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THE INFLUENCE OF NUTRIENT- AND TASTANT-INDUCED GUT-BRAIN SIGNALS ON APPETITE, EMOTIONS AND GASTROINTESTINAL SYMPTOMS

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical sciences

November 2018

Acknowledgements – dankwoord

Bedankt.

Een simpel woord, maar zelden meer op zijn plaats dan aan het einde van een doctoraat aangezien de afgelopen 4 jaar zeker geen one-woman-show waren. Deze pagina's zijn voor jullie, want zonder jullie was dit PhD project niet mogelijk geweest.

Eerst en vooral wil ik mijn promotor, Prof. Jan Tack, bedanken. Beste Jan, zonder jou zou er van dit PhD geen sprake geweest zijn. Bedankt om mij de opportuniteit te geven dit project aan te vatten en tot een goed einde te brengen in uw labo. Bedankt voor het vertrouwen en de kansen die u mij gegeven heeft in de voorbije 4 jaar. Uw onuitputtelijke energie en enthousiasme voor uw projecten en het onderzoek van ons allen zijn bewonderenswaardig!

Ik wil uiteraard ook mijn co-promotoren, Prof. Lukas Van Oudenhove en dr. Jessica Biesiekierski, bedanken. Lukas, bedankt voor alle steun tijdens de afgelopen 4 jaar en eerder ook tijdens mijn Master thesis project. Jouw immense drive voor onderzoek werkt enorm aanstekelijk, net als jouw otaku-SAS-level (ik geef toe dat ik dit ook soms verwenst heb ☺). Ik wil je vooral ook bedanken om te allen tijde in mij te geloven, wat er ook gebeurde. Ik neem veel van jouw advies zeker mee in mijn verdere carrière. My PhD would not have been the same without my other co-promotor. Jess, thanks for always supporting and encouraging me throughout this rollercoaster. Early mornings at MR8, weekends filled with study visits, extraction days in the lab, my first oral presentation in San Francisco or that one morning at Brussels Airport (will never forget ☺), … you were always by my side. Despite your hectic new research carrier down under, you never let me down during the past months and I would like to express my sincere gratitude for that. Besides, I would like to thank you for your support beyond the supervisor-student relationship. I could always count on you when I needed a hug or a peptalk. I could not have wished for a better supervisor and supportive friend than that crazy, pink-haired, Aussie Bersa.

My sincere gratitude goes out to my external jury members, Prof. Masclee and Prof. Beglinger for taking the time and effort to review my doctoral thesis. It is a great honour that my PhD is critically reviewed by such experts in the field of neuro-gastroenterology research. Ik wil ook graag mijn interne juryleden, Prof. Vandenbulcke en Prof. Van den Bergh, bedanken die mij gedurende de voorbije 4 jaar hebben bijgestaan met goede raad en waardevolle bemerkingen. Prof. Van Diest, u wil ik van harte bedanken om in een latere fase ook deel uit te maken van mijn jury en aanwezig te zijn op mijn publieke verdediging.

During the past 4 years, I was proudly part of the LaBGAS team. Giao, Nathalie, Fons, Anne-Christin, Imke, Egbert, Eddy, Daniëlle and Boushra, thank you for contributing both scientifically and personally to my PhD project. It was a pleasure working with all of you and I enjoyed our LaBGAS motto: "work hard, eat hard, drink hard" every time. Giao, bedankt om mij de wondere wereld van SPM te leren kennen. Ik heb altijd heel veel waarde gehecht aan je eerlijke en no-nonsens advies. Nathalie, bedankt om mij reeds als master thesis student onder je vleugels te nemen, om me in de laatste momenten altijd een hart onder de riem te steken en natuurlijk ook voor alle momenten daartussen. Fons, I cannot express my gratitude enough for your help with the phMRI analyses. Sometimes I still don't understand the Fons-working-way ☺, but our collaboration has been of great value. Anne, thank you for bringing that German Pünktlichkeit to the lab. I enjoyed working with you very much and definitely enjoyed your guiding skills at our LabBGAS-goes-Basel weekend!

