter of cell culture.

5.3.3 Formation of NABBs

NABBs with and without the CCK1R were made, by combining the belt protein and the lipids in the presence of detergent. Measurement of the protein concentration in the different fractions obtained after detergent removal using the pierce detergent removal resin, indicated that most of the protein was present in fractions 3 and 4. These fractions were combined and analyzed with western blot against the 1D4- and His_6-tag (Figure 5.9) of the CCK1R and zap1 protein respectively. For empty NABBs, only a band for zap1 can be seen. However, for CCK1R-NABBs a band for zap1 and for the CCK1R is present, indicating the formation of CCK1R-NABBs.

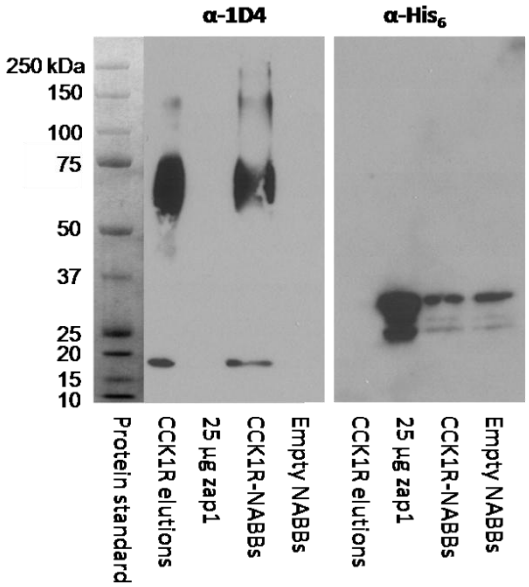


Figure 5.9. Western blots indicating formation of NABBs with and without receptor. Western blots were performed against the 1D4 tag of the CCK1R and the His-tag of zap1. 20 µl of NABBs were loaded on the gel. As a reference, 1 µl of the CCK1R elution and 25 ng of zap1 were also loaded on the gel.

5.3.4 Binding of FCCK to CCK1R-NABBs

An experiment to check binding of FCCK to CCK1R-NABBs was performed without the presence of devazepide as a stabilizing agent for the receptor, as no benefit of adding devazepide could be

proven in preliminary experiments. To evaluate whether the CCK1R incorporated into the NABBs was functional, it was tried to visualize binding to the CCK1R in NABBs with FCCK. Therefore, 1 nM of FCCK was added to the CCK1R-NABBs with or without coincubation of 10 µM of CCK-8S as a competitor. It was expected to see less fluorescence on the beads when the sample was coincubated with CCK-8S. However, the opposite occurred. The sample where no competitor was used showed no fluorescence (Figure 5.10). In contrast, the sample that was coincubated with a competitor did show fluorescence and even single molecules could be observed (Figure 5.10). Apparently, FCCK loses its fluorescence when bound to the receptor. When a competitor is present, the FCCK is free floating or sticking to the NABBs and thus visible. This suggests functional binding of FCCK to the CCK1R in NABBs.

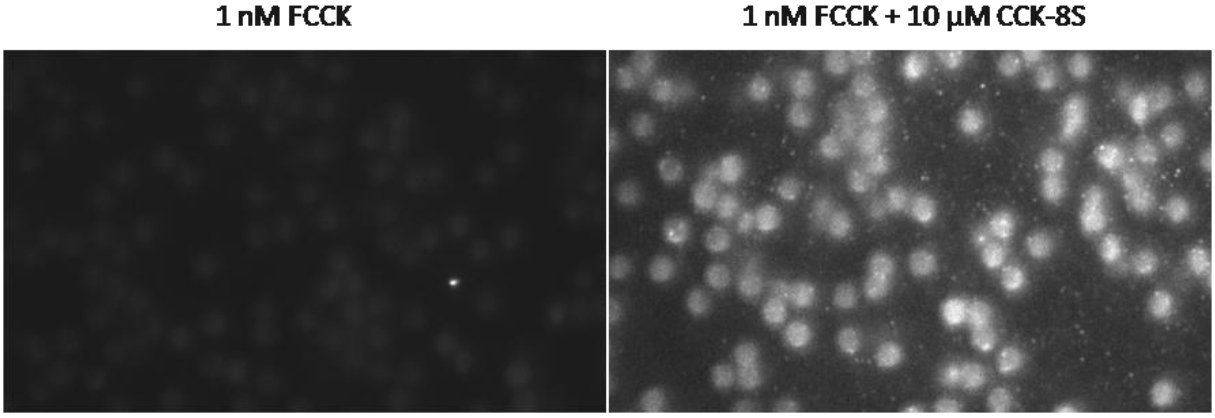


Figure 5.10. Dynabeads that hold CCK1R-NABBs in presence of 1 nM FCCK with or without coincubation with 10 µM of CCK-8S.

5.4 Discussion

From the calcium flux and receptor internalization assay, it could be confirmed that the CCK1R is functionally expressed in the HEK-293T cells. The necessary concentration to visualize receptor internalization, 1 µM of FCCK was very high in comparison with the EC_{50} of the calcium flux (7 nM) of this ligand. This is in agreement with a study of Wu et al. (*299*) where a concentration of 1 µM CCK-8S was used to visualize internalization of labeled CCK1R receptor, while the EC_{50} value for calcium flux was around 0.3 nM. As FCCK appeared to be able to fully activate the CCK1R with only a 100-fold lower affinity than CCK-8S, this ligand can be used in future experiments for the examination of ligand-receptor binding.

Furthermore, the purified zap1 protein and the CCK1R were sufficiently clean to be further incorporated into NABBs. The western blot showing bands of both CCK1R and zap1 for CCK1R-NABBs,

and only a band of zap1 for empty NABBs indicates the formation of CCK1R-NABBs. In a study of Szecowka et al. (*268*) on the purification of the CCK1R from pancreas, it is reported that after solubilization, the CCK1R does retain its affinity and specifity for its ligands. At this point, there is some evidence that the CCK1R in NABBs retains its functionality as it appears to be able to bind to FCCK. However, the evidence is indirect because FCCK appears to lose its fluorescence when bound to the receptor. We hypothesize that the fluorophore gets quenched and therefore is no longer fluorescent when bound to the receptor. The experiment should be repeated using a fluorescent labeled CCK1R ligand with another fluorophore.

In continuation of this project, bioactive peptides that bind to the CCK1R can be purified using the CCK1R-NABBs. The CCK1R-NABBs can be incubated with the peptide mixture and unbound peptides can be washed away. Consequently the bound peptides will be eluted using high-affinity CCK1R ligands, such as CCK-8S, and can be identified in the eluens by mass spectroscopy (MS). This procedure will need to be optimized in terms of ligands and buffers used for elution. CCK is very well suited to elute the bound peptides, however it might interfere with MS-identification. Therefore, small molecule ligands are the preferred choice for elution, as they are structurally distinct from peptides and will greatly simplify MS identification. Nevertheless, it should also be taken into account that these might have allosteric binding sites and therefore might not be able to elute the peptides. Also, if the peptides are rather hydrophobic, they might stick to the NABBs themselves, as was seen for FCCK. A lot of process optimization in terms of buffers and ligands used for elution is still necessary.

A similar concept has been previously described by Borch et al. (*29*) to identify proteins that interact with membrane glycolipids. This technique can not only be used to screen for bioactive peptides binding to the CCK1R, but can also be applied to other (pharmaceutical) components. Next to the use of CCK1R-NABBs as a selective screening tool for ligands, they can also be used to further elucidate the 3D structure of this receptor.

Chapter 6

Conclusions, general discussion and future perspectives

Chapter 6 Conclusions, general discussion and future perspectives

6.1 Main outcomes of this work

This PhD thesis contains 4 corner stones, i.e. method development for *in vitro* detection of CCK1R activation, screening of protein hydrolysates for CCK1R activity, purification and identification of the bioactive peptides and *in vivo* evaluation of the active peptides. These 4 parts will be briefly summarized below.

6.1.1 *In vitro* method development for calcium flux measurement using pure compounds

In vitro CCK1R activation was measured in CHO cells functionally expressing this receptor. When the CCK1R is activated, an intracellular calcium flux is induced which can be visualized with a cell permeable fluorescent dye. We found that the best homologous distribution of the fluorescent dye was obtained when it was incubated with the cells at 19 °C. Performing the experiment at a lower temperature (31 °C) than 37 °C, slowed down the metabolism of the fluorescent dye and allowed to perform stable calcium flux measurements within 80 min after incubation. For quantification of this calcium flux, a population average technique using a fluorescence plate reader was optimized and subsequently compared with a single-cell approach using confocal microscopy. With both strategies, dose–response curves were generated for the natural agonist CCK-8S, the partial agonist JMV-180 as well as the antagonist lorglumide. Significant differences were found between the ligands and a strong correspondence was observed between both methods in terms of maximum response and median effect concentrations. Both methods were highly sensitive and proved to be complementary: whereas the plate reader assay allowed faster, high-throughput screening, the confocal microscopy identified single-cell variations and revealed factors that reduce specificity and sensitivity, such as background fluorescence of the sample. Calcium fluxes were monitored kinetically during 34 s after sample addition. It was observed that increasing concentrations of the agonists resulted in higher calcium peaks and faster response times. CCK-8S had an EC_{50} value between 20 to 50 pM, while that of the partial agonist JMV-180 was 1000-fold higher and the calcium fluxes induced by the latter compound were decreased to less than 40% in comparison with CCK-8S. The calcium flux induced by JMV-180 appeared to be exemplary for the protein hydrolysates. A concentration of 40 µM of the antagonist lorglumide completely inhibited the calcium flux of 1 nM (concentration at which a maximum calcium flux was observed). Therefore this antagonist could be used as an extra control to

ensure whether true CCK1R-induced calcium fluxes are measured. The CHO-K1 cells showed no response to any of these compounds.

6.1.2 *In vitro* screening of protein hydrolysates with CCK1R activity

Four commercial crude soy hydrolysates as well as hydrolysates from purified soy and milk proteins were screened for CCK1R activity using the cell-based bioassay. Only the 7S and 11S soy hydrolysate showed a significant increase of the net fluorescence response. Interestingly, also the CHO-K1 cells showed a fluorescence increase when stimulated with these hydrolysates. This points at non-CCK1R-specific calcium fluxes, which might be induced by activation of other receptors such as receptor tyrosine kinase-type receptors or the thrombin receptor, on these cells (*57, 215, 226*). Ca^{2+} is often used as a second messenger in GPCR signaling (*143*). Furthermore, the tests were also repeated in the presence of the antagonist lorglumide and it was expected that the fluorescence increase of the CHO-CCK1R would fall back to the same level as that of CHO-K1 cells. The fluorescence increase of the CHO-CCK1R was reduced by lorglumide, however that of the CHO-K1 cells too. This might be an indication that lorglumide also inhibits other receptors. Fluorescence measurements were first performed using a fluorescence plate reader. When testing the commercial soy hydrolysates, very high background fluorescence of the hydrolysates themselves obscured the true values. Less background fluorescence was seen when testing hydrolysates from purified proteins, however it was decided to use confocal microscopy for further measurements to obtain more reliable results. The fluorescence plate reader was not suited to measure calcium induced fluorescence by complex matrices and can only be used to make a rough primary screen of more purified hydrolysates. The optical sectioning capacity of the confocal microscope allowed to measure true fluorescence increases at the cellular level by circumventing the autofluorescence of the sample and therefore became the preferred technique.

6.1.3 Purification and identification of bioactive peptides with CCK1R activity

The peptides present in the 7S and 11S hydrolysates were further purified by separating them in different molecular weight classes using gel filtration chromatography. All obtained peptide fractions showed significant *in vitro* CCK1R activity and peptides present in some of the most active fractions were identified. The electrostatic structure of one peptide (PALSWLR) showing best homology with CCK was analyzed and subsequently synthesized, but it showed no significant CCK1R activity.

In total, about 1000 peptides with a chain length from 7 to 32 amino acids have been identified in the soy protein hydrolysate fractions. It was tried to further purify the fractions after gel filtration using

RP-HPLC and the fractions with the highest absorbance were tested for CCK1R activity, but no activity could be measured. However this could be expected as the remaining quantities to perform the analysis were very low. Despite several attempts to identify the active peptide(s) using subsequent chromatographic techniques, the active peptide sequence(s) remained unknown. Therefore, a different approach to identify the active peptide(s) was developed. It was urged to embed the CCK1R in NABBs -a native-like bilayer membrane system kept together by a belt protein, zap1- for incorporation of GPCRs, as a means to selectively pull-down CCK1R-binding peptides from a peptide mixture. To this end, CCK1R and zap1 were purified after expression in HEK-293T cells and *E. coli* respectively using several different techniques. Western blot indicated incorporation of the CCK1R in the NABBs and preservation of functionality of the receptor was shown by binding with a fluorescent labeled CCK analog.

6.1.4 *In vivo* screening of 7S and 11S soy protein hydrolysate fractions

The set-up of the rat experiment as described in 4.2.7 was validated during preliminary experiments with the synthetic CCK1R agonist SR146131 (*24*) (Figure 4.1), using this compound it was confirmed that the set-up of the experiment was suited to measure an effect on food intake. The effect on food intake by the peptide fractions with a MW between 700 and 7000 Da from the 7S and 11S soy protein hydrolysates was evaluated in rats *in vivo*. As we could not demonstrate a significant difference in food intake between test and control groups, we speculate that the bioactive peptides may not reach their targets intact due to further breakdown or insufficient amounts needed for activity or low bioavailability. Furthermore it's also possible that, as food intake is regulated by different systems, that the effect on the CCK-signaling system was compensated by other systems.

6.2 Critical reflections

6.2.1 *Screening CCK1R-activating peptides*

Thus far, very promising *in vitro* data showing that bioactive peptides can activate the CCK1R has been obtained. *In vitro* calcium fluxes of CHO-CCK1R cells after stimulation with various protein hydrolysates have been monitored kinetically with a confocal microscope and per experiment (= addition of one sample in one well), 130 chronological images containing 100 to 150 cells were captured. This means that a tremendous amount of data has been collected. A critical issue in this project was to decide on how to process and summarize the data and which parameters to use to describe these data in such a way that a clear and distinct view would be obtained on how and to what extent CCK1R activation happens. All data were compared relative to the response of 1 nM

CCK-8S and corrected for background and non-specific responses by subtracting the response of CHO-K1 cells. This resulted in kinetic curves representing the relative fluorescence response, i.e. a fluorescent response truly evoked by CCK1R activation. Different parameters in terms of peak time, peak height, persistence of the signal and shape of the curve could be used to characterize the fluorescent response. The area under the relative fluorescence curves was considered as the most complete parameter to describe these curves as it contains information on peak height, peak time and signal persistence. However, a computer model describing the curves in terms of all the aforementioned parameters subsequently scoring these curves might suit even better and could be developed in continuation of this project.

6.2.2 Purification and identification of CCK1R-activating peptides

A major issue in this work is that thus far we have not been able to purify and identify the structure of the active peptide. In an attempt to identify the active peptide(s), as a sulfated tyrosine is very important for CCK1R activity and also the amino acid before and after tyrosine are important, (Figure 1.2 (bottom)) all peptides listed in Supplementary Table 1 containing a structure homologous to DYM (where D is replaced by E, S or T and M by C, I, L or T) were superimposed (using a sulfated tyrosine) with CCK-8S. However, no plausible candidate peptides could be found. This indicates that the active peptide(s) might have a completely different structure from CCK and/or even have another binding site on the receptor, which can explain the observed partial agonism. The development of the CCK1R-NABBs is a huge step to finally identify the active peptide, however, at first, more optimization of this technique is needed.

6.2.3 *In vivo* results and bioavailability

Since peptide mixtures clearly showing *in vitro* CCK1R activity have been obtained, it was decided to test the effect on food intake of these mixtures *in vivo*, but no effect on food intake was observed. Evidence exists that vagal afferents respond to amino acids and hydrolyzed protein (*234*), indicating once more that CCK1R-activating peptides might induce satiety through vagal afferents. The reason why no effect on food intake is seen in this project might be due to poor bioavailability of the peptides. The exact definition of bioavailability depends on the scientific context (*294*), but here it is meant as the fraction of a dose that reaches the CCK1R in the GI after oral administration. In this work we tried to induce satiety by mainly targeting the CCK1Rs located on vagal afferents in the GI. This means that the bioactive peptides may not be broken down in the GI after oral intake and need to be able to cross the intestinal wall intact, which might be a major obstacle. A clear cut-off value on the maximum peptide length that can cross the intestinal wall cannot be set, as this is dependent on

the chemical nature of the amino acids present in the peptide (*246*). Di- and tripeptides can be absorbed intact via a peptide-specific transport system e.g. PepT1, using an electrochemical proton gradient force. Peptides with a longer chain length can cross the epithelial wall via passive transport or actively through endocytosis or they even might pass between the tight junctions between two cells (*246*). We expect that a peptide that can activate the CCK1R will be at least eight amino acids long, as this is also for CCK the minimum required chain length for CCK1R activation. Despite a study that reports that biologically active peptides of up to 50 amino acids can be absorbed through the epithelial wall in small quantities, as shown for insulin (*236*), the cut-off chain length for food-borne peptides to be able to cross the epithelial wall is probably rather 8 to 12 amino acids (*246, 248*). When active peptides have been found, their ability to penetrate intact through the intestinal wall will have to be assessed.

Previously, the CCK1R has been targeted with pharmaceutical components, like SR146131 (*25*) from Sanofi-Aventis (Figure 4.1), which have good oral bioavailability. *In vitro* experiments showed that SR146131 is a highly potent (EC_{50} = 1.38 ± 0.06 nM) and selective CCK1R agonist. After oral administration to test animals, strong and significant reduction of food intake was seen. For example, after 3h a significant drop in the cumulative food intake of test rats compared to control rats was seen starting from the dose of 0.1 mg/kg, with an ED_{50} of 0.43 mg/kg BW. After 23 hours, the cumulative food intake of the test rats was still significantly reduced for doses starting from 0.3 mg/kg BW onwards and a maximum reduction of 63% at a dose of 3 mg/kg BW was obtained. Despite these promising results, severe side effects were seen in the different test animals. Mice showed a significant decrease in locomotor activity and increased turning behavior (*24*). To validate our set-up of the rat experiment to measure an effect on food intake as described in 4.2.7, we did some preliminary experiments with SR146131. Next to a significant reduction in food intake, decreased locomotor activity, increased turning behavior, back-arching and stretching were seen. To monitor these side effects, the test animals can be video filmed as mentioned before (*221*). Other physiological effects of CCK, like gall bladder emptying and gastric acid secretion should also be assessed. There are good indications that SR146131 is able to penetrate the blood-brain barrier (*24*), which is probably the explanation for the adverse effects seen. The advantage of using bioactive peptides as CCK1R agonists instead of pharmaceutical compounds is that they most likely will not show these side effects as they won't be able to cross the blood-brain barrier, if they even get there at all. No adverse effects were seen during the rat experiment in this study. It should also be taken into account that proteins from the *Leguminosae* family are a major source of food allergens, however these get mostly deactivated through hydrolysis (*40, 211*).

A difficulty in this project is that the CCK signaling system only induces satiety at the short term (*185*) and it is not sure yet that by influencing this system persistent weight loss can be measured in *in vivo* tests. For example, the pharmaceutical CCK agonist GI181771X significantly delays gastric emptying of solids and increases fasting gastric volumes in humans (*41*), however it appeared not to cause weight loss in overweight or obese human subjects (*138*). If this would be the case, research should focus on developing a combination of ingredients that affect several weight regulating mechanisms to tackle balancing out of one mechanism by the other.

6.3 Future perspectives

6.3.1 *In vitro* screening of more protein hydrolysates

More protein hydrolysates and peptides still need to be screened for their CCK1R-activating potential. A good candidate protein can be protein from pea, because like soy, pea is also a member of the *Leguminosae* family. Preliminary data support the hypothesis that the pea protein hydrolysate contains CCK1R activity to a similar extent as the soy protein (Figure 6.1). When using proteins from legumes, it has to be taken into account that these proteins might possess food allergens (*200*).

Preliminary experiments have also been carried out with whey protein hydrolysate, but this hydrolysate showed no significant CCK1R activity. Other candidate proteins are proteins from staple food such as potato and cassava. Potato proved to be very satiating (*123*) and contains up to 4% of protein (*39*). Potato proteins represent up to 25% of the waste stream of starch factories and therefore might form an interesting protein source for this research (*222*). The protein in cassava is especially present in the leaves and amounts up to 40%, however extraction is difficult and the yield is low due to the presence of tannins (*281*).