Ik wil graag ook de andere collega's (Ans, Nick, Wout, Karen, Florencia, Hannelore, Annelies, Jasper, Pieter, Dorien, Alison, Karlien, Eveline, Lien, Lucas, Ricard, Joran, Lieselot, ...) van onze onderzoeksgroep bedanken voor de samenwerking, hun kleine of grote bijdrage aan dit doctoraat maar vooral ook voor de leuke sfeer. Ik was met veel plezier een onderdeel van dit team op congressen in binnen- en buitenland, talrijke diners, pizza-lunches of hilarische weekendjes in de Ardennen. Joran en Eveline, bedankt voor al jullie hulp met de talloze RIA's en andere assays van dit project. Ans en Imke, vrouwkes, ik kan niet genoeg benadrukken hoeveel jullie steun en zelfs gewoon jullie aanwezigheid tijdens mijn doctoraat voor mij heeft betekend. Jullie stonden altijd voor mij klaar, wetenschappelijk maar vooral ook persoonlijk. Wijn-tandem-tripjes in Healdsburg, een shopping spree waar dan ook, ontelbare uitjes naar Baracca, wekelijkse sportieve momentjes maar ook een Whatsapp geschiedenis om U tegen te zeggen. Bedankt om samen met mij te lachen, maar ook om mij de beste schouders om op uit te huilen aan te bieden wanneer het nodig was. Ik sta er dan ook op dat we deze kleine dingen blijven verderzetten in de toekomst en ik beloof jullie dat ik ook altijd voor jullie klaar zal blijven staan als onze professionele wegen gescheiden zijn. Als uitbreiding hiervan wil ik graag "de vriendjes van Brecht"-groep bedanken. Charlotte en Charlotte, maar ook Eveline, bedankt voor toffe etentjes, lunches en leuke momenten die ik met jullie heb mogen delen in Leuven maar ook daarbuiten. Brecht, je relativeringsvermogen, humor en arsenaal aan hilarische uitspraken brachten echt een verademing naast de lange studiedagen. Prof. Vanuytsel, Tim, bedankt voor al je waardevolle raad en hulp bij het rekruteren van de NCGS patiënten. Ik waardeer het enorm dat je ondanks je drukke agenda altijd bereid was om vragen te beantwoorden of advies te geven wanneer ik er om vroeg. Annelies, jou wil ik bedanken voor je enorme inzet tijdens het laatste jaar. Door je zelfstandigheid en gedrevenheid, liet ik met het volste vertrouwen het gluten project aan je over en zag ik je als snel niet meer als mijn student, maar als mijn collega.

Een welgemeende dankjewel gaat uit naar Phyllis en Cindy voor hun praktische en administratieve ondersteuning.

Ook buiten mijn eigen onderzoeksgroep wil ik graag een aantal mensen bedanken.
De collega's van radiologie, Ron, Guido, Kris en Stefan, bedankt voor al de technische ondersteuning die jullie hebben geboden en voor jullie oneindige hulpvaardigheid wanneer ik voor 07h weer eens aan de lijn hing om te melden dat de scanner niet deed wat ik wilde of het infuus prikken niet van een leien dakje liep. Prof. Dupont, bedankt voor al je hulp met het programmeren van scripts voor onze fMRI analyses. Ik heb een enorme bewondering voor je kennis en ben enorm blij dat ik de kans heb gekregen om hiervan te mogen leren tijdens mijn PhD. Jonas, bedankt voor je hulp wanneer ik weer een "simpel" programma wou maken in Affect maar ook om jouw passie voor onderzoek met mij te delen tijdens mijn master thesis project. Bedankt aan de mensen van groep Prof. Kristin Verbeke om een luisterend oor te bieden en voor jullie leuke babbels en heerlijke gebakjes in onze bureau. Anja, jou wil ik enorm bedanken om je te ontfermen over de analyses van de intestinale permeabiliteit. Bedankt aan Prof. Inge Depoortere voor de ondersteuning bij de RIA's en het bitter project.

Natuurlijk stopt het niet bij de mensen die een wetenschappelijk steentje hebben bijgedragen, ook daarbuiten was dit PhD project niet mogelijk geweest zonder de onmetelijke steun van anderen.
Aan mijn vriendinnen en vrienden die elk op hun eigen manier steun geboden hebben of voor ontspanning gezorgd hebben in de voorbije 4 jaar: bedankt! Marlies, Sofie, 4LA-meisjes, vrienden van Jay, … bedankt voor leuke etentjes, drankjes, BBQ's, komen-eten diners, weekendjes weg, ... maar vooral voor jullie vriendschap. Claes, wat zou ik toch gedaan hebben zonder jouw ontelbare peptalks, voice berichtjes, foto's of speculaasjes en chocolaatjes? Je stond altijd voor mij klaar om naar mijn gezeur te luisteren wanneer het tegen stak en stond meteen paraat om me op te beuren, ondanks dat alles wat ik ratelde waarschijnlijk Chinees was. Ik waardeer onze vriendschap echt enorm. Vicky, onze levens zijn verschillend maar toch probeer je altijd te luisteren en begrip op te brengen. Je deur staat altijd open wanneer het nodig is en ik wil je nogmaals bedanken hiervoor. BMW-meisjes, Marthe, Charlotte, Daphne en Yasmine, ondanks dat onze wegen na onze studie gescheiden zijn, ben ik blij dat we elkaar niet uit het oog verloren zijn en ik kijk altijd met veel plezier uit naar onze diner dates. Yasmine, al tijdens onze master thesis stond jij altijd klaar met uitstekende goede raad en tot op de dag van vandaag, waardeer ik jouw professioneel advies en je mening nog altijd enorm. Stef, Jerre en mini-Bruce, avondwandelingen in de zomer of weekendjes weg waren altijd momenten om naar uit te kijken.

Graag wil ik me nu richten tot mijn lieve familie.

Mama, Papa, in de eerste plaats bedankt voor de kansen die jullie mij hebben gegeven, daarzonder zou ik hier nu niet gestaan hebben. Jullie hebben mij door dik en dun gesteund tijdens mijn studies en later ook tijdens mijn doctoraat, ook al was het vaak een ver-van-jullie-bed-show. Ik betwijfel of ik het vaak genoeg zeg, maar bedankt voor alle kleine en grote dingen die jullie voor mij doen en gedaan hebben, om mij te steunen in elke beslissing die ik neem, om mij op te vangen wanneer ik het nodig had, om vaak enthousiaster te zijn met wat ik bereikte dan ik zelf maar vooral ook gewoon bedankt om wie jullie zijn. Ik hou van jullie!

Guy, Patricia, bedankt om van mijn ouders gelukkige mensen te maken en vanuit de grond van mijn hart bedankt om voor mij klaar te staan en trots op mij te zijn alsof ik jullie eigen dochter ben. Maggy en Barto, bedankt voor de warme plek in jullie gezin. Talou, Nonke Raf, Aron, Minnie, bedankt voor de vaste gezelligheid bij oma en opa. Vanaf nu onderbreek ik deze niet meer voor studies, experimenten of PhDs. Hetzelfde geldt voor Tante Tina, Erwin en Victor!

Hiernaast wil ik uiteraard ook mijn grootouders bedanken. Meme en Bo, bedankt voor de warme plek voor en na de dagen op de middelbare school en lang daarna. Oma en opa, waar moet ik in godsnaam beginnen om jullie te bedanken? Dag in, dag uit staan jullie voor mij klaar met een helpende hand, een overvloed aan lekker eten en gebak, flauwe grapjes, briefjes tussen mijn kleren of in mijn koffer, sms'jes op elk ogenblik van de dag maar vooral met een hart van goud. Woorden schieten te kort om uit te drukken hoe hard ik dit apprecieer. Ik weet dat jullie trots zijn op mij maar ik ben ook enorm trots dat jullie erbij waren om mijn doctoraat te vieren. Ik hoop dat ik jullie op al de verdere stappen in mijn leven nog lang aan mijn zijde kan meenemen.