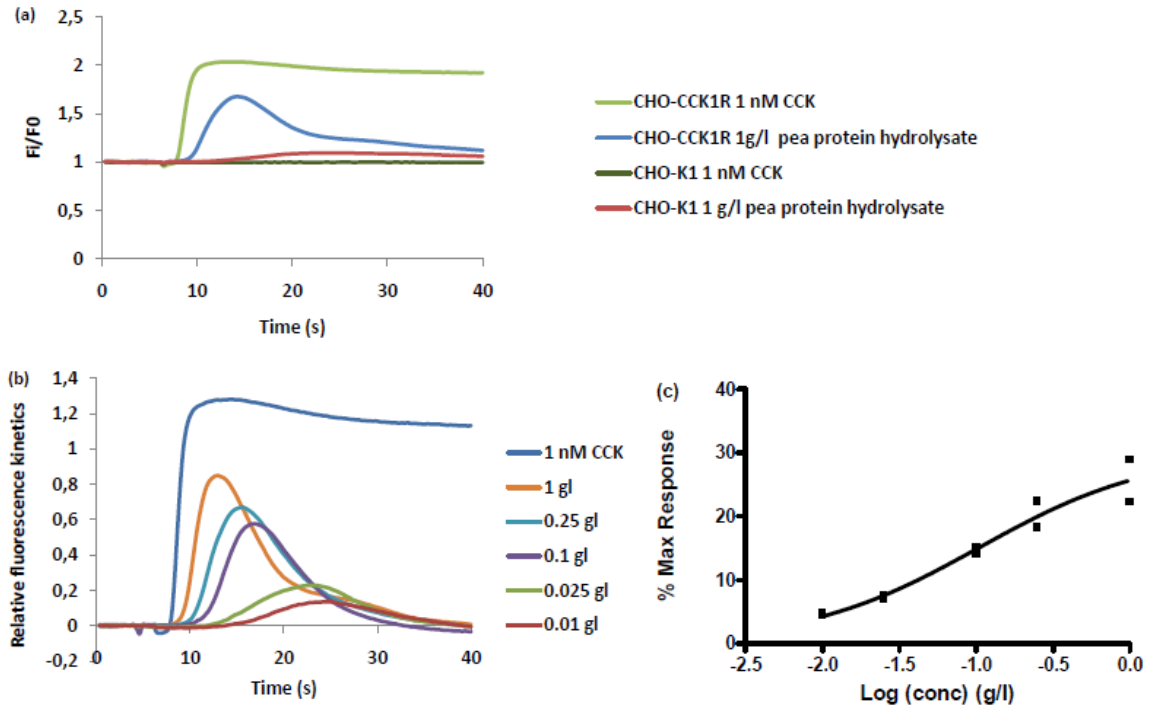


Figure 6.1. Fluorescence kinetics and dose-response curve for pea protein isolate hydrolyzed for 1 min with Promod 278P (Biocatalysts, Cardiff, UK representing CCK1R-mediated calcium fluxes in cell populations monitored with a resonant scanning confocal microscope). (a) Normalized fluorescence kinetics (Fi/F0) for CHO-CCK1R and CHO-K1. Results for sample as well as for 1 nM are shown. The curves represent the mean of 5 technical replicates. (b) Dose-dependent representative relative fluorescence kinetics (RF) of increasing concentrations of pea protein hydrolysate (0.01–1 g/l). The curves represent the mean of 5 technical replicates. (c) Dose–response curve for pea protein hydrolysate based on 2 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, i.e. the net response induced by 1 nM CCK-8S. The pea hydrolysates were obtained from Wageningen University and Research Centre, Food & Biobased Research, Group Bioactive Ingredients.

6.3.2 *Development of QSAR*

A rough 3D-model of the CCK1R (*7*), in which CCK-8S has been docked, exists. It could be tried to dock all the identified peptides into the CCK1R binding cavity as was done for sulfakinin (*258*) and select the peptide with the lowest binding energy for subsequent synthesis and *in vitro* evaluation. When the active peptides are identified and their *in vitro* activity is assessed, these data can be used to establish a QSAR (quantitative structure-activity relationship) from which possible new CCK1R agonistic structures might be deduced. However, before this strategy can be followed, the 3D model of the CCK1R has to be refined. This can be done using computer-assisted molecular modeling,

however the accuracy of such models remains a major issue (*7*). The crystal structure of the CCK1R would be very useful to this end and the list of known crystal structures from GPCRs is steadily growing, however GPCR-mediated signaling remains very complex. To gain a better understanding of this process, novel techniques and different approaches, e.g. color-coding of different proteins with different fluorescent probes and subsequent measurement by SMD-TIRF (small molecule detection – total internal reflection fluorescence), will be necessary (*127*).

6.3.3 *Bioavailability of the bioactive peptides*

6.3.3.1 Assesment of intestinal permeability

Several *in vitro* techniques exist to evaluate intestinal permeability. They can roughly be divided into two experimentally different categories: one based on uptake of the components into the system and a second one based on transport of the component through the system. Three different approaches for the latter category will be discussed here. One is the use of an everted intestinal sac, a small piece of intestine is cut off, one end is tied off and subsequently the "sac" is everted and filled with buffer containing the compound. Next, the serosal fluid (inside the sac) can be analyzed after a certain incubation period (*280*). The intestinal membrane of a frog seems to be very well suited as a model for human absorption (*278*). A second approach is based on the use of Ussing chambers or similar models, in which small sections of intestine are clamped between two glass chambers that are filled with buffer and appropriate nutrients, and supplied with 95% O_2. Transport of the compound through the tissue is then measured as a function of time (*280*). In a third approach, cultured cells are used to study intestinal permeation. Caco-2 cells, a cell line derived from a colon cancer adenocarcinoma, are widely used as they resemble to mature human enterocytes (*246*). They can be grown on porous filter supports and the permeation experiments can be directly performed in the original culture chambers. Caco-2 cells are very well suited for high-throughput screening with high reproducibility, but the disadvantage is that a caco-2 cell monolayer might be less permeable than human intestine (*280*). In the context of bioactive peptides, everted intestinal sacs might be the preferred method as this method is relatively inexpensive and simple to perform (*280*). For example, the effect of chain length on absorption of soy peptides was compared using rat everted intestinal sacs (*44*). Also, *in silico* methods for prediction of intestinal absorption are being developed and might be a great help to avoid peptide synthesis and reduce screening time and cost. These include computational models in which the physicochemical characteristics of the different peptides are taking into account. Recognition of key physicochemical properties then allows predictive screening (*4*).

6.3.3.2 Improvement of bioavailability

To improve the permeability of the intestinal wall, permeability enhancers such as surfactants, fatty acids, glycerides, steroidal detergents, acyl-carnitines and acyl-cholines, N-acetylated amino acids, and chitosans and other mucoadhesive polymers (*12*) can be added to the peptides. These compounds controllably and transiently open the tight-junctions between cells allowing the peptides to cross the epithelial wall via the transcellular route. However it should be avoided that toxic and unwanted compounds are being absorbed (*4*). The peptides can also be structurally or chemically modified to avoid enzymatic breakdown and to have a longer lifetime by pegylation, lipidation, glycosylation, addition of fatty acids or isoprenoids, N-methylation, amino acid substitution and formation of disulfide bonds (*246*). Furthermore, microencapsulation is a technique that is gaining general acceptance for improvement of bioavailability of bioactive compounds. It can be used to protect the peptides from harsh conditions in the GI and it allows controlled release of the peptides. Microencapsulation can also be used to mask the bad taste of the peptides (*152*).

6.3.4 *In vivo* screening

After *in vitro* (cell-based bioassay) and *ex vivo* (intestinal wall permeability) evaluation of the bioactive peptides, their effect on food intake and possible side effects can be evaluated *in vivo.* In a first phase, this will be done with rat experiments on the short and long term, and in a second phase with human intervention studies.

6.3.4.1 Short term experiments and evaluation of involved appetite regulating mechanism

During short term experiments, the effect of the bioactive compounds on food intake within one day is measured by measuring the amount of food that disappeared from the cage. Also other parameters like meal frequency and meal duration can be monitored (*291*). Video recording of the rats is possible and can be a very useful aid in assessing these data (*221*). As discussed before, appetite regulation is a complex process in which different mechanisms are involved. So when an effect on food intake is seen, despite the in vitro and the *ex vivo* results indicating that the CCK1R might be activated, it is unsure which appetite regulation mechanism(s) is/are altered. E.g. food intake might be reduced by stimulation of CCK production or even through another gastro-intestinal hormonal pathway. Therefore, some extra controls have to be built in. First, when the sample is given to the rats, the concentration in the blood of the different gut peptides and especially CCK should be measured within 10 to 30 min after sample addition and compared with the values of the control rats. The concentration of the gut peptides in the blood of the test rats should not be

different from that of the control rats. If this is the case, than CCK release is also at least partially involved. Secondly, to be sure that food intake reduction is induced via activation of CCK1Rs, the experiments should be repeated in combination with a CCK1R antagonist such as lorglumide (*219*). In summary, when no augmentation of CCK or other gut peptides in comparison to the control rats is seen after sample addition and the food intake reduction can be inhibited by a CCK1R antagonist, it can be concluded that the food intake reduction is caused by true CCK1R activation of the bioactive peptides. Also, the possibility that the CCK1R is activated in combination with an effect on other appetite regulation mechanisms should not be excluded. Furthermore, dose-response experiments will have to be carried out to determine the concentration for which an effect can be seen.

6.3.4.2 Long term effect on weight

It is well known that CCK affects appetite on the short term, i.e. within hours after a meal (*69*). The short term experiments reveal information about the effect of bioactive peptides on appetite within this time frame, but provide no information on what happens at the longer term. The long term goal is to see a decrease in weight or weight gain in the test animals compared to the control animals. These experiments can also be performed with standard laboratorium rats such as Sprague-Dawley or Long-Evans rats. An obesity-prone environment can be created for the rats by offering them fresh wet cookies, bread and sugar water in addition to standard laboratory chow (*243*). Interestingly, in a report of Swartz et al. (2010), obesity-prone rats seemed to reduce food intake more after intraperitoneal injection of CCK, compared to obesity-resistant rats. An explanation for this phenomenon might be the increased expression of CCK1Rs in these rats (*266*). However, these results are not conclusive (*165*). The rats should receive during a certain amount of time, e.g. 15 weeks, every day a certain dose of bioactive peptides and subsequently their body weight should be monitored on a day-to-day or week-to-week basis (*247*). Also during long term experiments, adverse effects should be monitored by daily inspection of the rats for signs of toxicity by e.g. measuring body temperature. Also the use of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, having a defect in the gene coding for the CCK1R, could be considered to evaluate the effect of product on CCK-induced satiety (*193*).

6.3.4.3 Human intervention studies and functional food matrix

The ultimate goal of this study is to attain a significant amount of weight loss in humans by oral uptake of CCK1R activating bioactive peptides. So after the *in vivo* experiments with animals, human intervention studies should be carried out. The designated experimental set-up to measure an effect of a product on food intake is the preload study design using within subject repeated measurements

and double-blind controlled conditions (*28*). The product under research can be administered to human subjects less than half an hour up to several hours before serving an accurately monitored test meal after which their spontaneous food intake and appetite ratings can be compared to the control group having received a placebo or no preload (*28, 59, 122, 154, 255, 306*). Appetite ratings can be validated using e.g. visual analogue scales (*209*). Also, instead of using test meals, subjects may self-report their food intake (*28*). This study can be preceded by a pilot study in which only a limited number of human subjects are included to confirm the effect on appetite and/or food intake, to estimate the desired dose and to calculate the sample size of the main intervention study (based on the magnitude of the effect on appetite/food intake and the standard deviation). Furthermore, the physiologically active dose should be reasonably ingestible (e.g. a couple of grams per day).

Also at this point, the food matrix (e.g. a yoghurt) in which the bioactive peptides will be incorporated for oral uptake has to be considered. It should mask the bitter taste of the peptides if any and it also may not interfere with the functioning of the peptides. The food matrix influences the bioaccessibility, i.e. the fraction which is released from the ingested matrix upon ingestion and which is available for absorption (*240*). The composition of the food matrix, the synergisms and antagonisms of the different compounds, but also physicochemical properties such as temperature, pH and texture of the matrix have an influence on the bioaccessibility. Also, the volume and the energy content of the food matrix affects physiological conditions in the GI which causes changes in the bioaccessibility of digested compounds. In the context of peptides, the hydrophilic or hydrophobic character of the peptides of interest will determine the composition of the food matrix (*229*).

6.3.5 Broader applications and opportunities

In this project, all the techniques used were focused on bioactive peptides, the CCK1R and satiety. The *in vitro* cell-based bioassay and the CCK1R-NABBs can also be used to screen for other food components. The CCK1R-activating potential of plant phenols can be investigated, as it has been indicated that plant constituents might influence the CCK1R signaling pathway (*147*). More generally, the *in vitro* cell-based bioassay and the CCK1R-NABBs can be used to screen for pharmaceutical CCK1R agonists, which do not necessarily need to have a(n) (sole) effect on satiety but which can also act on other CCK1R-mediated conditions such as gall stones and diabetes mellitus (see higher). Furthermore, this screening system might be applied in a parallel set-up to look for agonists and antagonists of the CCK2R and even for all kinds of other GPCRs too. Especially the use of NABBs in which a certain GPCR is incorporated as a pull-down system to identify components binding to the involved GPCR is a very innovative and promising technique.

6.4 Regulatory issues and social context

6.4.1 EFSA guidelines concerning marketing of bioactive peptides and safety thereof

The final application of this project is to market a functional food that can claim enhanced satiety. A claim that implies a relationship between consumption of a food product and health is called a "health claim". Such claim is subjected to the "Nutrition and health claims regulation", which came into legislation in January 2007 by the EU (Regulation (EC) No. 1924/2006 (*78*)). The food constituent on which the claim is made should have a beneficial physiological effect and claims should not:

- be ambiguous, false or misleading
- cause doubt on the safety and/or nutritional value of other foods
- give rise to excess consumption
- imply that a varied and balanced eating pattern is not sufficient to provide appropriate nutrient quantities
- mention changes in body functions that might frighten consumers

Health claims referring to an amount or rate of weight loss are not allowed, but claims related to appetite changes do are allowed and fall under article 13.1c of the nutrition and health claims regulation (*96*). These claims should be based on generally accepted scientific evidence, which is assessed and studied by EFSA. To review the evidence, EFSA addresses a number of questions (*74*):

- Studies performed on claimed food constituent? Food constituent well-defined and characterized?
- Design and quality of the study allows scientific substantiation of the claim?
- Study group representative of the population group?
- Study uses the appropriate outcome measure?

If the claim meets these criteria, EFSA evaluates the evidence to determine whether a cause-effect relationship is established. *In vitro* studies and animal studies can be used as supporting evidence and to explain plausible mechanisms, but profound human intervention studies on the target population are necessary in order to have a claim on appetite ratings approved and these need to show a sustained effect when the food is continuously consumed. It is also important that the dose of the active compound in the functional food on which the claim is made is in concordance with the dose used during the human-intervention studies. So after evaluation of the scientific evidence, EFSA provides an opinion whether a claim is sufficiently scientifically substantiated to the European Commission. The European Commission, together with member states representatives, makes the

final decision whether the health claim should be approved or rejected. No pre-established formula on what kind and to what extent studies have to be performed to substantiate a health claim, so each claim is assessed separately. This uncertainty concerning criteria in order to have a health claim approved is a big concern for the industry. (*74, 96*).

The safety of protein hydrolysates, protein hydrolysate fractions or bioactive peptides has to be assessed by the company trying to market that product. A special legislation exists that has to be applied when a company wants to market a 'novel food' (Regulation (EC) No 258/97 (*47*)). 'Novel foods' or 'novel food ingredients' are defined as 'foods and food ingredients that have not been used for human consumption to a significant degree in the EU before 15 May 1997 and are divided into several categories of which the following might be applicable to protein hydrolysates, fractions thereof or bioactive peptides:

> 'foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use;
> foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances.' (*47*)

When a new food product is developed, the safety assessment is generally based on the comparison with comparable traditional foods that have a documented history of safe use. When the safety of protein hydrolysates and fractions thereof is evaluated, following characteristics appear to form an appropriate benchmark (*242*):

- the documented history of safe consumption
- the data from safety studies and safety-related data from efficacy studies
- the amount of protein intake
- the amount of free amino-acid intake
- the safety aspects of the production processing, e.g. processing aids

6.4.2 Evaluation of regulatory issues in relation to CCK1R activating peptides

An example of a claim on a food product e.g. a yoghurt containing satiety-inducing CCK1R activating peptides could be the following:

"Soy peptides help to control excessive food intake".

It is clear that it is beneficial to constrain excessive food intake. To have this claim approved by EFSA, it is important to "sufficiently" define and characterize the active components. It is not clear however when a characterization is considered as sufficient. In the active soy protein hydrolysate fractions, a lot of peptides have been characterized and amino acid composition can be determined, but it is not clear whether it is necessary to identify the sequence of the active peptide.

Some examples for antihypertensive peptides and protein hydrolysates illustrating the extent of characterization exist. Lactotripeptides IPP and VVP (Evolus, Valio, Finland and AmealPeptide, Calpis Co., Japan) (*134*) are the subject of the health claim itself and thus are fully identified. However, dried bonito (*117*) and C12 peption (DMV, The Netherlands) (*277*) are protein hydrolysates of which only a certain amount of peptides have been identified. The main activity was attributed to the identified peptides and so EFSA considered these products as sufficiently characterized (*120*). From this it can be concluded that to get a claim approved, it is most likely that all the peptides contributing to the most activity will have to be identified.

Furthermore, a human-intervention study on overweight and/or obese people showing that continuous consumption of the food constituent causes a persistent reduced sense of hunger and/or reduced food intake is needed as described in paragraph 6.3.4.3. It seems appropriate to perform the study on overweight and/or obese subjects, however, the NDA still needs to decide whether extrapolation is possible to the (general) healthy population. Biological markers such as CCK blood concentration can be measured to support explanation of behavioral changes.

Also the safety of the protein hydrolysates will have to be assessed. A lot of data about the safety of protein hydrolysates exists, because they have been widely studied and used as a replacement for food protein causing allergy (*124*). Protein hydrolysates have also a wide history of use in patients with digestive disturbances and in sports nutrition (*172, 242*). Partially and extensively hydrolyzed proteins from a good quality source (e.g. soy, egg and milk) have a long history of safe use and no unacceptable side effects have been reported (*242*). Following Schaafsma (2009) (*242*), based on the safe replacement of protein by protein hydrolysates, protein hydrolysate intake levels are safe up to 126g/day. Concerning the amino acid uptake, an upper limit of 2.9% of methionine in the protein hydrolysates has been set, as too much methionine can cause undesirably high plasma levels of homocysteine, which might lead to hyperhomocysteinemia (*30, 239*). This 'average' content is comparable to the amount of methionine present in a 2:1 casein:soy mixture (*239*). Proteolytic enzymes (such as pepsin, trypsin and so on) are allowed in food and even do not need to be labeled

when they are considered as processing aids (*46*). So depending on the criteria described above, the isolated soy protein fractions would not fall under the novel foods legislation.

6.4.3 Social relevance of satiety-enhancing food products

Approximately 68% of the US population is overweight and more than 33% of the population suffers from obesity. In Europe, 32% of the population is overweight and 17% is obese (*100*). The average BMI value for adults in Flanders is 24.9 and lies on the border of overweight, 43% of the Flemish population is overweight and 11% is obese (*19*). The revenues of the European market for drugs against obesity totaled $ 668.1 million in 2008, these revenues are predicted to reach $1158,1 million in the year 2015. Moreover, the market for anti-obesity products is still growing, as the prevalence of obesity keeps on rising (*86*). Consumers are increasingly concerned about their health and are willing to pay extra for food with a health benefit. The sales of functional foods accounted for $ 26.4 million in 2005 (*17*). However, consumer acceptance of functional foods cannot be taken for granted and the trustworthiness of the health claim, taste and price of the product play an important role (*249, 285*). There's also variation in the consumer acceptance based on the type of food and the geographic context, so to assess the marketing possibilities of functional foods, these should be studied as being separate products within various food categories rather than as a homogenous group (*249*). Currently, the benefits of satiety-enhancing products for consumers remain under researched (*106*).

People who are on a diet experience malignancies such as preoccupation with eating and food, poor concentration, increased emotionality, irritability, dysphoria and deprivation. These factors might undermine diet compliance. A vast majority of food products having altered macronutrient composition, food structure, sensory impact or containing functional ingredients have proven their effect on short-term appetite regulation. However the sustainability of these effects to contribute to weight management remains a critical issue. This is also the case for functional foods increasing satiety. They might be a useful stepping stone for people to persist in their diet, but it is not clear what consumers expect from satiety-enhancing products. For example, the management of appetite in itself without a focus on weight management can be legitimate for consumers who find it hard to resist to the temptation of unhealthy food. Other consumers expect weight control from satiety-enhancing products, as it is assumed that changes in appetite result in long-term benefits for weight management (*106*).Nonetheless the clear demand for products helping consumers to manage their body weight (*106*), more efforts should be made in communicating that to successfully obtain and maintain a healthy weight, a healthy lifestyle and a balanced diet are necessary. Especially regular physical activity and a high intake of fibre are the main protective factors against obesity (*267*).

References

1. Adams, J. B.; Pyke, R. E.; Costa, J., et al., A double-blind, placebo-controlled study of a CCK-B receptor antagonist, CI-988, in patients with generalized anxiety disorder. *Journal of Clinical Psychopharmacology* **1995,** 15, (6), 428-434.

2. Allen, J. A.; Roth, B. L., Strategies to discover unexpected targets for drugs active at G protein-coupled receptors. *Annual Review of Pharmacology and Toxicology* **2011,** 51:, 117-44.

3. Allen, S. J.; Ribeiro, S.; Horuk, R.; Handel, T. M., Expression, purification and in vitro functional reconstitution of the chemokine receptor CCR1. *Protein Expression and Purification* **2009,** 66, (1), 73-81.

4. Antunes, F.; Andrade, F.; Ferreira, D.; Nielsen, H. M.; Sarmento, B., Models to predict intestinal absorption of therapeutic peptides and proteins. *Current Drug Metabolism* **2013,** 14, (1), 4-20.

5. Archer-Lahlou, E.; Escrieut, C.; Clerc, P., et al., Molecular mechanism underlying partial and full agonism mediated by the human cholecystokinin-1 receptor. *Journal of Biological Chemistry* **2005,** 280, (11), 10664-10674.

6. Archer-Lahlou, E.; Maigret, B.; Escrieut, C.; Pradayrol, L.; Fourmy, D., Rhodopsin crystal: new template yielding realistic models of G-protein-coupled receptors? *Trends in Pharmacological Sciences* **2003,** 24, (1), 36-40.

7. Archer-Lahlou, E.; Tikhonova, I.; Escrieut, C., et al., Modeled structure of a G-protein-coupled receptor: The cholecystokinin-1 receptor. *Journal of Medicinal Chemistry* **2005,** 48, (1), 180-191.

8. Arlander, S. J. H.; Dong, M. Q.; Ding, X. Q.; Pinon, D. I.; Miller, L. J., Key differences in molecular complexes of the cholecystokinin receptor with structurally related peptide agonist, partial agonist, and antagonist. *Molecular Pharmacology* **2004,** 66, (3), 545-552.

9. Asakawa, A.; Inui, A.; Kaga, T., et al., Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* **2003,** 52, (7), 947-952.

10. Astrup, A.; Rossner, S.; Van Goal, L., et al., Effects of liraglutide in the treatment of obesity: a randomised, double-blind, placebo-controlled study. *Lancet* **2009,** 374, (9701), 1606-1616.

11. Attwood, T. K.; Findlay, J. B. C., Fingerprinting G-protein coupled receptors. *Protein Engineering* **1994,** 7, (2), 195-203.

12. Aungst, B. J., Intestinal permeation enhancers. *Journal of Pharmaceutical Sciences* **2000,** 89, (4), 429-442.

13. Badman, M. K.; Flier, J. S., The gut and energy balance: Visceral allies in the obesity wars. *Science* **2005,** 307, (5717), 1909-1914.

14. Baggerman, G.; Cerstiaens, A.; De Loof, A.; Schoofs, L., Peptidomics of the larval *Drosophila melanogaster* central nervous system. *Journal of Biological Chemistry* **2002,** 277, (43), 40368-40374.

15. Baldwin, G. S.; Shulkes, A., CCK receptors and cancer. *Current Topics in Medicinal Chemistry* **2007,** 7, (12), 1232-1238.

16. Banerjee, S.; Huber, T.; Sakmar, T. P., Rapid incorporation of functional rhodopsin into nanoscale apolipoprotein bound bilayer (NABB) particles. *Journal of Molecular Biology* **2008,** 377, (4), 1067-1081.

17. Barton, C. L., Next generation nutraceuticals. *Global Business Insight* **2006**.

18. Batterham, R. L.; Cowley, M. A.; Small, C. J., et al., Gut hormone PYY3-36 physiologically inhibits food intake. *Nature* **2002,** 418, (6898), 650-654.

19. Bayingana, K.; Demarest, S.; Gisle, L., et al., Gezondheidsenquête door middel van Interview. Afdeling Epidemiologie. Wetenschappelijk Instituut Volkgsgezondheid. **2004**.

20. Berna, M. J.; Jensen, R. T., Role of CCK/gastrin receptors in gastrointestinal/metabolic diseases and results of human studies using gastrin/CCK receptor agonists/antagonists in these diseases. *Current Topics in Medicinal Chemistry* **2007,** 7, (12), 1211-1231.

21. Berna, M. J.; Tapia, J. A.; Sanch, V.; Jensen, R. T., Progress in developing cholecystokinin (CCK)/gastrin receptor ligands that have therapeutic potential. *Current Opinion in Pharmacology* **2007,** 7, (6), 583-592.

22. Berthoud, H.-R., The vagus nerve, food intake and obesity. *Regulatory Peptides* **2008,** 149, (1-3), 15-25.

23. Besanger, T. R.; Brennan, J. D., Entrapment of membrane proteins in sol-gel derived silica. *Journal of Sol-Gel Science and Technology* **2006,** 40, (2-3), 209-225.

24. Bignon, E.; Alonso, R.; Arnone, M., et al., SR146131: A new potent, orally active, and selective nonpeptide cholecystokinin subtype 1 receptor agonist. II: In vivo pharmacological characterization. *Journal of Pharmacology and Experimental Therapeutics* **1999,** 289, (2), 752-761.

25. Bignon, E.; Bachy, A.; Boigegrain, R., et al., SR146131: A new potent, orally active, and selective nonpeptide cholecystokinin subtype 1 receptor agonist. I: In vitro studies. *Journal of Pharmacology and Experimental Therapeutics* **1999,** 289, (2), 742-751.

26. Bishop, L. A.; Gerskowitch, V. P.; Hull, R. A. D.; Shankley, N. P.; Black, J. W., Combined dose-ratio analysis of cholecystokinin receptor antagonists, devazepide, lorglumide and loxiglumide in the guinea-pig gall-bladder. *British Journal of Pharmacology* **1992,** 106, (1), 61-66.

27. Blenau, W.; Baumann, A., Molecular and pharmacological properties of insect biogenic amine receptors: Lessons from *Drosophila melanogaster* and *Apis mellifera*. *Archives of Insect Biochemistry and Physiology* **2001,** 48, (1), 13-38.

28. Blundell, J.; de Graaf, C.; Hulshof, T., et al., Appetite control: methodological aspects of the evaluation of foods. *Obesity Reviews* **2010,** 11, (3), 251-270.

29. Borch, J.; Roepstorff, P.; Moller-Jensen, J., Nanodisc-based Co-immunoprecipitation for Mass Spectrometric Identification of Membrane-interacting Proteins. *Molecular & Cellular Proteomics* **2011,** 10, (7).

30. Bos, G. M. J.; Den Heijer, M., Hyperhomocysteinemia and venous thrombosis. *Seminars in Thrombosis and Hemostasis* **1998,** 24, (4), 387-391.

31. Bougatef, A.; Ravallec, R.; Nedjar-Arroume, N., et al., Evidence of in vivo satietogen effect and control of food intake of smooth hound (Mustelus mustelus) muscle protein hydrolysate in rats. *Journal of Functional Foods* **2010,** 2, (1), 10-16.

32. Bradford, M. M., Rapid and sensitive method for quantitation of microgram quantitites of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* **1976,** 72, (1-2), 248-254.

33. Bradwejn, J.; Koszycki, D., Cholecystokinin and panic disorder: Past and future clinical research strategies. *Scandinavian Journal of Clinical & Laboratory Investigation* **2001,** 61:, 19-27.

34. Brandon, D. L.; Friedman, M., Immunoassays of soy proteins. *Journal of Agricultural and Food Chemistry* **2002,** 50, (22), 6635-6642.

35. Brody, T.; Cravchik, A., *Drosophila melanogaster* G protein-coupled receptors. *Journal of Cell Biology* **2000,** 150, (2), F83-F88.

36. Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D., et al., CHARMM - A Program for macromolecular energy, minimization, and dynamics calculations. *Journal of Computational Chemistry* **1983,** 4, (2), 187-217.

37. Buchwald, H., Bariatric surgery for morbid obesity: Health implications for patients, health professionals and third-party payers. *Journal of the American College of Surgeons* **2005,** 200, (4), 593-604.

38. Burke, L. E.; Wang, J., Treatment strategies for overweight and obesity. *Journal of Nursing Scholarship* **2011,** 43, (4), 368-375.

39. Burlingame, B.; Mouille, B.; Charrondiere, R., Nutrients, bioactive non-nutrients and anti-nutrients in potatoes. *Journal of Food Composition and Analysis* **2009,** 22, (6), 494-502.

40. Cabanillas, B.; Pedrosa, M. M.; Rodriguez, J., et al., Influence of Enzymatic Hydrolysis on the Allergenicity of Roasted Peanut Protein Extract. *International Archives of Allergy and Immunology* **2012,** 157, (1), 41-50.

41. Castillo, E. J.; Delgado-Aros, S.; Camilleri, M., et al., Effect of oral CCK-1 agonist GI181771X on fasting and postprandial gastric functions in healthy volunteers. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2004,** 287, (2), G363-G369.

42. Cawston, E. E.; Miller, L. J., Therapeutic potential for novel drugs targeting the type 1 cholecystokinin receptor. *British Journal of Pharmacology* **2010,** 159, (5), 1009-1021.

43. Chau, I.; Cunningham, D.; Russell, C., et al., Gastrazole (JB95008), a novel CCK2/gastrin receptor antagonist, in the treatment of advanced pancreatic cancer: results from two randomised controlled trials. *British Journal of Cancer* **2006,** 94, (8), 1107-1115.

44. Chun, H.; Sasaki, M.; Fujiyama, Y.; Bamba, T., Effect of peptide chain length on absorption and intact transport of hydrolyzed soybean peptide in rat intestinal everted sac. *Journal of Clinical Biochemistry and Nutrition* **1996,** 21, (2), 131-140.

45. Clynen, E.; Schoofs, L., Peptidomic survey of the locust neuroendocrine system. *Insect Biochemistry and Molecular Biology* **2009,** 39, (8), 491-507.

46. Codex Alimintarius Commission, Inventory of processing aids. *Codex Alimentarius* **1992,** 1A, 1-39.

47. Commission of the European Communities, Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 Januari 1997 concerning novel foods and novel food ingredients. *Official Journal of the European Communities* **1997,** L43/1.

48. Considine, R. V.; Sinha, M. K.; Heiman, M. L., et al., Serum immunoreactive leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine* **1996,** 334, (5), 292-295.

49. Cooke, R. M.; Koglin, M.; Errey, J. C.; Marshall, F. H., Preparation of purified GPCRs for structural studies. *Biochemical Society Transactions* **2013,** 41, 185-190.

50. Cordier-Bussat, M.; Bernard, C.; Haouche, S., et al., Peptones stimulate cholecystokinin secretion and gene transcription in the intestinal cell line STC-1. *Endocrinology* **1997,** 138, (3), 1137-1144.

51. Cudennec, B.; Ravallec-Ple, R.; Courois, E.; Fouchereau-Peron, M., Peptides from fish and crustacean by-products hydrolysates stimulate cholecystokinin release in STC-1 cells. *Food Chemistry* **2008,** 111, (4), 970-975.

52. de Graaf, C.; Blom, W. A. M.; Smeets, P. A. M.; Stafleu, A.; Hendriks, H. F. J., Biomarkers of satiation and satiety. *American Journal of Clinical Nutrition* **2004,** 79, (6), 946-961.

53. De Silva, A.; Bloom, S. R., Gut hormones and appetite control: A focus on PYY and GLP-1 as therapeutic targets in obesity. *Gut and Liver* **2012,** 6, (1), 10-20.

54. de Tullio, P.; Delarge, J.; Pirotte, B., Therapeutic and chemical developments of cholecystokinin receptor ligands. *Expert Opinion on Investigational Drugs* **2000,** 9, (1), 129-146.

55. Degen, L.; Oesch, S.; Casanova, M., et al., Effect of peptide YY3-36 on food intake in humans. *Gastroenterology* **2005,** 129, (5), 1430-1436.

56. Delzenne, N.; Blundell, J.; Brouns, F., et al., Gastrointestinal targets of appetite regulation in humans. *Obesity Reviews* **2010,** 11, (3), 234-250.

57. Dickenson, J. M.; Hill, S. J., Transfected adenosine A(1) receptor-mediated modulation of thrombin-stimulated phospholipase C and phospholipase A(2) activity in CHO cells. *European Journal of Pharmacology* **1997,** 321, (1), 77-86.

58. Dickinson, P. S.; Stevens, J. S.; Rus, S., et al., Identification and cardiotropic actions of sulfakinin peptides in the American lobster *Homarus americanus*. *Journal of Experimental Biology* **2007,** 210, (13), 2278-2289.

59. Diepvens, K.; Haeberer, D.; Westerterp-Plantenga, M., Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. *International Journal of Obesity* **2008,** 32, (3), 510-518.

60. Ding, X. Q.; Dolu, V.; Hadac, E. M., et al., Refinement of the structure of the ligand-occupied cholecystokinin receptor using a photolabile amino-terminal probe. *Journal of Biological Chemistry* **2001,** 276, (6), 4236-4244.

61. Ding, X. Q.; Dolu, V.; Hadac, E. M.; Schuetz, M.; Miller, L. J., Disulfide bond structure and accessibility of cysteines in the ectodomain of the cholecystokinin receptor: Specific mono-reactive receptor constructs examine charge-sensitivity of loop regions. *Receptors & Channels* **2003,** 9, (2), 83-91.

62. Divirgilio, F.; Steinberg, T. H.; Silverstein, S. C., Inhibition of Fura-2 sequestration and secretion with organic anion transport blockers. *Cell Calcium* **1990,** 11, (2-3), 57-&.

63. Dong, M. Q.; Hadac, E. M.; Pinon, D. I.; Miller, L. J., Differential spatial approximation between cholecystokinin residue 30 and receptor residues in active and inactive conformations. *Molecular Pharmacology* **2005,** 67, (6), 1892-1900.

64. Dong, M. Q.; Lam, P. C. H.; Pinon, D. I.; Abagyan, R.; Miller, L. J., Elucidation of the Molecular Basis of Cholecystokinin Peptide Docking to Its Receptor Using Site-Specific Intrinsic Photoaffinity Labeling and Molecular Modeling. *Biochemistry* **2009,** 48, (23), 5303-5312.

65. Downer, K. E.; Haselton, A. T.; Nachman, R. J.; Stoffolano, J. G., 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* **2007,** 53, (1), 106-112.

66. Doyon, M.; Labrecque, J., Functional foods: a conceptual definition. *British Food Journal* **2008,** 110, (10-11), 1133-1149.

67. Druce, M. R.; Minnion, J. S.; Field, B. C. T., et al., Investigation of Structure-Activity Relationships of Oxyntomodulin (Oxm) Using Oxm Analogs. *Endocrinology* **2009,** 150, (4), 1712-1721.

68. Dufresne, M.; Escrieut, C.; Clerc, P., et al., Molecular cloning, developmental expression and pharmacological characterization of the CCKB gastrin receptor in the calf pancreas. *European Journal of Pharmacology* **1996,** 297, (1-2), 165-179.

69. Dufresne, M.; Seva, C.; Fourmy, D., Cholecystokinin and gastrin receptors. *Physiological Reviews* **2006,** 86, (3), 805-847.

70. Dulloo, A. G., Ephedrine, xanthines and prostaglandin-inhibitors - actions and interactions in the stimulation of thermogenesis. *International Journal of Obesity* **1993,** 17, S35-S40.

71. Dunlop, J.; Zhang, Y. X.; Evans, N., Full and partial agonist activity of c-terminal cholecystokinin peptides at the cloned human CCK-A receptor expressed in Chinese hamster ovary cells. *Peptides* **1997,** 18, (6), 865-868.

72. Duve, H.; Thorpe, A.; Scott, A. G., et al., The sulfakinins of the blowfly *Calliphora vomitoria* - peptide isolation, gene cloning and expression studies. *European Journal of Biochemistry* **1995,** 232, (2), 633-640.

73. East, P. D.; Hales, D. F.; Cooper, P. D., 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 & Cell* **1997,** 29, (3), 347-354.

74. EFSA, Guidance on the scientific requirements for health claims related to appetite ratings, weight managment, and blood glucose concentrations. *EFSA Journal* **2012,** 10, (3), 2604.

75. EMEA, European public assessment report (EPAR) Acomplia. Epar summary for the public. EMEA/H/C/666. **2007**.

76. EMEA, Questions and answers on the suspension of medicines containing sibutramine. EMEA/H/A-107/1256 **2010**.

77. Escrieut, C.; Gigoux, V.; Archer, E., et al., The biologically crucial C terminus of cholecystokinin and the non-peptide agonist SR-146,131 share a common binding site in the human CCK1 receptor - Evidence for a crucial role of Met-121 in the activation process. *Journal of Biological Chemistry* **2002,** 277, (9), 7546-7555.

78. European Commission, Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. *Official Journal of the European Union* **2006,** L404, 9-25.

79. Eysselein, V. E.; Eberlein, G. A.; Hesse, W. H., et al., Molecular variants of cholecystokinin after endogenous stimulation in humans - A time study. *American Journal of Physiology* **1990,** 258, (6), 951-957.

80. Farooqi, I. S.; Matarese, G.; Lord, G. M., et al., Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *Journal of Clinical Investigation* **2002,** 110, (8), 1093-1103.

81. FDA, FDA Drug Safety Communication: FDA Recommends Against the Continued Use of Meridia (sibutramine). **2010,** http://www.fda.gov/Drugs/DrugSafety/ucm228746.htm (as consulted on 8 Feb 2013)

82. Foltz, M.; Ansems, P.; Schwarz, J., et al., Protein hydrolysates induce CCK release from enteroendocrine cells and act as partial agonists of the CCK1 receptor. *Journal of Agricultural and Food Chemistry* **2008,** 56, (3), 837-843.

83. Foord, S. M.; Bonner, T. I.; Neubig, R. R., et al., International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacological Reviews* **2005,** 57, (2), 279-288.

84. Foucaud, M.; Archer-Lahlou, E.; Marco, E., et al., Insights into the binding and activation sites of the receptors for cholecystokinin and gastrin. *Regulatory Peptides* **2008,** 145, (1-3), 17-23.

85. Fredriksson, R.; Lagerstrom, M. C.; Lundin, L. G.; Schioth, H. B., The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular Pharmacology* **2003,** 63, (6), 1256-1272.

86. Frost; Sullivan, European Lifestyle Disorders Therapeutics Market, M3F8-52. **2009**.

87. Galas, M. C.; Lignon, M. F.; Rodriguez, M., et al., Structure-activity relationship studies on cholecystokinin - analogs with partial agonist activity. *American Journal of Physiology* **1988,** 254, (2), G176-G182.

88. Gard, A. L.; Lenz, P. H.; Shaw, J. R.; Christie, A. E., Identification of putative peptide paracrines/hormones in the water flea *Daphnia pulex* (Crustacea; Branchiopoda; Cladocera) using transcriptomics and immunohistochemistry. *General and Comparative Endocrinology* **2009,** 160, (3), 271-287.

89. Gaudreau, P.; Lavigne, G. J.; Quirion, R., Cholecystokinin antagonists proglumide, lorglumide and benzotript, but not L-364,718, interact with brain opioid binding-sites. *Neuropeptides* **1990,** 16, (1), 51-55.

90. Geraedts, M. C. P.; Troost, F. J.; Fischer, M.; Edens, L.; Saris, W. H. M., Direct induction of CCK and GLP-1 release from murine endocrine cells by intact dietary proteins. *Molecular Nutrition & Food Research* **2011,** 55, (3), 476-484.

91. Ghesquiere, B.; Colaert, N.; Helsens, K., et al., *In vitro* and *in vivo* protein-bound tyrosine nitration characterized by diagonal chromatography. *Molecular & Cellular Proteomics* **2009,** 8, (12), 2642-2652.