Mijn laatste woord van dank gaat uit naar mijn vriend en rots in de branding. Jay, je hebt me de afgelopen 8 jaar zien groeien doorheen mijn studies en mijn PhD, met de nodige struggles die erbij kwamen kijken. Waarschijnlijk heb jij soms even hard afgezien van dit doctoraat als ikzelf ☺. Je nuchtere kijk op de dingen heeft me meer dan eens vooruitgeholpen. Ik wil je bedanken voor je onuitputtelijke steun en geduld maar ook voor je begrip wanneer mijn weekends weer eens met wetenschap in plaats van met quality time gevuld waren. Ik zie u graag en ik kijk enorm uit naar alles wat de toekomst nog voor ons in petto heeft.

Julie

Sint-Truiden, oktober 2018

List of abbreviations

ACC	Anterior cingulate cortex
AgRP	Agouti-related peptide
AIC	Akaike's information criterion
ARC	Arcuate nucleus
ASL	Arterial spin labelling
ATI	α-amylase trypsin inhibitors
BA	Brodmann area
BED	Binge eating disorder
BMI	Body mass index
BOLD	Blood oxygenation level-dependent
CAR	Cortisol awakening response
CART	Cocaine- and amphetamine regulated transcript
CBT	Cognitive behavioural therapy
CCK	Cholecystokinin
CNS	Central nervous system
CSF	Cerebrospinal fluid
DB	Denatonium Benzoate
DEBQ	Dutch eating behaviour questionnaire
dlPFC	Dorsolateral prefrontal cortex
DPP4	Dipeptidyl peptidase IV
EDI	Eating disorder inventory
EEC	Enteroendocrine cell
EPI	Echo planar imaging
FGID	Functional gastrointestinal disorders
FID	Food incentive delay (task)

fMRI	Functional magnetic resonance imaging
FODMAP	Fermentable oligo-, di-, and mono-saccharides and polyols
FWHM	Full width at half maximum
GFD	Gluten-free diet
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal
GLM	General linear model
GLP-1	Glucagon-like peptide-1
GOAT	Ghrelin O-acyltransferase
GPCR	G-protein coupled receptor
HC	High-calorie
HLA	Human leukocyte antigen
HPA	Hypothalamus-pituitary-adrenal
HPLC	High performance liquid chromatography
hsCRP	High sensitive C-reactive protein
HV	Healthy volunteers
IBS	Irritable bowel syndrome
IgA-tTg	Tissue transglutaminase, Immunoglobulin A
ITI	Intertrial interval
LC	Low-calorie
LMR	Lactulose-mannitol ratio
MMC	Migrating motor complex
MNI	Montreal neurological institute
NA	Negative Affect
NCGS	Non-coeliac gluten sensitivity
NCS	Non-caloric sweeteners

NF	Non-food
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OFC	Orbitofrontal cortex
PA	Positive affect
pACC	Perigenual anterior cingulate cortex
PAG	Periaqueductal gray
PANAS	Positive and negative affect schedule
PBN	Parabrachial nucleus
PFC	Prefrontal cortex
phMRI	Pharmacological magnetic resonance imaging
PHQ	Patient health questionnaire
PMSF	Phenylmethanesulfonyl fluoride
POMC	Pro-opiomelanocortin
POMS	Profile of mood state
PVN	Paraventricular nucleus
PYY	Peptide YY
QHCl	Quinine hydrochloride
rCBF	Regional cerebral blood flow
RIA	Radioimmunoassay
ROI	Regions of interest
rsFC	Resting state functional connectivity
SAM	Self-assessment manikin
SEM	Standard error of the mean
SMA	Supplementary motor area
SP	Snack points