92. Gibbs, B. F.; Zougman, A.; Masse, R.; Mulligan, C., Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Research International* **2004,** 37, (2), 123-131.

93. Gigoux, V.; Escrieut, C.; Fehrentz, J. A., et al., Arginine 336 and asparagine 333 of the human cholecystokinin-A receptor binding site interact with the penultimate aspartic acid and the C-terminal amide of cholecystokinin. *Journal of Biological Chemistry* **1999,** 274, (29), 20457-20464.

94. Gigoux, V.; Escrieut, C.; Silvente-Poirot, S., et al., 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* **1998,** 273, (23), 14380-14386.

95. Gigoux, V.; Maigret, B.; Escrieut, C., et al., Arginine 197 of the cholecystokinin-A receptor binding site interacts with the sulfate of the peptide agonist cholecystokinin. *Protein Science* **1999,** 8, (11), 2347-2354.

96. Gilsenan, M. B., Nutrition & health claims in the European Union: A regulatory overview. *Trends in Food Science & Technology* **2011,** 22, (10), 536-542.

97. Giragossian, C.; Mierke, D. F., Intermolecular interactions between cholecystokinin-8 and the third extracellular loop of the cholecystokinin A receptor. *Biochemistry* **2001,** 40, (13), 3804-3809.

98. Giragossian, C.; Mierke, D. F., Intermolecular interactions between cholecystokinin-8 and the third extracellular loop of the cholecystokinin-2 receptor. *Biochemistry* **2002,** 41, (14), 4560-4566.

99. Giragossian, C.; Mierke, D. F., Determination of ligand-receptor interactions of cholecystokinin by nuclear magnetic resonance. *Life Sciences* **2003,** 73, (6), 705-713.

100. Global_Business_Insights, Obesity drug market in de US & EU. **2008**.

101. Gotthardt, M.; Behe, P.; Beuter, D., et al., Improved tumour detection by gastrin receptor scintigraphy in patients with metastasised medullary thyroid carcinoma. *European Journal of Nuclear Medicine and Molecular Imaging* **2006,** 33, (11), 1273-1279.

102. Guyenet, S. J.; Schwartz, M. W., Regulation of food Intake, energy balance, and body fat mass: Implications for the pathogenesis and treatment of obesity. *Journal of Clinical Endocrinology & Metabolism* **2012,** 97, (3), 745-755.

103. Haber, E. P.; Procopio, J.; Carvalho, C. R. O., et al., New insights into fatty acid modulation of pancreatic beta-cell function. In *International Review of Cytology - a Survey of Cell Biology, Vol 248*, Jeon, K. W., Ed. Elsevier Academic Press Inc: San Diego, **2006**; Vol. 248, pp 1-+.

104. Hadac, E. M.; Ji, Z. S.; Pinon, D. I., et al., A peptide agonist acts by occupation of a monomeric G protein-coupled receptor: Dual sites of covalent attachment to domains near TM1 and TM7 of the same molecule make biologically significant domain-swapped dimerization unlikely. *Journal of Medicinal Chemistry* **1999,** 42, (12), 2105-2111.

105. Hadac, E. M.; Pinon, D. I.; Ji, Z. S., et al., Direct identification of a second distinct site of contact between cholecystokinin and its receptor. *Journal of Biological Chemistry* **1998,** 273, (21), 12988-12993.

106. Halford, J. C. G.; Harrold, J. A., Satiety-enhancing products for appetite control: science and regulation of functional foods for weight management. *Proceedings of the Nutrition Society* **2012,** 71, (2), 350-362.

107. Hamm, H. E., The many faces of G protein signaling. *Journal of Biological Chemistry* **1998,** 273, (2), 669-672.

108. Han, L. K.; Takaku, T.; Li, J.; Kimura, Y.; Okuda, H., Anti-obesity action of oolong tea. *International Journal of Obesity* **1999,** 23, (1), 98-105.

109. Hand, K. V.; Bruen, C. M.; O'Halloran, F.; Giblin, L.; Green, B. D., Acute and chronic effects of dietary fatty acids on cholecystokinin expression, storage and secretion in enteroendocrine STC-1 cells. *Molecular Nutrition & Food Research* **2010,** 54, S93-S103.

110. Hargrave, K. M.; Li, C. L.; Meyer, B. J., et al., Adipose depletion and apoptosis induced by trans-10, cis-12 conjugated linoleic acid in mice. *Obesity Research* **2002,** 10, (12), 1284-1290.

111. Harikumar, K. G.; Clain, J.; Pinon, D. I.; Dong, M. Q.; Miller, L. J., Distinct molecular mechanisms for agonist peptide binding to types A and B cholecystokinin receptors demonstrated using fluorescence spectroscopy. *Journal of Biological Chemistry* **2005,** 280, (2), 1044-1050.

112. Harikumar, K. G.; Gao, F.; Pinon, D. I.; Miller, L. J., Use of multidimensional fluorescence resonance energy transfer to establish the orientation of cholecystokinin docked at the type A cholecystokinin receptor. *Biochemistry* **2008,** 47, (36), 9574-9581.

113. Harikumar, K. G.; Pinon, D. I.; Miller, L. J., Fluorescence characteristics of hydrophobic partial agonist probes of the cholecystokinin receptor. *Bioscience Reports* **2006,** 26, (2), 89-100.

114. Harikumar, K. G.; Pinon, D. I.; Wessels, W. S., et al., Measurement of intermolecular distances for the natural agonist peptide docked at the cholecystokinin receptor expressed in situ using fluorescence resonance energy transfer. *Molecular Pharmacology* **2004,** 65, (1), 28-35.

115. Harikumar, K. G.; Pinon, D. L.; Wessels, W. S.; Prendergast, F. G.; Miller, L. J., Environment and mobility of a series of fluorescent reporters at the amino terminus of structurally related peptide agonists and antagonists bound to the cholecystokinin receptor. *Journal of Biological Chemistry* **2002,** 277, (21), 18552-18560.

116. Harshini, S.; Nachman, R. J.; Sreekumar, S., In vitro release of digestive enzymes by FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina arenosella* (Walk.). *Peptides* **2002,** 23, (10), 1759-1763.

117. Hartmann, R.; Meisel, H., Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology* **2007,** 18, (2), 163-169.

118. Helsens, K.; Colaert, N.; Barsnes, H., et al., ms_lims, a simple yet powerful open source laboratory information management system for MS-driven proteomics. *Proteomics* **2010,** 10, (6), 1261-1264.

119. Herranz, R., Cholecystokinin antagonists: Pharmacological and therapeutic potential. *Medicinal Research Reviews* **2003,** 23, (5), 559-605.

120. Herregods, G. Activity and purification of ACE inhibitory peptides from animal by-products: *in vitro* and *in vivo* evaluation. PhD thesis, Ghent University, Ghent, Belgium, **2011**.

121. Hewes, R. S.; Taghert, P. H., Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Research* **2001,** 11, (6), 1126-1142.

122. Hira, T.; Mori, N.; Nakamori, T., et al., Acute effect of soybean beta-conglycinin hydrolysate ingestion on appetite sensations in healthy humans. *Appetite* **2012,** 57, (3), 765-768.

123. Holt, S. H. A.; Miller, J. C. B.; Petocz, P.; Farmakalidis, E., A satiety index of common foods. *European Journal of Clinical Nutrition* **1995,** 49, (9), 675-690.

124. Host, A.; Halken, S., Hypoallergenic formulas - when, to whom and how long: after more than 15 years we know the right indication! *Allergy* **2004,** 59, 45-52.

125. Hsu, C.-L.; Yen, G.-C., Induction of cell apoptosis in 3T3-L1 pre-adipocytes by flavonoids is associated with their antioxidant activity. *Molecular Nutrition & Food Research* **2006,** 50, (11), 1072-1079.

126. Huang, S. C.; Yu, D. H.; Wank, S. A., et al., Importance of sulfation of gastrin or cholecystokinin (CCK) on affinity for gastrin and CCK receptors. *Peptides* **1989,** 10, (4), 785-789.

127. Huber, T.; Sakmar, T. P., Escaping the flatlands: new approaches for studying the dynamic assembly and activation of GPCR signaling complexes. *Trends in Pharmacological Sciences* **2011,** 32, (7), 410-419.

128. Huda, M. S. B.; Wilding, J. P. H.; Pinkney, J. H., Gut peptides and the regulation of appetite. *Obesity Reviews* **2006,** 7, (2), 163-182.

129. Huppi, K.; Siwarski, D.; Pisegna, J. R.; Wank, S., Chromosomal localization of the gastric and brain receptors for cholecystokinin (CCKAR and CCKBR) in human and mouse. *Genomics* **1995,** 25, (3), 727-729.

130. Husson, S. J.; Lindemans, M.; Janssen, T.; Schoofs, L., Comparison of *Caenorhabditis elegans* NLP peptides with arthropod neuropeptides. *Trends in Parasitology* **2009,** 25, (4), 171-181.

131. Hwang, J. T.; Park, I. J.; Shin, J. I., et al., Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. *Biochemical and Biophysical Research Communications* **2005,** 338, (2), 694-699.

132. Imeryuz, N.; Yegen, B. C.; Bozkurt, A., et al., Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **1997,** 273, (4), G920-G927.

133. Janssen, T.; Meelkop, E.; Lindemans, M., et al., Discovery of a cholecystokinin-gastrin-like signaling system in nematodes. *Endocrinology* **2008,** 149, (6), 2826-2839.

134. Jauhiainen, T.; Vapaatalo, H.; Poussa, T., et al., Lactobacillus helveticus fermented milk lowers blood pressure in hypertensive subjects in 24-h ambulatory blood pressure measurement. *American Journal of Hypertension* **2005,** 18, (12), 1600-1605.

135. Ji, B.; Bi, Y.; Simeone, D.; Mortensen, R. M.; Logsdon, C. D., Human pancreatic acinar cells lack functional responses to cholecystokinin and gastrin. *Gastroenterology* **2001,** 121, (6), 1380-1390.

136. Ji, Z. S.; Hadac, E. M.; Henne, R. M., et al., Direct identification of a distinct site of interaction between the carboxyl-terminal residue of cholecystokinin and the type A cholecystokinin receptor using photoaffinity labeling. *Journal of Biological Chemistry* **1997,** 272, (39), 24393-24401.

137. Johnsen, A. H.; Duve, H.; Davey, M.; Hall, M.; Thorpe, A., Sulfakinin neuropeptides in a crustacean - isolation, identification and tissue localization in the tiger prawn *Penaeus monodon*. *European Journal of Biochemistry* **2000,** 267, (4), 1153-1160.

138. Jordan, J.; Greenway, F. L.; Leiter, L. A., et al., Stimulation of cholecystokinin-A receptors with GI181771X does not cause weight loss in overweight or obese patients. *Clinical Pharmacology & Therapeutics* **2008,** 83, (2), 281-287.

139. Kakade, M. L.; Rackis, J. J.; McGhee, J. E.; Puski, G., Determination of trypsin-inhibitor activity of soy products - collaborative analysis of an improved procedure. *Cereal Chemistry* **1974,** 51, (3), 376-382.

140. Katsuura, G.; Asakawa, A.; Inui, A., Roles of pancreatic polypeptide in regulation of food intake. *Peptides* **2002,** 23, (2), 323-329.

141. Kawada, T.; Sekiguchi, T.; Sakai, T.; Aoyama, M.; Satake, H., Neuropeptides, Hormone Peptides, and Their Receptors in Ciona intestinalis: An Update. *Zoological Science* **2010,** 27, (2), 134-153.

142. Kennedy, K.; Gigoux, V.; Escrieut, C., et al., Identification of two amine acids of the human cholecystokinin-A receptor that interact with the N-terminal moiety of cholecystokinin. *Journal of Biological Chemistry* **1997,** 272, (5), 2920-2926.

143. Kiselyov, K.; Shin, D. M.; Muallem, S., Signalling specificity in GPCR-dependent Ca^{2+} signalling. *Cellular Signalling* **2003,** 15, (3), 243-253.

144. Kissileff, H. R.; Pisunyer, F. X.; Thornton, J.; Smith, G. P., C-terminal octapeptide of cholecystokinin decreases food-intake in man. *American Journal of Clinical Nutrition* **1981,** 34, (2), 154-160.

145. Kitts, D. D.; Weiler, K., Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design* **2003,** 9, (16), 1309-1323.

146. Knepp, A. M.; Grunbeck, A.; Banerjee, S.; Sakmar, T. P.; Huber, T., Direct Measurement of Thermal Stability of Expressed CCR5 and Stabilization by Small Molecule Ligands. *Biochemistry* **2012,** 50, (4), 502-511.

147. Köhnke, R.; Lindbo, A.; Larsson, T., et al., Thylakoids promote release of the satiety hormone cholecystokinin while reducing insulin in healthy humans. *Scandinavian Journal of Gastroenterology* **2009,** 44, (6), 712-719.

148. Kolakowski, L. F., GCRDb - A G-protein-coupled receptor database. *Receptors & Channels* **1994,** 2, (1), 1-7.

149. Komarnytsky, S.; Cook, A.; Raskin, I., Potato protease inhibitors inhibit food intake and increase circulating cholecystokinin levels by a trypsin-dependent mechanism. *International Journal of Obesity* **2011,** 35, (2), 236-243.

150. Korbonits, M.; Trainer, P. J.; Little, J. A., et al., Leptin levels do not change acutely with food administration in normal or obese subjects, but are negatively correlated with pituitary-adrenal activity. *Clinical Endocrinology* **1997,** 46, (6), 751-757.

151. Korhonen, H.; Pihlanto, A., Food-derived bioactive peptides - Opportunities for designing future foods. *Current Pharmaceutical Design* **2003,** 9, (16), 1297-1308.

152. Kuang, S. S.; Oliveira, J. C.; Crean, A. M., Microencapsulation as a tool for incorporating bioactive ingredients into food. *Critical Reviews in Food Science and Nutrition* **2010,** 50, (10), 951-968.

153. Kubiak, T. M.; Larsen, M. J.; Burton, K. J., et al., Cloning and functional expression of the first *Drosophila melanogaster* sulfakinin receptor DSK-R1. *Biochemical and Biophysical Research Communications* **2002,** 291, (2), 313-320.

154. Lam, S.; Moughan, P. J.; Awati, A.; Morton, H. R., The influence of whey protein and glycomacropeptide on satiety in adult humans. *Physiology & Behavior* **2009,** 96, (1), 162-168.

155. Langin, D., Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome. *Pharmacological Research* **2006,** 53, (6), 482-491.

156. Lankiewicz, S.; Lobitz, N.; Wetzel, C. H. R., et al., Molecular cloning, functional expression, and pharmacological characterization of 5-hydroxytryptamine(3) receptor cDNA and its splice variants from guinea pig. *Molecular Pharmacology* **1998,** 53, (2), 202-212.

157. Lateef, D. M.; Washington, M. C.; Sayegh, A. I., The short term satiety peptide cholecystokinin reduces meal size and prolongs intermeal interval. *Peptides* **2011,** 32, (6), 1289-1295.

158. Lee, Y. M.; Beinborn, M.; McBride, E. W., et al., The human brain cholecystokinin-B/gastrin receptor - cloning and characterization. *Journal of Biological Chemistry* **1993,** 268, (11), 8164-8169.

159. Lefranc, F.; Chaboteaux, C.; Belot, N., et al., Determination of RNA expression for cholecystokinin/gastrin receptors (CCKA,CCKB and CCKC) in human tumors of the central and peripheral nervous system. *International Journal of Oncology* **2003,** 22, (1), 213-219.

160. Li, Y.; Hao, Y. B.; Owyang, C., High-affinity CCK-A receptors on the vagus nerve mediate CCK-stimulated pancreatic secretion in rats. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **1997,** 273, (3), 679-685.

161. Li, Y.; Hao, Y. B.; Owyang, C., Diazepam-binding inhibitor mediates feedback regulation of pancreatic secretion and postprandial release of cholecystokinin. *Journal of Clinical Investigation* **2000,** 105, (3), 351-359.

162. Liddle, R. A., Regulation of cholecystokinin secretion in humans. *Journal of Gastroenterology* **2000,** 35, 181-187.

163. Lin, J. K.; Lin-Shiau, S. Y., Mechanisms of hypolipidemic and anti-obesity effects of tea and tea polyphenols. *Molecular Nutrition & Food Research* **2006,** 50, (2), 211-217.

164. Liu, C.; Wang, H. L.; Cui, Z. M., et al., Optimization of extraction and isolation for 11S and 7S globulins of soybean seed storage protein. *Food Chemistry* **2007,** 102, (4), 1310-1316.

165. Londono, M.; Tellez, L. A.; Gutierrez, R., Physiological low doses of leptin and cholecystokinin induces body weight-loss in juvenile and lean, but not in adult-obese rats. *International Journal of Peptide Research and Therapeutics* **2012,** 18, (1), 77-88.

166. Lovick, T. A., CCK as a modulator of cardiovascular function. *Journal of Chemical Neuroanatomy* **2009,** 38, (3), 176-184.

167. Madsen, L.; Petersen, R. K.; Kristiansen, K., Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **2005,** 1740, (2), 266-286.

168. Maestro, J. L.; Aguilar, R.; Pascual, N., et al., Screening of antifeedant activity in brain extracts led to the identification of sulfakinin as a satiety promoter in the German cockroach - are arthropod sulfakinins homologous to vertebrate gastrins-cholecystokinins? *European Journal of Biochemistry* **2001,** 268, (22), 5824-5830.

169. Makovec, F.; Bani, M.; Chiste, R., et al., Different peripheral and central antagonistic activity of new glutaramic acid-derivatives on satiety induced by cholecystokinin in rats. *Regulatory Peptides* **1986,** 16, (3-4), 281-290.

170. Malgaroli, A.; Milani, D.; Meldolesi, J.; Pozzan, T., Fura-2 measurement of cytosolic free Ca-2+ in monolayers and suspensions of various types of animal-cells. *Journal of Cell Biology* **1987,** 105, (5), 2145-2155.

171. Maljaars, P. W. J.; Peters, H. P. F.; Mela, D. J.; Masclee, A. A. M., Ileal brake: A sensible food target for appetite control. A review. *Physiology & Behavior* **2008,** 95, (3), 271-281.

172. Manninen, A. H., Protein hydrolysates in sports nutrition. *Nutrition & Metabolism* **2009,** 6, 5.

173. Marchal-Victorion, S.; Vionnet, N.; Escrieut, C., et al., Genetic, pharmacological and functional analysis of cholecystokinin-1 and cholecystokinin-2 receptor polymorphism in type 2 diabetes and obese patients. *Pharmacogenetics* **2002,** 12, (1), 23-30.

174. Mather, S. J.; McKenzie, A. J.; Sosabowski, J. K., et al., Selection of radiolabeled gastrin analogs for peptide receptor-targeted radionuclide therapy. *Journal of Nuclear Medicine* **2007,** 48, (4), 615-622.

175. Mayer, J.; Thomas, D. W., Regulation of food intake and obesity. *Science* **1967,** 156, 328-337.

176. McKay, R. M.; McKay, J. P.; Suh, J. M.; Avery, L.; Graff, J. M., Tripeptidyl peptidase II promotes fat formation in a conserved fashion. *Embo Reports* **2007,** 8, (12), 1183-1189.

177. McVeigh, P.; Leech, S.; Marks, N. J.; Geary, T. G.; Maule, A. G., Gene expression and pharmacology of nematode NLP-12 neuropeptides. *International Journal for Parasitology* **2006,** 36, (6), 633-640.

178. Meldal, B. H. M.; Debenham, N. J.; De Ley, P., et al., An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. *Molecular Phylogenetics and Evolution* **2007,** 42, (3), 622-636.

179. Meyering-Vos, M.; Muller, A., RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*. *Journal of Insect Physiology* **2007,** 53, (8), 840-848.

180. Miederer, S. E.; Lindstaedt, H.; Kutz, K.; Mayershofer, R., Efficient treatment of gastric-ulcer with proglumide (milid) in outpatients (double-blind trial). *Acta Hepato-Gastroenterologica* **1979,** 26, (4), 314-318.

181. Miller, L. J.; Gao, F., Structural basis of cholecystokinin receptor binding and regulation. *Pharmacology & Therapeutics* **2008,** 119, (1), 83-95.

182. Minta, A.; Kao, J. P. Y.; Tsien, R. Y., Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem* **1989,** 264:, 8171-8178.

183. Mita, K.; Kasahara, M.; Sasaki, S., et al., The genome sequence of silkworm, *Bombyx mori*. *DNA Research* **2004,** 11, (1), 27-35.

184. Moon, H.-S.; Lee, H.-G.; Choi, Y.-J.; Kim, T.-G.; Cho, C.-S., Proposed mechanisms of (-)-epigallocatechin-3-gallate for anti-obesity. *Chemico-Biological Interactions* **2007,** 167, (2), 85-98.

185. Moran, T. H., Cholecystokinin and satiety: Current perspectives. *Nutrition* **2000,** 16, (10), 858-865.

186. Moran, T. H.; Kinzig, K. P., Gastrointestinal satiety signals - II. Cholecystokinin. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2004,** 286, (2), G183-G188.

187. Morisset, J.; Julien, S.; Laine, J., Localization of cholecystokinin receptor subtypes in the endocrine pancreas. *Journal of Histochemistry & Cytochemistry* **2003,** 51, (11), 1501-1513.

188. Muenzberg, H., Leptin-signaling pathways and leptin resistance. In *Frontiers in Eating and Weight Regulation*, Langhans, W. G. N., Ed. **2010**; Vol. 63, pp 123-132.

189. Nachman, R. J.; Giard, W.; Favrel, P., et al., Insect myosuppressins and sulfakinins stimulate release of the digestive enzyme alpha-amylase in two invertebrates: The scallop *Pecten maximus* and insect *Rhynchophorus ferrugineus*. *Annals of the New York Academy of Sciences* **1997,** 814:, 335-338.

190. Nagpal, R.; Behare, P.; Rana, R., et al., Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food & Function* **2011,** 2, (1), 18-27.

191. Nakai, M.; Fukui, Y.; Asami, S., et al., Inhibitory effects of oolong tea polyphenols on pancreatic lipase in vitro. *Journal of Agricultural and Food Chemistry* **2005,** 53, (11), 4593-4598.

192. Nakajima, S.; Hira, T.; Tsubata, M.; Takagaki, K.; Hara, H., Potato Extract (Potein) Suppresses Food Intake in Rats through Inhibition of Luminal Trypsin Activity and Direct Stimulation of Cholecystokinin Secretion from Enteroendocrine Cells. *Journal of Agricultural and Food Chemistry* **2011,** 59, (17), 9491-9496.

193. Nakamura, H.; Kihara, Y.; Tashiro, M., et al., Defects of cholecystokinin (CCK)-A receptor gene expression and CCK-A receptor-mediated biological functions in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Journal of Gastroenterology* **1998,** 33, (5), 702-709.

194. Nichols, R., Isolation and expression of the *Drosophila* drosulfakinin neural peptide gene-product, DSK-I. *Molecular and Cellular Neuroscience* **1992,** 3, (4), 342-347.

195. Nichols, R., The first nonsulfated sulfakinin activity reported suggests nsDSK acts in gut biology. *Peptides* **2007,** 28, (4), 767-773.

196. Nichols, R.; Bendena, W. G.; Tobe, S. S., Myotropic peptides in *Drosophila melanogaster* and the genes that encode them. *Journal of Neurogenetics* **2002,** 16, (1), 1-28.

197. Nichols, R.; Lim, I. A., Spatial and temporal immunocytochemical analysis of drosulfakinin (Dsk) gene products in the *Drosophila melanogaster* central nervous system. *Cell and Tissue Research* **1996,** 283, (1), 107-116.

198. Nichols, R.; Manoogian, B.; Walling, E.; Mispelon, M., Plasticity in the effects of sulfated and nonsulfated sulfakinin on heart contractions. *Frontiers in Bioscience* **2009,** 14:, 4035-4043.

199. Nichols, R.; Schneuwly, S. A.; Dixon, J. E., Identification and characterization of a *Drosophila* homolog to the vertebrate neuropeptide cholecystokinin. *Journal of Biological Chemistry* **1988,** 263, (25), 12167-12170.

200. Nicolaou, N.; Custovic, A., Molecular diagnosis of peanut and legume allergy. *Current Opinion in Allergy and Clinical Immunology* **2011,** 11, (3), 222-228.

201. Nishi, T.; Hara, H.; Asano, K.; Tomita, F., The soybean beta-conglycinin beta 51-63 fragment suppresses appetite by stimulating cholecystokinin release in rats. *Journal of Nutrition* **2003,** 133, (8), 2537-2542.

202. Nishi, T.; Hara, H.; Tomita, F., Soybean beta-conglycinin peptone suppresses food intake and gastric emptying by increasing plasma cholecystokinin levels in rats. *Journal of Nutrition* **2003,** 133, (2), 352-357.

203. Noble, F.; Wank, S. A.; Crawley, J. N., et al., International union of pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. *Pharmacological Reviews* **1999,** 51, (4), 745-781.

204. Notredame, C.; Higgins, D. G.; Heringa, J., T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology* **2000,** 302, (1), 205-217.

205. Ohkoshi, E.; Miyazaki, H.; Shindo, K., et al., Constituents from the leaves of nelumbo nucifera stimulate lipolysis in the white adipose tissue of mice. *Planta Medica* **2007,** 73, (12), 1255-1259.

206. Okuno, M.; Kajiwara, K.; Imai, S., et al., Perilla oil prevents the excessive growth of visceral adipose tissue in rats by down-regulating adipocyte differentiation. *Journal of Nutrition* **1997,** 127, (9), 1752-1757.

207. Padwal, R. S.; Majumdar, S. R., Drug treatments for obesity: orlistat, sibutramine, and rimonabant. *Lancet* **2007,** 369, (9555), 71-77.

208. Pande, A. C.; Greiner, M.; Adams, J. B.; Lydiard, R. B.; Pierce, M. W., Placebo-controlled trial of the CCK-B antagonist, CI-988, in panic disorder. *Biological Psychiatry* **1999,** 46, (6), 860-862.

209. Parker, B. A.; Sturm, K.; MacIntosh, C., et al., Relation between food intake and visual analogue scale ratings of appetite and other sensations in healthy older and young subjects. *European Journal of Clinical Nutrition* **2004,** 58, (2), 212-218.

210. Pellegrini, M.; Mierke, D. F., Molecular complex of cholecystokinin-8 and N-terminus of the cholecystokinin A receptor by NMR spectroscopy. *Biochemistry* **1999,** 38, (45), 14775-14783.

211. Penas, E.; Prestamo, G.; Polo, F.; Gomez, R., Enzymatic proteolysis, under high pressure of soybean whey: Analysis of peptides and the allergen Gly m 1 in the hydrolysates. *Food Chemistry* **2006,** 99, (3), 569-573.

212. Peter, S. A. S.; D'Amato, M.; Beglinger, C., CCK1 antagonists: Are they ready for clinical use? *Digestive Diseases* **2006,** 24, (1-2), 70-82.

213. Philip, G. K.; Creevey, C. J.; McInerney, J. O., The Opisthokonta and the Ecdysozoa may not be clades: Stronger support for the grouping of plant and animal than for animal and fungi and stronger support for the Coelomata than Ecdysozoa. *Molecular Biology and Evolution* **2005,** 22, (5), 1175-1184.

214. Picot, L.; Ravallec, R.; Fouchereau-Peron, M., et al., Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture* **2010,** 90, (11), 1819-1826.

215. Piiper, A.; Stryjek-Kaminska, D.; Zeuzem, S., Epidermal growth factor activates phospholipase C-gamma(1) via G(i1-2) proteins in isolated pancreatic acinar membranes. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **1997,** 272, (5), G1276-G1284.

216. Poosti, R.; Di Malta, L.; Gagne, D., et al., The third intracellular loop of the rat and mouse cholecystokinin-A receptors is responsible for different patterns of gene activation. *Molecular Pharmacology* **2000,** 58, (6), 1381-1388.

217. Potter, R. M.; Harikumar, K. G.; Wu, S. V.; Miller, L. J., Differential sensitivity of types 1 and 2 cholecystokinin receptors to membrane cholesterol. *Journal of Lipid Research* **2012,** 53, (1), 137-148.

218. Predel, R.; Nachman, R. J.; Gade, G., Myostimulatory neuropeptides in cockroaches: structures, distribution, pharmacological activities, and mimetic analogs. *Journal of Insect Physiology* **2001,** 47, (4-5), 311-324.

219. Pupovac, J.; Anderson, G. H., Dietary peptides induce satiety via cholecystokinin-A and peripheral opioid receptors in rats. *Journal of Nutrition* **2002,** 132, (9), 2775-2780.

220. Racotta, I. S.; Leblanc, J.; Richard, D., The effect of caffeine an food-intake in rats - involvement of corticotropin-releasing factor and the sympathoadrenal system. *Pharmacology Biochemistry and Behavior* **1994,** 48, (4), 887-892.

221. Raedt, R.; Clinckers, R.; Mollet, L., et al., Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. *Journal of Neurochemistry* **2011,** 117, (3), 461-469.

222. Ralet, M. C.; Gueguen, J., Potato proteins: composition, recovery and functional properties. *Sciences Des Aliments* **1999,** 19, (2), 147-165.

223. Ravallec-Ple, R.; Van Wormhoudt, A., Secretagogue activities in cod (*Gadus morhua*) and shrimp (*Penaeus aztecus*) extracts and alcalase hydrolysates determined in AR4-2J pancreatic tumour cells. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **2003,** 134, (4), 669-679.

224. Rayalam, S.; Della-Fera, M. A.; Baile, C. A., Phytochemicals and regulation of the adipocyte life cycle. *Journal of Nutritional Biochemistry* **2008,** 19, (11), 717-726.

225. Raybould, H. E., Mechanisms of CCK signaling from gut to brain. *Current Opinion in Pharmacology* **2007,** 7, (6), 570-574.

226. Rebres, R. A.; Roach, T. I. A.; Fraser, I. D. C., et al., Synergistic Ca^{2+} responses by G alpha(i)- and G alpha(q)-coupled G-protein-coupled receptors require a single PLC beta isoform that is sensitive to both G beta gamma and G alpha(q). *Journal of Biological Chemistry* **2011,** 286, (2), 942-951.

227. Rehfeld, J. F.; Friis-Hansen, L.; Goetze, J. P.; Hansen, T. V. O., The biology of cholecystokinin and gastrin peptides. *Current Topics in Medicinal Chemistry* **2007,** 7, (12), 1154-1165.

228. Rehfeld, J. F.; Sun, G.; Christensen, T.; Hillingso, J. G., The predominant cholecystokinin in human plasma and intestine is cholecystokinin-33. *Journal of Clinical Endocrinology & Metabolism* **2001,** 86, (1), 251-258.

229. Rein, M. J.; Renouf, M.; Cruz-Hernandez, C., et al., Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. *British Journal of Clinical Pharmacology* **2012,** 75, (3), 588-602.

230. Reubi, J. C., Targeting CCK receptors in human cancers. *Current Topics in Medicinal Chemistry* **2007,** 7, (12), 1239-1242.

231. Reubi, J. C.; Waser, B.; Laderach, U., et al., Localization of cholecystokinin A and cholecystokinin B-gastrin receptors in the human stomach. *Gastroenterology* **1997,** 112, (4), 1197-1205.

232. Richards, S.; Gibbs, R. A.; Gerardo, N. M., et al., Genome Sequence of the Pea Aphid Acyrthosiphon pisum. *Plos Biology* **2010,** 8, (2).

233. Rindi, G.; Grant, S. G. N.; Yiangou, Y., et al., Development of neuroendocrine tumors in the gastrointestinal-tract of transgenic mice - heterogeneity of hormone expression. *American Journal of Pathology* **1990,** 136, (6), 1349-1363.

234. Ritter, R. C., Gastrointestinal mechanisms of satiation for food. *Physiology & Behavior* **2004,** 81, (2), 249-273.

235. Rivera-Barreno, R.; del Castillo-Vaquero, A.; Salido, G. M.; Gonzalez, A., Effect of cinnamtannin B-1 on cholecystokinin-8-evoked responses in mouse pancreatic acinar cells. *Clinical and Experimental Pharmacology and Physiology* **2010,** 37, (10), 980-988.

236. Roberts, P. R.; Burney, J. D.; Black, K. W.; Zaloga, G. P., Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion* **1999,** 60, (4), 332-337.

237. Rocchia, W.; Alexov, E.; Honig, B., Extending the applicability of the nonlinear Poisson-Boltzmann equation: Multiple dielectric constants and multivalent ions. *Journal of Physical Chemistry B* **2001,** 105, (28), 6507-6514.

238. Sali, A.; Blundell, T. L., Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology* **1993,** 234, (3), 779-815.

239. Sarwar, G.; Peace, R. W., The protein-quality of some enteral products is inferior to that of casein as assessed by rat growth methods and digestibility-corrected amino-acid scores. *Journal of Nutrition* **1994,** 124, (11), 2223-2232.

240. Saura-Calixto, F.; Serrano, J.; Goni, I., Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chemistry* **2007,** 101, (2), 492-501.

241. Savelkoul, F.; Vanderpoel, A. F. B.; Tamminga, S., The presence and inactivation of trypsin inhibitors, tannins, lectins and amylase inhibitors in legume seeds during germination. A review. *Plant Foods for Human Nutrition* **1992,** 42, (1), 71-85.

242. Schaafsma, G., Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *European Journal of Clinical Nutrition* **2009,** 63, (10), 1161-1168.

243. Schallert, T.; Whishaw, I. Q., 2 types of aphagia and 2 types of sensorimotor impairment after lateral hypothalamic-lesions - observations in normal weight, dieted, and fattened rats. *Journal of Comparative and Physiological Psychology* **1978,** 92, (4), 720-741.

244. Schmitz, F.; Goke, M. N.; Otte, J. M., et al., Cellular expression of CCK-A and CCK-B/gastrin receptors in human gastric mucosa. *Regulatory Peptides* **2001,** 102, (2-3), 101-110.

245. Schwartz, M. W.; Woods, S. C.; Porte, D.; Seeley, R. J.; Baskin, D. G., Central nervous system control of food intake. *Nature* **2000,** 404, (6778), 661-671.

246. Segura-Campos, M.; Chel-Guerrero, L.; Betancur-Ancona, D.; Hernandez-Escalante, V. M., Bioavailability of bioactive Peptides. *Food Reviews International* **2011,** 27, (3), 213-226.

247. Shim, W. S.; Back, H.; Seo, E. K.; Lee, H. T.; Shim, C. K., Long-term administration of an aqueous extract of dried, immature fruit of Poncirus trifoliata (L.) Raf. suppresses body weight gain in rats. *Journal of Ethnopharmacology* **2009,** 126, (2), 294-299.

248. Shimizu, M.; Tsunogai, M.; Arai, S., Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides* **1997,** 18, (5), 681-687.

249. Siro, I.; Kapolna, E.; Kapolna, B.; Lugasi, A., Functional food. Product development, marketing and consumer acceptance-A review. *Appetite* **2008,** 51, (3), 456-467.

250. Slizyte, R.; Mozuraityte, R.; Martinez-Alvarez, O., et al., Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (Gadus morhua) backbones. *Process Biochemistry* **2009,** 44, (6), 668-677.

251. Slon-Usakiewicz, J. J.; Ng, W.; Dai, J. R.; Pasternak, A.; Redden, P. R., Frontal affinity chromatography with MS detection (FAC-MS) in drug discovery. *Drug Discovery Today* **2005,** 10, (6), 409-416.

252. Smeets, R. L. L.; Fouraux, M. A.; van Emst-de Vries, S. E.; De Pont, J.; Willems, P., Protein kinase C-mediated inhibition of transmembrane signalling through CCKA and CCKB receptors. *British Journal of Pharmacology* **1998,** 123, (6), 1189-1197.

253. Smeets, R. L. L.; Garner, K. M.; Hendriks, M., et al., Recovery from TPA inhibition of receptor-mediated Ca^{2+} mobilization is paralleled by down-regulation of protein kinase C-alpha in CHO cells expressing the CCK-A receptor. *Cell Calcium* **1996,** 20, (1), 1-9.

254. Smeets, R. L. L.; Garner, K. M.; Hendriks, M., et al., Recovery from TPA inhibition of receptor-mediated Ca^{2+} mobilization is paralleled by down-regulation of protein kinase C-alpha in CHO cells expressing the CCK-A receptor. *Cell Calcium* **1996,** 20, (1), 1-9.

255. Smith, C. E.; Mollard, R. C.; Luhovyy, B. L.; Anderson, G. H., The effect of yellow pea protein and fibre on short-term food intake, subjective appetite and glycaemic response in healthy young men. *British Journal of Nutrition* **2012,** 108, S74-S80.

256. Smith, J. P.; Verderame, M. F.; McLaughlin, P., et al., Characterization of the CCK-C (cancer) receptor in human pancreatic cancer. *International Journal of Molecular Medicine* **2002,** 10, (6), 689-694.

257. Sramek, J. J.; Kramer, M. S.; Reines, S. A.; Cutler, N. R., Pilot-study of a CCKB antagonist in patients with panic disorder - preliminary findings. *Anxiety* **1994,** 1, (3), 141-143.

258. Staljanssens, D.; Azari, E. K.; Christiaens, O., et al., The CCK(-like) receptor in the animal kingdom: Functions, evolution and structures. *Peptides* **2011,** 32, (3), 607-619.

259. Staljanssens, D.; De Vos, W. H.; Willems, P.; Van Camp, J.; Smagghe, G., Time-resolved quantitative analysis of CCK1 receptor-induced intracellular calcium increase. *Peptides* **2012,** 34, 219-225.

260. Staljanssens, D.; Van Camp, J.; Herregods, G., et al., Antihypertensive effect of insect cells: In vitro and in vivo evaluation. *Peptides* **2011,** 32, (3), 526-530.

261. Strader, A. D.; Woods, S. C., Gastrointestinal hormones and food intake. *Gastroenterology* **2005,** 128:, 175-191.

262. Sufian, K.; Hira, T.; Miyashita, K., et al., Pork peptone stimulates cholecystokinin secretion from enteroendocrine cells and suppresses appetite in rats. *Bioscience Biotechnology and Biochemistry* **2006,** 70, (8), 1869-1874.

263. Sufian, M. K. N. B.; Hira, T.; Asano, K.; Hara, H., Peptides derived from dolicholin, a phaseolin-like protein in country beans (Dolichos lablab), potently stimulate cholecystokinin secretion

from Enteroendocrine STC-1 cells. *Journal of Agricultural and Food Chemistry* **2007,** 55, (22), 8980-8986.

264. Sufian, M. K. N. B.; Hira, T.; Nakamori, T., et al., Soybean beta-conglycinin bromelain hydrolysate stimulates cholecystokinin secretion by enteroendocrine STC-1 cells to suppress the appetite of rats under meal-feeding conditions. *Bioscience Biotechnology and Biochemistry* **2011,** 75, (5), 848-853.

265. Suzuki, K.; Simpson, K. A.; Minnion, J. S.; Shillito, J. C.; Bloom, S. R., The role of gut hormones and the hypothalamus in appetite regulation. *Endocrine Journal* **2010,** 57, (5), 359-372.

266. Swartz, T. D.; Duca, F. A.; Covasa, M., Differential feeding behavior and neuronal responses to CCK in obesity-prone and -resistant rats. *Brain Research* **2010,** 1308, 79-86.

267. Swinburn, B. A.; Caterson, I.; Seidell, J. C.; James, W. P. T., Diet, nutrition and the prevention of excess weight gain and obesity. *Public Health Nutrition* **2004,** 7, (1A), 123-146.

268. Szecowka, J.; Hallden, G.; Goldfine, I. D.; Williams, J. A., Purification of the pancreatic cholecystokinin receptor. *Regulatory Peptides* **1989,** 24, (3), 215-224.

269. Szewczyk, J. R.; Laudernan, C., CCK1R agonists: A promising target for the pharmacological treatment of obesity. *Current Topics in Medicinal Chemistry* **2003,** 3, (8), 837-854.

270. Tang, C. W.; Biemond, I.; Lamers, C., Cholecystokinin receptors in human pancreas and gallbladder muscle: A comparative study. *Gastroenterology* **1996,** 111, (6), 1621-1626.

271. Thielecke, F.; Boschmann, M., The potential role of green tea catechins in the prevention of the metabolic syndrome - A review. *Phytochemistry* **2009,** 70, (1), 11-24.

272. Thompson, J. D.; Higgins, D. G.; Gibson, T. J., CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **1994,** 22, (22), 4673-4680.

273. Thorn, P.; Petersen, O. H., Calcium oscillations in pancreatic acinar-cells, evoked by the cholecystokinin analog JMV-180, depend on functional inositol 1,4,5-trisphosphate receptors. *Journal of Biological Chemistry* **1993,** 268, (31), 23219-23221.

274. Titz, A., The borderline between medicinal products and food supplements. *Pharmaceuticals Policy and Law* **2005,** 8, 37-4949.