SPM	Statistical Parametric Mapping
STPI	State trait personality inventory
TAS1R	Taste 1 receptor
TAS2R	Taste 2 receptor
TE	Echo time
TLR4	Toll-like receptor 4
TR	Repetition time
TRC	Taste receptor cell
VAS	Visual analogue scale
VTA	Ventral tegmental area
WM	White matter

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CHAPTER 1

General introduction

Part of this content has been published in a slightly different form as: Weltens N*, **Iven J***, Van Oudenhove L°, Kano M°. The gut-brain axis in health neuroscience: implications for functional gastrointestinal disorders and appetite regulation. *Ann N Y Acad Sci*. 2018 Sep;1428(1):129-150.
* These authors contributed equally to this work as first authors. ° These authors contributed equally to this work as senior authors.

1 General introduction

1.1 Brain-gut axis

"Gut feelings", "having butterflies in your stomach", "having a knot in your bowel" or "trust your gut" are commonly used expressions, indicating that a strong connection between our gut, our brain, and our emotions is widely recognised. This interaction between the brain and the digestive tract was first studied during the 19th and early 20th century and since then, a two-way relationship has been assumed (1). The bidirectional, neurohumoral signalling system between the gut and the brain enabling this interaction, is referred to as the brain-gut axis. This system allows information regarding the physiological state of the body to be transferred, not only from the gut to the brain, but also brain-to-gut (e.g. alteration of gastrointestinal (GI) function by stress and emotions) (2). More broadly, the brain-gut axis, as part of a larger interoceptive system, enables coordination between peripheral processes and a person's overall physical and emotional state (3). This coordination occurs via integration of afferent, homeostatic gut-brain signals with exteroceptive sensory signals and ongoing cognitive-affective processes (1, 4).

1.1.1 The brain-gut axis in the regulation of food-intake

Adequate food intake is crucial for maintaining energy levels and, hence, survival. Food intake is regulated by a homeostatic system signalling energy balance, which is part of the brain-gut axis (5). One of the most potent drivers of the consumption of food is its rewarding nature. The rewarding value of food is not only dependent on its exteroceptive properties (vision, smell, taste), but also on the energy status of the body; the same food items can be rewarding when one is hungry, but can be less pleasant to consume after satiation. This reflects a complex interplay between the homeostatic system and a reward-driven, hedonic signalling circuit (6).

1.1.1.1 Homeostatic control of feeding

The hypothalamus and several brainstem nuclei (including the nucleus of the solitary tract (NTS) and parabrachial nucleus (PBN)) are considered key homeostatic brain areas (7). These brain areas receive information related to the body's nutritional needs and energy resources from neural and endocrine peripheral signals conveyed through vagal and spinal afferents that innervate the proximal and distal part of the GI tract, respectively (8).

GI afferent nerve fibres terminate within the wall of the GI tract and are classified as mesenteric, serosal, muscular, ganglionic or mucosal endings depending on their terminal distribution, which also determines their functionality. Mucosal afferents respond to distortion of the mucosal epithelium or to chemicals present in the lumen of the gut, and muscular afferents respond to stretch and distention.

Consequently, these two types of afferents are indispensable in the regulation of chemo- and mechanosensitivity of the GI tract, respectively (9). A combination of signals from these visceral afferents allows information regarding the luminal content (e.g. food) and mechanical state (e.g. gastric distention, motility) to be transferred to the central nervous system (CNS). Primary afferent fibres that innervate essentially all tissues of the body terminate in the most superficial layer (lamina I) of the spinal and trigeminal dorsal horns, which project to autonomic cell columns of the spinal cord and then to homeostatic integration sites in the brainstem. The main target of lamina I fibres is the PBN (the main brainstem homeostatic integration site), which then projects to the periaqueductal gray (PAG; the homeostatic motor centre) and the thalamus. Together with afferent information transmitted via the NTS, a direct representation of the internal state of the body is created (1, 3, 10) (Figure 1.1). The homeostatic afferent information in the thalamus is further transmitted via two complementary routes (i.e. the medial thalamic nuclei and the basal ventral medial nucleus of the thalamus) to the anterior cingulate cortex (ACC) and insular cortex, respectively. These regions are primarily involved in encoding affective-motivational and sensory-discriminative aspects of visceral sensory information, respectively, corresponding to the simultaneous generation of both a sensation and a motivation (3, 10).