275. Torfs, P.; Baggerman, G.; Meeusen, T., et al., Isolation, identification, and synthesis of a disulfated sulfakinin from the central nervous system of an arthropod, the white shrimp *Litopenaeus vannamei*. *Biochemical and Biophysical Research Communications* **2002,** 299, (2), 312-320.

276. Torgerson, J. S.; Hauptman, J.; Boldrin, M. N.; Sjostrom, L., Xenical in the prevention of diabetes in obese subjects (XENDOS) study. *Diabetes Care* **2004,** 27, (1), 155-161.

277. Townsend, R. R.; McFadden, C. B.; Ford, V.; Cadee, J. A., A randomized, double-blind, placebo-controlled trial of casein protein hydrolysate (C12 peptide) in human essential hypertension. *American Journal of Hypertension* **2004,** 17, (11), 1056-1058.

278. Trapani, G.; Franco, M.; Trapani, A., et al., Frog intestinal sac: A new *in vitro* method for the assessment of intestinal permeability. *Journal of Pharmaceutical Sciences* **2004,** 93, (12), 2909-2919.

279. Tsunoda, Y.; Yoshida, H.; Owyang, C., Structural requirements of CCK analogues to differentiate second messengers and pancreatic secretion. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **1996,** 271, (1), G8-G19.

280. Tukker, J. J., *In vitro* methods for the assessment of permeability. In *Oral drug absorption*, J.B., D.; Lennernäs, H., Eds. Marcel Dekker: New York, **2000**; pp 51-72.

281. Urribarri, L.; Chacon, D.; Gonzalez, O.; Ferrer, A., Protein extraction and enzymatic hydrolysis of ammonia-treated cassava leaves (*Manihot esculenta* Crantz). *Applied Biochemistry and Biotechnology* **2009,** 153, (1-2), 94-102.

282. van Heerden, F. R., Hoodia gordonii: A natural appetite suppressant. *Journal of Ethnopharmacology* **2008,** 119, (3), 434-437.

283. van Heerden, F. R.; Horak, R. M.; Maharaj, V. J., et al., An appetite suppressant from Hoodia species. *Phytochemistry* **2007,** 68, (20), 2545-2553.

284. Verbaeys, I.; Leon-Tamariz, F.; Buyse, J., et al., PEGylated cholecystokinin prolongs satiation in rats: dose dependency and receptor involvement. *British Journal of Pharmacology* **2007,** 152, (3), 396-403.

285. Verbeke, W., Consumer acceptance of functional foods: socio-demographic, cognitive and attitudinal determinants. *Food Quality and Preference* **2005,** 16, (1), 45-57.

286. Vercruysse, L.; Smagghe, G.; Herregods, G.; Van Camp, J., ACE inhibitory activity in enzymatic hydrolysates of insect protein. *Journal of Agricultural and Food Chemistry* **2005,** 53, (13), 5207-5211.

287. Verdich, C.; Flint, A.; Gutzwiller, J. P., et al., A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. *Journal of Clinical Endocrinology & Metabolism* **2001,** 86, (9), 4382-4389.

288. von Lengerke, T.; Krauth, C., Economic costs of adult obesity: A review of recent European studies with a focus on subgroup-specific costs. *Maturitas* **2011,** 69, (3), 220-229.

289. Wei, Z.; Baggerman, G.; Nachman, R. J., et al., Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*. *Journal of Insect Physiology* **2000,** 46, (9), 1259-1265.

290. Weinstock, G. M.; Robinson, G. E.; Gibbs, R. A., et al., Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **2006,** 443, (7114), 931-949.

291. West, D. B.; Fey, D.; Woods, S. C., Cholecystokinin persistently suppresses meal size but not food-intake in free-feeding rats. *American Journal of Physiology* **1984,** 246, (5), R776-R787.

292. Westerterp-Plantenga, M. S.; Lejeune, M.; Kovacs, E. M. R., Body weight loss and weight maintenance in relation to habitual caffeine intake and green tea supplementation. *Obesity Research* **2005,** 13, (7), 1195-1204.

293. WHO, Obesity and overweight. Fact sheet N°311. **2012,** http://www.who.int/mediacentre/factsheets/fs311/en/ (as consulted on 8 Feb 2013)

294. Wienk, K. J. H.; Marx, J. J. M.; Beynen, A. C., The concept of iron bioavailability and its assessment. *European Journal of Nutrition* **1999,** 38, (2), 51-75.

295. Willems, P.; Vanemstdevries, S. E.; Vanos, C. H.; Depont, J., Dose-dependent recruitment of pancreatic acinar-cells during receptor-mediated calcium mobilization. *Cell Calcium* **1993,** 14, (2), 145-159.

296. Wolf, Y. I.; Rogozin, I. B.; Koonin, E. V., Coelomata and not ecdysozoa: Evidence from genome-wide phylogenetic analysis. *Genome Research* **2004,** 14, (1), 29-36.

297. Wolfram, S.; Wang, Y.; Thielecke, F., Anti-obesity effects of green tea: From bedside to bench. *Molecular Nutrition & Food Research* **2006,** 50, (2), 176-187.

298. Wren, A. M.; Bloom, S. R., Gut hormones and appetite control. *Gastroenterology* **2007,** 132, (6), 2116-2130.

299. Wu, S. V.; Harikumar, K. G.; Burgess, R. J.; Reeve, J. R.; Miller, L. J., Effects of cholecystokinin-58 on type 1 cholecystokinin receptor function and regulation. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2008,** 295, (3), G641-G647.

300. Yan, E. C. Y.; Kazmi, M. A.; Ganim, Z., et al., Retinal counterion switch in the photoactivation of the G protein-coupled receptor rhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* **2003,** 100, (16), 9262-9267.

301. Yang, J.-Y.; Della-Fera, M. A.; Hartzell, D. L., et al., Esculetin induces apoptosis and inhibits adipogenesis in 3T3-L1 cells. *Obesity* **2006,** 14, (10), 1691-1699.

302. Yang, J.-Y.; Della-Fera, M. A.; Rayalam, S., et al., Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sciences* **2008,** 82, (19-20), 1032-1039.

303. Yule, D. I.; Tseng, M. J.; Williams, J. A.; Logsdon, C. D., A cloned CCK-A receptor transduces multiple signals in response to full and partial agonists. *American Journal of Physiology* **1993,** 265, (5), 999-1004.

304. Yule, D. I.; Williams, J. A., CCK antagonists reveal that CCK-8 and JMV-180 interact with different sites on the rat pancreatic acinar cell CCK(A) receptor. *Peptides* **1994,** 15, (6), 1045-1051.

305. Yun, J. W., Possible anti-obesity therapeutics from nature - A review. *Phytochemistry* **2010,** 71, (14-15), 1625-1641.

306. Zafar, T. A.; Waslien, C.; AlRaefaei, A.; Alrashidi, N.; AlMahmoud, E., Whey protein sweetened beverages reduce glycemic and appetite responses and food intake in young females. *Nutrition Research* **2013,** 33, (4), 303-310.

Supplementary data

Supplementary Table 1: Overview of identified peptides in Fraction E and F of 7SH and fraction F of 11SH. All peptides were identified with >99% reliability (see M&M paragraph 4.2.5). Ace: acetylation of the N-terminus. The more space the peptide comprises in the table, the more often it has been identified.

Accession number	Start position of peptide	End position of peptide	Peptide
7SH Frac E			
P33784	1	7	Ace-MLNIIHR-COOH
P13916	113	127	NH2-QEEEHEQREEQEWPR-COOH
	152	165	NH2-QFPFPRPPHQKEER-COOH
	192	208	NH2-NKNPFLFGSNRFETLFK-COOH
	194		NH2-NPFLFGSNRFETLFK-COOH
	198	206	NH2-FGSNRFETL-COOH
	221	232	NH2-NQRSPQLQNLRD-COOH
	224	231	NH2-SPQLQNLR-COOH
		232	NH2-SPQLQNLRD-COOH
		233	NH2-SPQLQNLRDY-COOH
		234	NH2-SPQLQNLRDYR-COOH
	246	255	NH2-LLPNHADADY-COOH
	267	277	NH2-SLVNNDDRDSY-COOH
	278	286	NH2-RLQSGDALR-COOH
	285	293	NH2-LRVPSGTTY-COOH
	287	305	NH2-VPSGTTYYVVNPDNNENLR-COOH
	294		NH2-YVVNPDNNENLR-COOH
		306	NH2-YVVNPDNNENLRL-COOH
	295		NH2-VVNPDNNENLRL-COOH
	319	339	NH2-FESFFLSSTEAQQSYLQGFSR-COOH
	323	333	NH2-FLSSTEAQQSY-COOH
		334	NH2-FLSSTEAQQSYL-COOH
	350	360	NH2-FEEINKVLFSR-COOH
	356	370	NH2-VLFSREEGQQQGEQR-COOH

	358		NH2-FSREEGQQQGEQR-COOH
		373	NH2-FSREEGQQQGEQRLQE-COOH
	359	370	NH2-SREEGQQQGEQR-COOH
		371	NH2-SREEGQQQGEQRL-COOH
		373	NH2-SREEGQQQGEQRLQE-COOH
	371	385	NH2-LQESVIVEISKEQIR-COOH
	377		NH2-VEISKEQIR-COOH
	397	409	NH2-KTISSEDKPFNLR-COOH
	398		NH2-TISSEDKPFNLR-COOH
	400		NH2-SSEDKPFNLR-COOH
	410	422	NH2-SRDPIYSNKLGKF-COOH
	422	429	NH2-FFEITPEK-COOH
		434	NH2-FFEITPEKNPQLR-COOH
	423	433	NH2-FEITPEKNPQL-COOH
		434	NH2-FEITPEKNPQLR-COOH
		435	NH2-FEITPEKNPQLRD-COOH
	424	434	NH2-EITPEKNPQLR-COOH
	441	455	NH2-SIVDMNEGALLLPHF-COOH
	445		NH2-MNEGALLLPHF-COOH
	446		NH2-NEGALLLPHF-COOH
		459	NH2-NEGALLLPHFNSKA-COOH
	448	455	NH2-GALLLPHF-COOH
	450		NH2-LLLPHF-COOH
	464	474	NH2-VINEGDANIEL-COOH
	525	536	NH2-FAIGINAENNQR-COOH
		537	NH2-FAIGINAENNQRN-COOH
	526	536	NH2-AIGINAENNQR-COOH
	527		NH2-IGINAENNQR-COOH
	537	568	NH2-NFLAGSQDNVISQIPSQVQELAFPGSAQAVEK-COOH
	557	565	NH2-LAFPGSAQA-COOH
		568	NH2-LAFPGSAQAVEK-COOH
		569	NH2-LAFPGSAQAVEKL-COOH

	575	584	NH2-ESYFVDAQPK-COOH
	578		NH2-FVDAQPK-COOH
P11827	110	118	NH2-QPHQEEEHE-COOH
	188	204	NH2-DKESQESEGSESQREPR-COOH
	208	217	NH2-NKNPFHFNSK-COOH
		218	NH2-NKNPFHFNSKR-COOH
	219	224	NH2-FQTLFK-COOH
	223	231	NH2-FKNQYGHVR-COOH
	239	247	NH2-RSQQLQNLR-COOH
	240		NH2-SQQLQNLR-COOH
	240	248	NH2-SQQLQNLRD-COOH
		249	NH2-SQQLQNLRDY-COOH
	251	261	NH2-ILEFNSKPNTL-COOH
	254		NH2-FNSKPNTL-COOH
	262	272	NH2-LLPHHADADYL-COOH
	283	293	NH2-TLVNNDDRDSY-COOH
	295	302	NH2-LQSGDALR-COOH
	310	321	NH2-YVVNPDNDENLR-COOH
		322	NH2-YVVNPDNDENLRM-COOH
	344	353	NH2-LAIPVNKPGR-COOH
		354	NH2-LAIPVNKPGRF-COOH
	345		NH2-AIPVNKPGRF-COOH
	354	374	NH2-FESFFLSSTQAQQSYLQGFSK-COOH
	358	369	NH2-FLSSTQAQQSYL-COOH
	359	368	NH2-LSSTQAQQSY-COOH
	375	384	NH2-NILEASYDTK-COOH
		390	NH2-NILEASYDTKFEEINK-COOH
	378	385	NH2-EASYDTKF-COOH
	386	393	NH2-EEINKVLF-COOH
	391	405	NH2-VLFGREEGQQQGEER-COOH
	393		NH2-FGREEGQQQGEER-COOH
	394	406	NH2-GREEGQQQGEERL-COOH
	406	416	NH2-LQESVIVEISK-COOH

	432	441	NH2-KTISSEDKPF-COOH
	433		NH2-TISSEDKPF-COOH
	445	453	NH2-SRDPIYSNK-COOH
		454	NH2-SRDPIYSNKL-COOH
		456	NH2-SRDPIYSNKLGK-COOH
		457	NH2-SRDPIYSNKLGKL-COOH
	447	456	NH2-DPIYSNKLGK-COOH
	477	485	NH2-VDMNEGALF-COOH
		528	NH2-QQQEEQPLEVR-COOH
	518		
	518	529	NH2-QQQEEQPLEVRK-COOH
	559	570	NH2-FAFGINAENNQR-COOH
	560		NH2-AFGINAENNQR-COOH
	561		NH2-FGINAENNQR-COOH
	599	605	NH2-DIENLIK-COOH
	604	612	NH2-IKSQSESYF-COOH
	606	620	NH2-SQSESYFVDAQPQQK-COOH
		624	NH2-SQSESYFVDAQPQQKEEGN-COOH
	609	620	NH2-ESYFVDAQPQQK-COOH
	611	624	NH2-YFVDAQPQQKEEGN-COOH
	612	620	NH2-FVDAQPQQK-COOH
		624	NH2-FVDAQPQQKEEGN-COOH
	613		NH2-VDAQPQQKEEGN-COOH
P25974	24	37	NH2-LKVREDENNPFYFR-COOH
	62	70	NH2-RSPQLENLR-COOH
	63		NH2-SPQLENLR-COOH
		71	NH2-SPQLENLRD-COOH
		72	NH2-SPQLENLRDY-COOH
	126	144	NH2-IPAGTTYYLVNPHDHQNLK-COOH
	134	143	NH2-LVNPHDHQNL-COOH
	231	243	NH2-KTISSEDEPFNLR-COOH

	232		NH2-TISSEDEPFNLR-COOH
	244	255	NH2-SRNPIYSNNFGK-COOH
	269	292	NH2-DLDIFLSSVDINEGALLLPHFNSK-COOH
	279	289	NH2-INEGALLLPHF-COOH
	318	329	NH2-QKQEEEPLEVQR-COOH
	320		NH2-QEEEPLEVQR-COOH
	371	382	NH2-NFLAGEKDNVVR-COOH
	373		NH2-LAGEKDNVVR-COOH
		383	NH2-LAGEKDNVVRQ-COOH
	374	382	NH2-AGEKDNVVR-COOH
	387	402	NH2-QVQELAFPGSAQDVER-COOH
	391		NH2-LAFPGSAQDVER-COOH
		403	NH2-LAFPGSAQDVERL-COOH
	407	420	NH2-QRESYFVDAQPQQK-COOH
	409	427	NH2-ESYFVDAQPQQKEEGSKGR-COOH
P04776	37	51	NH2-NALKPDNRIESEGGL-COOH
	39		NH2-LKPDNRIESEGGL-COOH
	44		NH2-RIESEGGL-COOH
	51	62	NH2-LIETWNPNNKPF-COOH
	111	120	NH2-FEEPQQPQQR-COOH
	192	206	NH2-FLKYQQEQGGHQSQK-COOH
	193	205	NH2-LKYQQEQGGHQSQ-COOH
		206	NH2-LKYQQEQGGHQSQK-COOH
	194		NH2-KYQQEQGGHQSQK-COOH
	209	221	NH2-HQQEEENEGGSIL-COOH
	222	233	NH2-SGFTLEFLEHAF-COOH

	225		NH2-TLEFLEHAF-COOH
	233	241	NH2-FSVDKQIAK-COOH
	323	332	NH2-HNIGQTSSPD-COOH
	343	356	NH2-TATSLDFPALSWLR-COOH
	355	361	NH2-LRLSAEF-COOH
	371	383	NH2-VPHYNLNANSIIY-COOH
	375	388	NH2-NLNANSIIYALNGR-COOH
	401	411	NH2-VFDGELQEGRV-COOH
	409	417	NH2-GRVLIVPQN-COOH
		418	NH2-GRVLIVPQNF-COOH
	435	452	NH2-KTNDTPMIGTLAGANSLL-COOH
		445	NH2-KTNDTPMIGTL-COOH
	481	492	NH2-KFLVPPQESQKR-COOH
	483		NH2-LVPPQESQKR-COOH
	485		NH2-PPQESQKR-COOH
P04405	34	48	NH2-NALKPDNRIESEGGF-COOH
	36		NH2-LKPDNRIESEGGF-COOH
	49	59	NH2-IETWNPNNKPF-COOH
	78	87	NH2-RPSYTNGPQE-COOH
		89	NH2-RPSYTNGPQEIY-COOH
	88	97	NH2-IYIQQGNGIF-COOH
		98	NH2-IYIQQGNGIFG-COOH
	90	97	NH2-IQQGNGIF-COOH
	108	117	NH2-YQEPQESQQR-COOH
	108		NH2-YQEPQESQQR-COOH
	109		NH2-QEPQESQQR-COOH
	139	147	NH2-IAVPTGVAW-COOH
		159	NH2-IAVPTGVAWWMYNNEDTPVVA-COOH
	148		NH2-WMYNNEDTPVVA-COOH
	149		NH2-MYNNEDTPVVA-COOH
	160	168	NH2-VSIIDTNSL-COOH
		169	NH2-VSIIDTNSLE-COOH
		172	NH2-VSIIDTNSLENQL-COOH
		177	NH2-VSIIDTNSLENQLDQMPR-COOH
	162	172	NH2-IIDTNSLENQL-COOH
	166		NH2-NSLENQL-COOH
		175	NH2-NSLENQLDQM-COOH
		177	NH2-NSLENQLDQMPR-COOH
	167		NH2-SLENQLDQMPR-COOH
	169		NH2-ENQLDQMPR-COOH

	172		NH2-LDQMPR-COOH
	180	188	NH2-YLAGNQEQE-COOH
		189	NH2-YLAGNQEQEF-COOH
		192	NH2-YLAGNQEQEFLKY-COOH
	190	204	NH2-LKYQQQQQGGSQSQK-COOH
	217	225	NH2-ILSGFAPEF-COOH
	226	233	NH2-LKEAFGVN-COOH
		234	NH2-LKEAFGVNM-COOH
		235	NH2-LKEAFGVNMQ-COOH
	228	238	NH2-EAFGVNMQIVR-COOH
	260	267	NH2-RVTAPAMR-COOH
	312	322	NH2-RQNIGQNSSPD-COOH
	335	342	NH2-TSLDFPAL-COOH
	345	351	NH2-LKLSAQY-COOH
	389	398	NH2-ERVFDGELQE-COOH
	391	401	NH2-VFDGELQEGGV-COOH
		402	NH2-VFDGELQEGGVL-COOH
	401	408	NH2-VLIVPQNF-COOH
	402		NH2-LIVPQNF-COOH
	414	425	NH2-SQSDNFEYVSFK-COOH
	425	435	NH2-KTNDRPSIGNL-COOH
	426		NH2-TNDRPSIGNL-COOH
	443	454	NH2-NALPEEVIQHTF-COOH
	446		NH2-PEEVIQHTF-COOH
	449		NH2-VIQHTF-COOH
	473	481	NH2-LVPPQESQR-COOH
P11828	81	90	NH2-RPSYTNAPQE-COOH
	91	100	NH2-IYIQQGSGIF-COOH
		101	NH2-IYIQQGSGIFG-COOH
	111	117	NH2-FEEPQQK-COOH
		119	NH2-FEEPQQKGQ-COOH
	163	172	NH2-IDTNSFQNQL-COOH
		175	NH2-IDTNSFQNQLDQM-COOH
		177	NH2-IDTNSFQNQLDQMPR-COOH
	169		NH2-QNQLDQMPR-COOH
	190	202	NH2-LQYQPQKQQGGTQ-COOH
		205	NH2-LQYQPQKQQGGTQSQK-COOH
	439	450	NH2-NALPEEVIQQTF-COOH
	471	477	NH2-PPKESQR-COOH

	38	52	NH2-NALEPDHRVESEGGL-COOH
	92	98	NH2-IIIAQGK-COOH
	112	120	NH2-FEEPQEQSN-COOH
		121	NH2-FEEPQEQSNR-COOH
	126	137	NH2-SQKQQLQDSHQK-COOH
		139	NH2-SQKQQLQDSHQKIR-COOH
	138	146	NH2-IRHFNEGDV-COOH
		147	NH2-IRHFNEGDVL-COOH
	171	180	NH2-LDTSNFNNQL-COOH
		185	NH2-LDTSNFNNQLDQTPR-COOH
		186	NH2-LDTSNFNNQLDQTPRV-COOH
	177	185	NH2-NNQLDQTPR-COOH
		186	NH2-NNQLDQTPRV-COOH
	187	200	NH2-FYLAGNPDIEYPET-COOH
	198	208	NH2-PETMQQQQQQK-COOH
	237	247	NH2-FLAQSFNTNED-COOH
	238	246	NH2-LAQSFNTNE-COOH
P02858		247	NH2-LAQSFNTNED-COOH
	260	269	NH2-KQIVTVEGGL-COOH
	261		NH2-QIVTVEGGL-COOH
	276	284	NH2-WQEQQDEDE-COOH
	362	369	NH2-RPRQEEPR-COOH
		371	NH2-RPRQEEPRER-COOH
	412	419	NH2-NSLTLPAL-COOH
		420	NH2-NSLTLPALR-COOH
		421	NH2-NSLTLPALRQ-COOH
	414		NH2-LTLPALRQ-COOH
	485	497	NH2-FVVAEQAGEQGFE-COOH
	486		NH2-VVAEQAGEQGFE-COOH
		498	NH2-VVAEQAGEQGFEY-COOH
	502	511	NH2-KTHHNAVTSY-COOH
	517	523	NH2-RAIPSEV-COOH
		528	NH2-RAIPSEVLAHSY-COOH
	517	528	NH2-RAIPSEVLAHSY-COOH
	518		NH2-AIPSEVLAHSY-COOH
	529	537	NH2-NLRQSQVSE-COOH
	531	538	NH2-RQSQVSEL-COOH
	539	555	NH2-KYEGNWGPLVNPESQQG-COOH
		557	NH2-KYEGNWGPLVNPESQQGSP-COOH

		558	NH2-KYEGNWGPLVNPESQQGSPR-COOH
	545		NH2-GPLVNPESQQGSPR-COOH
	548	557	NH2-VNPESQQGSP-COOH
		558	NH2-VNPESQQGSPR-COOH
P04347	54	64	NH2-IETWNSQHPEL-COOH
	93	99	NH2-IIVVQGK-COOH
	113	122	NH2-FEKPQQQSSR-COOH
	127	137	NH2-SQQQLQDSHQK-COOH
		139	NH2-SQQQLQDSHQKIR-COOH
	171	185	NH2-LDTSNFNNQLDQNPR-COOH
		186	NH2-LDTSNFNNQLDQNPRV-COOH
	177	185	NH2-NNQLDQNPR-COOH
		186	NH2-NNQLDQNPRV-COOH
	242	251	NH2-NTNEDTAEKL-COOH
	468	476	NH2-KTHHNAVSS-COOH
		477	NH2-KTHHNAVSSY-COOH
	495	502	NH2-NLGQSQVR-COOH
		503	NH2-NLGQSQVRQ-COOH
	504	516	NH2-LKYQGNSGPLVNP-COOH
	505		NH2-KYQGNSGPLVNP-COOH
P0C7L3	26	33	Ace-ADDLAAIP-COOH
Q00184	20	27	NH2-PSKLLPVL-COOH
7SH Frac F			
P33784	1	7	Ace-MLNIIHR-COOH
P13916	152	165	NH2-QFPFPRPPHQKEER-COOH
	190	197	NH2-HKNKNPFL-COOH
	192	198	NH2-NKNPFLF-COOH
		202	NH2-NKNPFLFGSNR-COOH
	198	203	NH2-FGSNRF-COOH
		206	NH2-FGSNRFETL-COOH
	203	208	NH2-FETLFK-COOH
		213	NH2-FETLFKNQYGR-COOH
	214	219	NH2-IRVLQR-COOH
	224	231	NH2-SPQLQNLR-COOH
		232	NH2-SPQLQNLRD-COOH
	267	277	NH2-SLVNNDDRDSY-COOH
	278	286	NH2-RLQSGDALR-COOH
	285	293	NH2-LRVPSGTTY-COOH
		294	NH2-LRVPSGTTYY-COOH
	294	305	NH2-YVVNPDNNENLR-COOH
	294	306	NH2-YVVNPDNNENLRL-COOH

	319	339	NH2-FESFFLSSTEAQQSYLQGFSR-COOH
	323	334	NH2-FLSSTEAQQSYL-COOH
	379	385	NH2-ISKEQIR-COOH
	397	409	NH2-KTISSEDKPFNLR-COOH
	398		NH2-TISSEDKPFNLR-COOH
	416	422	NH2-SNKLGKF-COOH
	422	429	NH2-FFEITPEK-COOH
		434	NH2-FFEITPEKNPQLR-COOH
	446	455	NH2-NEGALLLPHF-COOH
	448		NH2-GALLLPHF-COOH
	450		NH2-LLLPHF-COOH
	525	536	NH2-FAIGINAENNQR-COOH
	526		NH2-AIGINAENNQR-COOH
	537	568	NH2-NFLAGSQDNVISQIPSQVQELAFPGSAQAVEK-COOH
	569	574	NH2-LLKNQR-COOH
	575	584	NH2-ESYFVDAQPK-COOH
P11827	117	125	NH2-HEQKEEHEW-COOH
	156	162	NH2-EEEKHEW-COOH
	208	217	NH2-NKNPFHFNSK-COOH
		218	NH2-NKNPFHFNSKR-COOH
	218	224	NH2-RFQTLFK-COOH
	219		NH2-FQTLFK-COOH
		231	NH2-FQTLFKNQYGHVR-COOH
	223		NH2-FKNQYGHVR-COOH
	224		NH2-KNQYGHVR-COOH
	239	247	NH2-RSQQLQNLR-COOH
	240		NH2-SQQLQNLR-COOH
		249	NH2-SQQLQNLRDY-COOH
		250	NH2-SQQLQNLRDYR-COOH
	295	302	NH2-LQSGDALR-COOH
	345	354	NH2-AIPVNKPGRF-COOH
	354	374	NH2-FESFFLSSTQAQQSYLQGFSK-COOH
	358	368	NH2-FLSSTQAQQSY-COOH
		369	NH2-FLSSTQAQQSYL-COOH
	378	385	NH2-EASYDTKF-COOH
	379		NH2-ASYDTKF-COOH
	380		NH2-SYDTKF-COOH
	386	393	NH2-EEINKVLF-COOH
	432	441	NH2-KTISSEDKPF-COOH
	445	452	NH2-SRDPIYSN-COOH
		453	NH2-SRDPIYSNK-COOH

		456	NH2-SRDPIYSNKLGK-COOH
	447	453	NH2-DPIYSNK-COOH
	480	489	NH2-NEGALFLPHF-COOH
	485	493	NH2-FLPHFNSKA-COOH
	509	517	NH2-VGIKEQQQR-COOH
	518	528	NH2-QQQEEQPLEVR-COOH
	559	570	NH2-FAFGINAENNQR-COOH
	560		NH2-AFGINAENNQR-COOH
	561		NH2-FGINAENNQR-COOH
	571	577	NH2-NFLAGSK-COOH
	604	611	NH2-IKSQSESY-COOH
		612	NH2-IKSQSESYF-COOH
	606	624	NH2-SQSESYFVDAQPQQKEEGN-COOH
P25974	25	37	NH2-KVREDENNPFYFR-COOH
	26		NH2-VREDENNPFYFR-COOH
	28		NH2-EDENNPFYFR-COOH
	62	70	NH2-RSPQLENLR-COOH
	63		NH2-SPQLENLR-COOH
		73	NH2-SPQLENLRDYR-COOH
	117	125	NH2-NLHPGDAQR-COOH
	133	143	NH2-YLVNPHDHQNL-COOH
	244	252	NH2-SRNPIYSNN-COOH
		253	NH2-SRNPIYSNNF-COOH
		255	NH2-SRNPIYSNNFGK-COOH
	246		NH2-NPIYSNNFGK-COOH
	371	382	NH2-NFLAGEKDNVVR-COOH
P04776	91	97	NH2-IYIQQGK-COOH
	111	120	NH2-FEEPQQPQQR-COOH
	135	141	NH2-NFREGDL-COOH
		151	NH2-NFREGDLIAVPTGVAWW-COOH
	193	206	NH2-LKYQQEQGGHQSQK-COOH
	233	241	NH2-FSVDKQIAK-COOH
	345	356	NH2-TSLDFPALSWLR-COOH
	350	357	NH2-PALSWLRL-COOH
	358	366	NH2-SAEFGSLRK-COOH
	361		NH2-FGSLRK-COOH
	370	383	NH2-FVPHYNLNANSIIY-COOH

	422	430	NH2-ARSQSDNFE-COOH
	476	481	NH2-NNNPFK-COOH
P04405	41	48	NH2-RIESEGGF-COOH
	49	59	NH2-IETWNPNNKPF-COOH
	78	87	NH2-RPSYTNGPQE-COOH
		89	NH2-RPSYTNGPQEIY-COOH
	88	97	NH2-IYIQQGNGIF-COOH
	88	98	NH2-IYIQQGNGIFG-COOH
	89	97	NH2-YIQQGNGIF-COOH
		98	NH2-YIQQGNGIFG-COOH
	90	97	NH2-IQQGNGIF-COOH
	108	117	NH2-YQEPQESQQR-COOH
	134	148	NH2-REGDLIAVPTGVAWW-COOH
	139	147	NH2-IAVPTGVAW-COOH
		159	NH2-IAVPTGVAWWMYNNEDTPVVA-COOH
	148	157	NH2-WMYNNEDTPV-COOH
		159	NH2-WMYNNEDTPVVA-COOH
		158	NH2-WMYNNEDTPVV-COOH
	160	172	NH2-VSIIDTNSLENQL-COOH
		177	NH2-VSIIDTNSLENQLDQMPR-COOH
	169		NH2-ENQLDQMPR-COOH
	180	188	NH2-YLAGNQEQE-COOH
		189	NH2-YLAGNQEQEF-COOH
		192	NH2-YLAGNQEQEFLKY-COOH
	219	225	NH2-SGFAPEF-COOH
	226	233	NH2-LKEAFGVN-COOH
		234	NH2-LKEAFGVNM-COOH
	227	233	NH2-KEAFGVN-COOH
	335	344	NH2-TSLDFPALWL-COOH
	340	347	NH2-PALWLLKL-COOH
	345	351	NH2-LKLSAQY-COOH
	346		NH2-KLSAQY-COOH
	360	366	NH2-FVPHYTL-COOH
	361	370	NH2-VPHYTLNANS-COOH
	414	425	NH2-SQSDNFEYVSFK-COOH
	449	455	NH2-VIQHTFN-COOH
P11828	91	100	NH2-IYIQQGSGIF-COOH
		101	NH2-IYIQQGSGIFG-COOH
	92	100	NH2-YIQQGSGIF-COOH
	147	159	NH2-YWMYNNEDTPVVA-COOH

	190	196	NH2-LQYQPQK-COOH
P02858	38	45	NH2-NALEPDHR-COOH
	56	64	NH2-WNSQHPELK-COOH
	57		NH2-NSQHPELK-COOH
	83	90	NH2-PSYSPYPR-COOH
	92	98	NH2-IIIAQGK-COOH
	112	120	NH2-FEEPQEQSN-COOH
	138	147	NH2-IRHFNEGDVL-COOH
	156	167	NH2-WTYNTGDEPVVA-COOH
	177	185	NH2-NNQLDQTPR-COOH
		186	NH2-NNQLDQTPRV-COOH
	302	308	NH2-RPSHGKR-COOH
	398	404	NH2-ADFYNPK-COOH
	422	428	NH2-FQLSAQY-COOH
	433	443	NH2-KNGIYSPHWNL-COOH
	438	445	NH2-SPHWNLNA-COOH
		447	NH2-SPHWNLNANS-COOH
	502	511	NH2-KTHHNAVTSY-COOH
	517	528	NH2-RAIPSEVLAHSY-COOH
	538	547	NH2-LKYEGNWGPL-COOH
	539		NH2-KYEGNWGPL-COOH
	548	558	NH2-VNPESQQGSPR-COOH
P04347	93	99	NH2-IIVVQGK-COOH
	177	185	NH2-NNQLDQNPR-COOH
		186	NH2-NNQLDQNPRV-COOH
	468	477	NH2-KTHHNAVSSY-COOH
	469		NH2-THHNAVSSY-COOH
	476	482	NH2-SYIKDVF-COOH
	494	502	NH2-YNLGQSQVR-COOH
		503	NH2-YNLGQSQVRQ-COOH
	495	502	NH2-NLGQSQVR-COOH
		503	NH2-NLGQSQVRQ-COOH
Q9X2V9	494	499	NH2-ILNLYR-COOH
Q46731	362	367	NH2-RLLQLR-COOH
11SH Frac E			
P13916	197	206	NH2-LFGSNRFETL-COOH
	224	231	NH2-SPQLQNLR-COOH
	267	274	NH2-SLVNNDDR-COOH
		277	NH2-SLVNNDDRDSY-COOH
		278	NH2-SLVNNDDRDSYR-COOH
	278	286	NH2-RLQSGDALR-COOH
	294	305	NH2-YVVNPDNNENLR-COOH
		306	NH2-YVVNPDNNENLRL-COOH

	319	333	NH2-FESFFLSSTEAQQSY-COOH
		337	NH2-FESFFLSSTEAQQSYLQGF-COOH
	320	333	NH2-ESFFLSSTEAQQSY-COOH
	323		NH2-FLSSTEAQQSY-COOH
	397	409	NH2-KTISSEDKPFNLR-COOH
	398		NH2-TISSEDKPFNLR-COOH
	422	429	NH2-FFEITPEK-COOH
		432	NH2-FFEITPEKNPQ-COOH
		434	NH2-FFEITPEKNPQLR-COOH
	423		NH2-FEITPEKNPQLR-COOH
	525	536	NH2-FAIGINAENNQR-COOH
	526		NH2-AIGINAENNQR-COOH
	575	584	NH2-ESYFVDAQPK-COOH
P11827	93	103	NH2-EEDEGEQPRPF-COOH
	110	120	NH2-QPHQEEEHEQK-COOH
	239	247	NH2-RSQQLQNLR-COOH
	240		NH2-SQQLQNLR-COOH
	251	259	NH2-ILEFNSKPN-COOH
		261	NH2-ILEFNSKPNTL-COOH
	263	270	NH2-LPHHADAD-COOH
	280	293	NH2-AILTLVNNDDRDSY-COOH
	283		NH2-TLVNNDDRDSY-COOH
	294	302	NH2-NLQSGDALR-COOH
	295		NH2-LQSGDALR-COOH
	344	353	NH2-LAIPVNKPGR-COOH
	345		NH2-AIPVNKPGR-COOH
	354	366	NH2-FESFFLSSTQAQQ-COOH
	358	368	NH2-FLSSTQAQQSY-COOH
	375	384	NH2-NILEASYDTK-COOH
	385	393	NH2-FEEINKVLF-COOH
	406	416	NH2-LQESVIVEISK-COOH
	432	441	NH2-KTISSEDKPF-COOH
	433		NH2-TISSEDKPF-COOH
	445	453	NH2-SRDPIYSNK-COOH
		456	NH2-SRDPIYSNKLGK-COOH

	518	528	NH2-QQQEEQPLEVR-COOH
	530	540	NH2-YRAELSEQDIF-COOH
	560	570	NH2-AFGINAENNQR-COOH
	599	605	NH2-DIENLIK-COOH
	606	620	NH2-SQSESYFVDAQPQQK-COOH
	609		NH2-ESYFVDAQPQQK-COOH
	612		NH2-FVDAQPQQK-COOH
P25974	28	37	NH2-EDENNPFYFR-COOH
	62	70	NH2-RSPQLENLR-COOH
	63		NH2-SPQLENLR-COOH
		72	NH2-SPQLENLRDY-COOH
		73	NH2-SPQLENLRDYR-COOH
	117	125	NH2-NLHPGDAQR-COOH
	126	144	NH2-IPAGTTYYLVNPHDHQNLK-COOH
	134		NH2-LVNPHDHQNLK-COOH
	158	172	NH2-YDDFFLSSTQAQQSY-COOH
		176	NH2-YDDFFLSSTQAQQSYLQGF-COOH
	159	172	NH2-DDFFLSSTQAQQSY-COOH
	186	194	NH2-HSEFEEINR-COOH
	205	215	NH2-QQEGVIVELSK-COOH
	231	243	NH2-KTISSEDEPFNLR-COOH
	232	240	NH2-TISSEDEPF-COOH
	232	243	NH2-TISSEDEPFNLR-COOH
	244	253	NH2-SRNPIYSNNF-COOH
		255	NH2-SRNPIYSNNFGK-COOH
	320	329	NH2-QEEEPLEVQR-COOH
	332	348	NH2-AELSEDDVFVIPAAYPF-COOH
	371	382	NH2-NFLAGEKDNVVR-COOH
	374		NH2-AGEKDNVVR-COOH
	387	402	NH2-QVQELAFPGSAQDVER-COOH
	407	420	NH2-QRESYFVDAQPQQK-COOH
	409	425	NH2-ESYFVDAQPQQKEEGSK-COOH
P04776	44	51	NH2-RIESEGGL-COOH
	51	62	NH2-LIETWNPNNKPF-COOH
	91	97	NH2-IYIQQGK-COOH
	111	120	NH2-FEEPQQPQQR-COOH

	193	206	NH2-LKYQQEQGGHQSQK-COOH
	207	221	NH2-GKHQQEEENEGGSIL-COOH
	209		NH2-HQQEEENEGGSIL-COOH
	222	233	NH2-SGFTLEFLEHAF-COOH
	225		NH2-TLEFLEHAF-COOH
	233	238	NH2-FSVDKQ-COOH
		241	NH2-FSVDKQIAK-COOH
	242	254	NH2-NLQGENEGEDKGA-COOH
		255	NH2-NLQGENEGEDKGAI-COOH
		256	NH2-NLQGENEGEDKGAIV-COOH
		257	NH2-NLQGENEGEDKGAIVT-COOH
		253	NH2-NLQGENEGEDKG-COOH
	263	271	NH2-SVIKPPTDE-COOH
		278	NH2-SVIKPPTDEQQQRPQE-COOH
	264	271	NH2-VIKPPTDE-COOH
	321	332	NH2-LRHNIGQTSSPD-COOH
	322		NH2-RHNIGQTSSPD-COOH
	323		NH2-HNIGQTSSPD-COOH
		334	NH2-HNIGQTSSPDIY-COOH
		335	NH2-HNIGQTSSPDIYN-COOH
		340	NH2-HNIGQTSSPDIYNPQAGS-COOH
		341	NH2-HNIGQTSSPDIYNPQAGSV-COOH
		342	NH2-HNIGQTSSPDIYNPQAGSVT-COOH
	323	344	NH2-HNIGQTSSPDIYNPQAGSVTTA-COOH
		343	NH2-HNIGQTSSPDIYNPQAGSVTT-COOH
	333	342	NH2-IYNPQAGSVT-COOH
		344	NH2-IYNPQAGSVTTA-COOH
	357	365	NH2-LSAEFGSLR-COOH
		366	NH2-LSAEFGSLRK-COOH
	401	410	NH2-VFDGELQEGR-COOH
		411	NH2-VFDGELQEGRV-COOH
	403		NH2-DGELQEGRV-COOH
	411	423	NH2-VLIVPQNFVVAAR-COOH
	435	443	NH2-KTNDTPMIG-COOH
		445	NH2-KTNDTPMIGTL-COOH
	436		NH2-TNDTPMIGTL-COOH

	444	451	NH2-TLAGANSL-COOH
		452	NH2-TLAGANSLL-COOH
	483	492	NH2-LVPPQESQKR-COOH
	485	491	NH2-PPQESQK-COOH
P04405	33	41	NH2-LNALKPDNR-COOH
	34		NH2-NALKPDNR-COOH
	41	48	NH2-RIESEGGF-COOH
	42	59	NH2-IESEGGFIETWNPNNKPF-COOH
	49		NH2-IETWNPNNKPF-COOH
	78	86	NH2-RPSYTNGPQ-COOH
		87	NH2-RPSYTNGPQE-COOH
		88	NH2-RPSYTNGPQEI-COOH
		89	NH2-RPSYTNGPQEIY-COOH
	88	97	NH2-IYIQQGNGIF-COOH
		98	NH2-IYIQQGNGIFG-COOH
	90	97	NH2-IQQGNGIF-COOH
	90	98	NH2-IQQGNGIFG-COOH
	98	107	NH2-GMIFPGCPST-COOH
	108	117	NH2-YQEPQESQQR-COOH
	133	147	NH2-FREGDLIAVPTGVAW-COOH
		139	NH2-FREGDLI-COOH
	134	147	NH2-REGDLIAVPTGVAW-COOH
	139		NH2-IAVPTGVAW-COOH
	148	157	NH2-WMYNNEDTPV-COOH
		159	NH2-WMYNNEDTPVVA-COOH