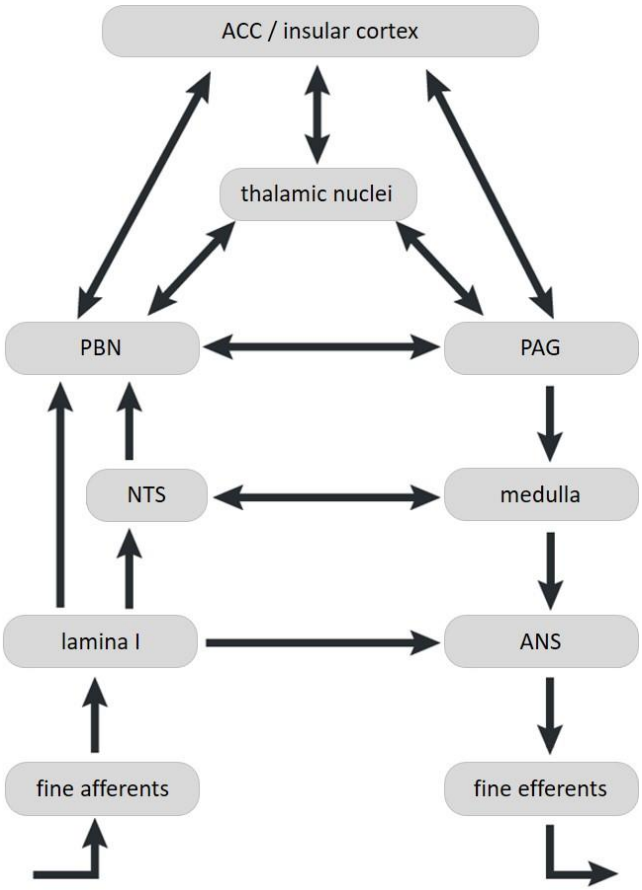

Figure 1.1 Neural homeostatic signalling
Small-diameter afferent fibres that signal the physiological condition of the body terminate in lamina I of the spinal and trigeminal dorsal horns. Lamina I neurons project to the nucleus of the solitary tract (NTS) within the brainstem. The main target is the parabrachial nucleus (PBN), which projects to the periaqueductal gray (PAG). Finally, the information is send, through thalamic nuclei to the anterior cingulate cortex (ACC) and insular cortex. Adapted from Craig et al. (3).

In addition to this viscerosensory pathway, gut-brain signalling mechanisms also include a number of peptide hormones, secreted by enteroendocrine cells (EEC) throughout the GI tract in response to food intake (anorexigenic hormones, e.g. glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY)) to induce satiety, or in response to fasting to initiate food intake (orexigenic, e.g. ghrelin) (11). Detailed information on the different gut peptides can be found in the following section 1.1.1.3. As previously mentioned, the hypothalamus has a prominent role in the central regulation of appetite. Besides neural signals, hormonal signals also directly and indirectly converge in this brain area (12). The primary site of action for gut hormones is the arcuate nucleus (ARC) in the ventral hypothalamus. Due to its anatomical location near the median eminence (where the blood-brain-barrier is incomplete), it is easily accessible for circulating levels of hormones (13). Within the ARC, two distinct subpopulations of neurons are implicated in the regulation of feeding behaviour, with opposing effects on food intake. The first subset of neurons are co-expressing agouti-related peptide