	149		NH2-MYNNEDTPVVA-COOH
	150		NH2-YNNEDTPVVA-COOH
	160	167	NH2-VSIIDTNS-COOH
		168	NH2-VSIIDTNSL-COOH
		169	NH2-VSIIDTNSLE-COOH
		172	NH2-VSIIDTNSLENQL-COOH
	162	168	NH2-IIDTNSL-COOH
		169	NH2-IIDTNSLE-COOH
		172	NH2-IIDTNSLENQL-COOH
	163		NH2-IDTNSLENQL-COOH
	166		NH2-NSLENQL-COOH
		175	NH2-NSLENQLDQM-COOH
		177	NH2-NSLENQLDQMPR-COOH
	169		NH2-ENQLDQMPR-COOH
	178	191	NH2-RFYLAGNQEQEFLK-COOH
	179		NH2-FYLAGNQEQEFLK-COOH
	180	188	NH2-YLAGNQEQE-COOH
		189	NH2-YLAGNQEQEF-COOH
	181		NH2-LAGNQEQEF-COOH
		191	NH2-LAGNQEQEFLK-COOH
		192	NH2-LAGNQEQEFLKY-COOH
	182	189	NH2-AGNQEQEF-COOH
	207	218	NH2-QQEEENEGSNIL-COOH
	216	225	NH2-NILSGFAPEF-COOH
	217		NH2-ILSGFAPEF-COOH
	219		NH2-SGFAPEF-COOH
		226	NH2-SGFAPEFL-COOH
		227	NH2-SGFAPEFLK-COOH
	226	233	NH2-LKEAFGVN-COOH
		234	NH2-LKEAFGVNM-COOH
		235	NH2-LKEAFGVNMQ-COOH
	227	233	NH2-KEAFGVN-COOH

		235	NH2-KEAFGVNMQ-COOH
	228	238	NH2-EAFGVNMQIVR-COOH
	239	251	NH2-NLQGENEEEDSGA-COOH
	312	322	NH2-RQNIGQNSSPD-COOH
		324	NH2-RQNIGQNSSPDIY-COOH
		334	NH2-RQNIGQNSSPDIYNPQAGSITTA-COOH
	313		NH2-QNIGQNSSPDIYNPQAGSITTA-COOH
	323		NH2-IYNPQAGSITTA-COOH
		333	NH2-IYNPQAGSITT-COOH
	335	342	NH2-TSLDFPAL-COOH
	367	378	NH2-NANSIIYALNGR-COOH
	369		NH2-NSIIYALNGR-COOH
	389	395	NH2-ERVFDGE-COOH
		396	NH2-ERVFDGEL-COOH
		398	NH2-ERVFDGELQE-COOH
	391		NH2-VFDGELQE-COOH
		402	NH2-VFDGELQEGGVL-COOH
	401	407	NH2-VLIVPQN-COOH
		408	NH2-VLIVPQNF-COOH
	402		NH2-LIVPQNF-COOH
	414	424	NH2-SQSDNFEYVSF-COOH
		425	NH2-SQSDNFEYVSFK-COOH
	425	434	NH2-KTNDRPSIGN-COOH
		439	NH2-KTNDRPSIGNLAGAN-COOH
		441	NH2-KTNDRPSIGNLAGANSL-COOH
		435	NH2-KTNDRPSIGNL-COOH
	426	438	NH2-TNDRPSIGNLAGA-COOH
		439	NH2-TNDRPSIGNLAGAN-COOH
		441	NH2-TNDRPSIGNLAGANSL-COOH
		435	NH2-TNDRPSIGNL-COOH
	440	454	NH2-SLLNALPEEVIQHTF-COOH
	443		NH2-NALPEEVIQHTF-COOH
	446		NH2-PEEVIQHTF-COOH
	466	481	NH2-NNNPFSFLVPPQESQR-COOH
	473		NH2-LVPPQESQR-COOH
	475		NH2-PPQESQR-COOH
P11828	81	90	NH2-RPSYTNAPQE-COOH
		91	NH2-RPSYTNAPQEI-COOH
	91	100	NH2-IYIQQGSGIF-COOH
	93		NH2-IQQGSGIF-COOH
	139	145	NH2-IAVPTGF-COOH

	163	172	NH2-IDTNSFQNQL-COOH
		175	NH2-IDTNSFQNQLDQM-COOH
	169	177	NH2-QNQLDQMPR-COOH
	190	196	NH2-LQYQPQK-COOH
	241	251	NH2-KLQGENEEEEK-COOH
	309	328	NH2-HNIGQTSSPDIFNPQAGSIT-COOH
		330	NH2-HNIGQTSSPDIFNPQAGSITTA-COOH
	445	451	NH2-VIQQTFN-COOH
P02858	38	45	NH2-NALEPDHR-COOH
		52	NH2-NALEPDHRVESEGGL-COOH
	45		NH2-RVESEGGL-COOH
	57	63	NH2-NSQHPEL-COOH
		64	NH2-NSQHPELK-COOH
	92	98	NH2-IIIAQGK-COOH
	112	120	NH2-FEEPQEQSN-COOH
		121	NH2-FEEPQEQSNR-COOH
	138	147	NH2-IRHFNEGDVL-COOH
	140	146	NH2-HFNEGDV-COOH
	158	167	NH2-YNTGDEPVVA-COOH
	177	185	NH2-NNQLDQTPR-COOH
		186	NH2-NNQLDQTPRV-COOH
	188	197	NH2-YLAGNPDIEY-COOH
	189		NH2-LAGNPDIEY-COOH
	260	266	NH2-KQIVTVE-COOH
		269	NH2-KQIVTVEGGL-COOH
	261		NH2-QIVTVEGGL-COOH
	276	284	NH2-WQEQQDEDE-COOH
	290	301	NH2-DEDEQIPSHPPR-COOH
	292		NH2-DEQIPSHPPR-COOH
	315	330	NH2-DEDEDKPRPSRPSQGK-COOH
	486	496	NH2-VVAEQAGEQGF-COOH
		497	NH2-VVAEQAGEQGFE-COOH
		498	NH2-VVAEQAGEQGFEY-COOH
	487	497	NH2-VAEQAGEQGFE-COOH
	502	511	NH2-KTHHNAVTSY-COOH

	503		NH2-THHNAVTSY-COOH
	517	523	NH2-RAIPSEV-COOH
	531	538	NH2-RQSQVSEL-COOH
	545	558	NH2-GPLVNPESQQGSPR-COOH
	548	557	NH2-VNPESQQGSP-COOH
		558	NH2-VNPESQQGSPR-COOH
P04347	54	64	NH2-IETWNSQHPEL-COOH
	93	99	NH2-IIVVQGK-COOH
	113	119	NH2-FEKPQQQ-COOH
		120	NH2-FEKPQQQS-COOH
		122	NH2-FEKPQQQSSR-COOH
	172	185	NH2-DTSNFNNQLDQNPR-COOH
	176		NH2-FNNQLDQNPR-COOH
	177		NH2-NNQLDQNPR-COOH
	188	200	NH2-YLAGNPDIEHPET-COOH
		201	NH2-YLAGNPDIEHPETM-COOH
		208	NH2-YLAGNPDIEHPETMQQQQQQK-COOH
	189	200	NH2-LAGNPDIEHPET-COOH
		201	NH2-LAGNPDIEHPETM-COOH
	190		NH2-AGNPDIEHPETM-COOH
	251	257	NH2-LRSPDDE-COOH
		258	NH2-LRSPDDER-COOH
	275	283	NH2-WQEQEDEDE-COOH
	327	336	NH2-PSRPEQQEPR-COOH
	468	477	NH2-KTHHNAVSSY-COOH
	469		NH2-THHNAVSSY-COOH
	494	502	NH2-YNLGQSQVR-COOH
	495		NH2-NLGQSQVR-COOH
	504	513	NH2-LKYQGNSGPL-COOH
		516	NH2-LKYQGNSGPLVNP-COOH
	505	513	NH2-KYQGNSGPL-COOH
		516	NH2-KYQGNSGPLVNP-COOH
	506		NH2-YQGNSGPLVNP-COOH
	507		NH2-QGNSGPLVNP-COOH

Summary

Obesity is a world-wide health problem with tremendous health care costs (*288*). Weight maintenance is a complex system in which different mechanisms are involved (*13*). One of these mechanisms involves the cholecystokinin receptor type 1 (CCK1R). The CCK1R is a GPCR (G-protein coupled receptor) localized in the gastrointestinal tract that induces a feeling of satiety upon activation by its natural hormone cholecystokinin (CCK). Bioactive peptides, which can be released from food protein, can mimic the effect of CCK and have an influence on satiety. Such peptides could be used as a satiating ingredient in the development of new functional foods for the prevention and treatment of obesity.

We set up a cell-based bioassay in 96 well-plates to screen for such bioactive peptides that can activate the CCK1R, based on the fluorescent visualization of a Ca-flux when the receptor is activated. Fluorescence measurements were done using a plate reader and a confocal microscope and the assay was benchmarked with CCK-8S (sulfated octapeptide), JMV-180 and lorglumide. The confocal microscope appeared to be the preferred measuring device when complex samples had to be measured, as measurements with the plate reader could easily be biased by background fluorescence of the sample. Screening of different food protein hydrolysates showed that some food protein hydrolysates , such as soy protein hydrolysates, possess significant CCK1R activity.

The peptides in the active soy protein hydrolysates were separated by use of size exclusion chromatography, the CCK1R activity of the resulting fractions was re-evaluated and significant *in vitro* CCK1R activity was found. The effect on food intake of the active fractions with a physiological relevant molecular weight was evaluated *in vivo* with rats, but no significant effect could be measured. The amino acid sequences of the peptides present in some promising fractions was identified, however it remained not possible to identify which particular peptide(s) accounted for the CCK1R activity as more than 100 peptides were still present in the fractions. Hence, a highly-selective tool to extract and identify the active peptides was necessary. Therefore, a first onset was made to incorporate the CCK1R into NABBs (nanoscale apo-lipoprotein bound bilayer particles), a unique native-like bilayer membrane system for incorporation of GPCRs, as such nanoparticles could be used as an affinity-selection-mass spectrometry technique to identify CCK1R-binding peptides. Functional incorporation of the CCK1R in NABBs was shown by binding with a fluorescent labeled CCK analog.

Samenvatting

Obesitas is een wereldwijd gezondheidsprobleem met enorme kosten voor de gezondheidszorg (*288*). Gewichtsbehoud is een complex systeem waarin verschillende mechanismen betrokken zijn (*13*). Eén van deze mechanismen heeft betrekking tot de cholecystokinine receptor type 1 (CCK1R). De CCK1R is een GPCR (G-proteïne gekoppelde receptor) gelokaliseerd in het maag-darmkanaal, die een gevoel van verzadiging induceert na activatie door het natuurlijke ligand cholecystokinine (CCK). Bioactieve peptiden, die vrijgesteld kunnen worden uit voedingseiwit, zouden het effect van CCK kunnen nabootsen en zo een invloed hebben op verzadiging. Dergelijke peptiden kunnen worden gebruikt als een verzadigend ingrediënt in de ontwikkeling van nieuwe functionele voedingsmiddelen voor de preventie en behandeling van obesitas. Om te screenen naar peptiden die de CCK1R kunnen activeren werd een cell-based bioassay opgezet in 96-well platen. Activatie van de CCK1R veroorzaakt een Ca-flux in de cel die kan gevisualiseerd worden als een fluorescent signaal. Fluorescentie metingen werden gedaan met behulp van een plaatlezer en een confocale microscoop en de assay werd gestandaardiseerd met CCK-8S (gesulfateerd octapeptide), JMV-180 en lorglumide. De confocale microscoop bleek het meest aangewezen toestel voor meting van complexe stalen aangezien de metingen met de plaatlezer bemoeilijkt werden door de achtergrondfluorescentie van het staal. Verschillende hydrolysaten van voedingseiwit werden gescreend en sommigen, zoals soja-eiwit hydrolysaten, vertoonden significante CCK1R activiteit. De peptiden in de werkzame soja-eiwit hydrolysaten werden gescheiden door middel van gelpermeatiechromatografie, de CCK1R-activiteit van de verkregen fracties werd opnieuw getest en deze vertoonden significante *in vitro* CCK1R activiteit. Het effect op de voedselinname van de actieve fracties met een fysiologisch relevante moleculaire massa werd *in vivo* onderzocht bij ratten, maar geen significant effect kon worden aangetoond. De aminozuursequentie van de peptiden in enkele veelbelovende fracties werd geïdentificeerd, maar het was niet mogelijk om die/dat peptide(n) dat verantwoordelijk is voor de CCK1R activiteit te identificeren, aangezien nog meer dan 100 peptiden aanwezig waren in de fracties. Daarom werd een eerste aanzet gedaan tot het incorporeren van de CCK1R in NABBs (apo-lipoproteïne gebonden bilayer nanopartikels), een unieke natieve bilayer voor incorporatie van GPCRs. Zulke nanodeeltjes kunnen gebruikt worden om CCK1R-bindende peptiden te identificeren door selectie op basis van affiniteit gekoppeld aan massaspectrometrie. De functionele incorporatie van de CCK1R in NABBs werd aangetoond door binding met een fluorescent gemerkt CCK analoog.

Curriculum Vitae Dorien Staljanssens

University address:

Faculty of Bioscience Engineering

Department of Crop Protection

Department of Food Safety and Food Quality

Coupure Links 653

9000 Gent, Belgium

Phone: 0032 9 264 61 48

Dorien.Staljanssens@UGent.be

Nationality: Belgian

Date of birth: 26th of August 1986

Education

2009 – present	**PhD** "Bioactive peptides with CCK1R activity as an aid in the battle against obesity" Under the supervision of Prof. Dr. ir. Guy Smagghe (Department of Crop protection) and Prof. Dr. ir. John Van Camp (Department of Food Safety and Food Quality), **Ghent University**.
2007-2009	**MSc Bioscience Engineering: Food Science and Nutrition** Final year dissertation: "Food protein from insect cells as a source for antihypertensive peptides: *in vitro* and *in vivo* evaluation", Ghent University
2004-2007	**BSc Bioscience Engineering: Chemistry and Food Technology,** Ghent University
1998-2004	Secondary school - main subject: **Latin and mathematics,** OLVP, Bornem, Belgium

Foreign experience

Sep-Dec 2012 and Mar-Jun 2013 I did research in context of my PhD under supervision of Prof. Sakmar at **Rockefeller University** (New York, NY, US)

Publications in international scientific journals with peer-review

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.

STALJANSSENS, D., SMAGGHE, G. & VAN CAMP, J. (2012) Protein-derived bioactives affecting CCK-induced satiety. *Agro Food Industry high-tech,* 23(2), 6-8.

STALJANSSENS, D., VAN CAMP, J., BILLIET, A., DE MEYER, T., AL SHUKOR, N., DE VOS, W. H. & SMAGGHE, G. (2012) Screening of soy and milk protein hydrolysates for their ability to activate the CCK1 receptor. *Peptides,* 34, 226-231.

STALJANSSENS, D., DE VOS, W. H., WILLEMS, P., VAN CAMP, J. & SMAGGHE, G. (2012) Time-resolved quantitative analysis of CCK1 receptor-induced intracellular calcium increase. *Peptides,* 34, 219-225.

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.

STALJANSSENS, D., VAN CAMP, J., HERREGODS, G., DHAENENS, M., DEFORCE, D., VAN DE VOORDE, J. & SMAGGHE, G. (2011) Antihypertensive effect of insect cells: In vitro and in vivo evaluation. *Peptides,* 32**,** 526-530.

Presentations

STALJANSSENS, D., SMAGGHE, G., DE VOS, W.H., GEVAERT, K., SAKMAR, T., VAN CAMP, J. (2012) Potential Activation of Cholecystolinin Receptor by Bioactive Peptides in Protein Nutrients Center for Basic and Translational Research on Disorders of the Digestive System Symposium, New York, United States, October 22, 2012. (poster presentation)

STALJANSSENS, D., VAN CAMP, J., HUYBREGTS, L., VAN AMERONGEN, A., DE VOS, W., GEVAERT, K., BOON, P., RAEDT, R. & SMAGGHE, G. Purification of CCK1R-binding bioactive peptides and evaluation of their effects on rat food intake. 26th Conference of European Comparative Endocrinologists, Zürich, Switzerland, August 21-25, 2012. (oral presentation)

STALJANSSENS, D., DE VOS, W.H., VAN CAMP, J. & SMAGGHE, G. Screening of protein hydrolysates for their potential to activate the CCK1 receptor. 36th FEBS Congress: Biochemistry for tomorrows medicine, Torino, Italy, June 25-30, 2011. (poster presentation)

STALJANSSENS, D., SMAGGHE, G., De Vos, W.H. &Van Camp, J. Screening tool for bioactive peptides with CCK1R activity. NutrEvent. Lille, France, June 15-16, 2011. (poster presentation)

STALJANSSENS, D., DE VOS, W.H., WILLEMS, P., VAN CAMP, J. & SMAGGHE. G. Cell-based bioassay to screen for bioactive peptides with CCK1 receptor activity. 2nd annual meeting of Belgian nutrition society : microorganisms in human nutrition : exploring new pathways for health, Brussels, Belgium, April 29, 2011. (poster presentation)

STALJANSSENS, D., DE VOS, W.H., WILLEMS, P., VAN CAMP, J. & SMAGGHE G. Bioactive peptides with CCK-receptor activity as an aid in the battle against obesity. Annual Conference of International Society of Nutraceuticals and Functional Foods: Nutraceuticals, Functional Foods, and Dietary Supplements: Science, Methodologies, and Applications. Sanur, Indonesia, October 11-15, 2010. (oral presentation)

STALJANSSENS, D., DE VOS, W.H., WILLEMS, P., VAN CAMP, J. & SMAGGHE, G. Comparison of two techniques to measure a CCK1-receptor induced intracellular calcium increase. Advanced Light Microscopy Symposium. Ghent, Belgium, September 23-24, 2010. (poster presentation)

Research experience

- Set-up of cell-based bioassay to screen for bioactive peptides with CCK1R activity.
- Cell-free assay for measurement of ACE inhibition
- Confocal microscopyy
- Cell culture of vertebrate and invertebrate cell lines
- Protein/peptides chemistry and processing: protein purification by pH precipitation, enzymatic hydrolysis, determination of degree of hydrolysis, protein quantification with Kjeldahl/Bradford, SDS-PAGE, Western blot, size-exclusion gel filtration chromatography, GPCR purification
- *In vivo* experiments with rats: oral administration of sample with gavage, measurement of blood pressure and food intake
- Molecular work: subcloning, transformation, transfection
- Production of nanoscale apo-lipoprotein bound bilayer particles (NABBs) and incorporation of CCK1R

Tutoring of undergraduate students

Next to my own research, I have been a tutor for seven undergraduate students who needed to make their final year dissertation.

- 2010: Sarah De Schepper (KaHo Sint-Lieven)
- 2011: Nadin Al Shukor (UGent), Annelies Billiet (HoGent) and Jens Van Vynckt (KaHo Sint-Lieven)
- 2012: Hanne Vriens (UGent), Anneleen Schollier (HoGent) and Umman Tanriverdi (KaHo Sint-Lieven)

Grants and awards

- Full travel and accommodation grant from FWO (Research Foundation Flanders) for a long stay abroad at Rockefeller University, NY (Mar-Jun 2013)
- Full travel and accommodation grant from FWO (Research Foundation Flanders) for a long stay abroad at Rockefeller University, NY (Sep-Dec 2012)
- Full travel and accommodation grant for FEBS Young Scientists Forum and FEBS Congress from FEBS, Turin, Italy, June 2011.
- Award for best poster presentation on 2nd annual meeting of Belgian Nutrition Society, Brussels, Belgium, April 2011.
- Travel grant for Annual Conference of International Society of Nutraceuticals and Functional Foods from CWO (Commission for Scientific Research, Faculty of Bioscience Engineering, Ghent University), Sanur, Indonesia, October 2010.