(AgRP) and neuropeptide Y (NPY). AgRP/NPY neurons are activated by food deprivation and rapidly inhibited by food ingestion, and regulate feeding by incorporating excitatory and inhibitory inputs (14). Apart from the AgRP/NPY expressing neurons, a second important ARC neuronal population, which inhibits food intake upon activation, expresses pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (15). The paraventricular nucleus (PVN) in the hypothalamus, together with the ventral tegmental area (VTA), are important targets of these ARC neurons; in turn, they signal to higher reward-related brain areas such as the amygdala and nucleus accumbens to regulate appetite (16, 17). This confirms the idea that AgRP/NPY neurons translate information on the body's internal state (e.g. hunger) into sustained appetitive and consummatory behaviour via a downstream neural network integrating homeostatic and reward/cognitive mechanisms (18).

Similar to the ARC, the area postrema of the brainstem lacks a complete blood-brain barrier, thereby receiving humoral peripheral satiety signals. By virtue of these signals as well as its extensive vagal input, the brainstem serves as a second homeostatic integrator controlling food intake (12). A neural pathway between the brainstem and the hypothalamus exists, where the dorsal vagal complex (a complex of the dorsal motor nucleus, area postrema and the NTS in the caudal brainstem) is crucial in the processing and transmission of vagal afferent signals to the hypothalamus. This signalling system serves as an alternative pathway through which gut hormones can exert their (an-)orexigenic effects (13, 19).

Regardless of the route of transmission of information (neurally or humorally) to the hypothalamus, an efferent signal is generated and transmitted via the brainstem downwards to modulate appetite and GI function (12). For example, the PAG receives excitatory signalling from the prefrontal cortex and inhibitory signalling from the amygdala and projects to the rostroventral medulla, which modulates sensitivity of spinal dorsal horn neurons to afferent information from within the GI tract (9).

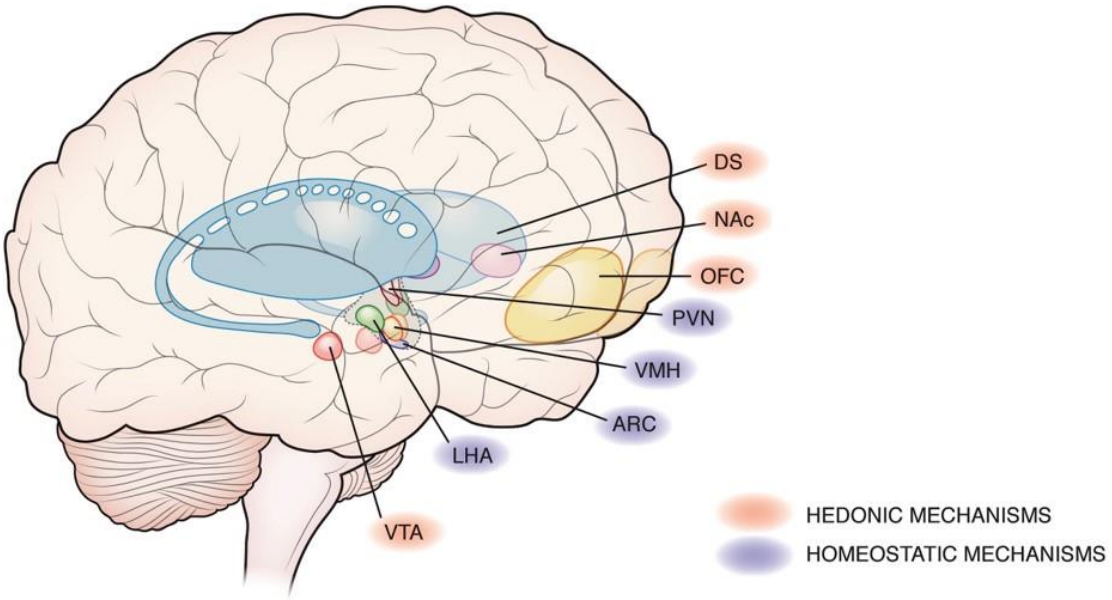

Figure 1.2 Brain areas associated with homeostatic and hedonic control of feeding.
VTA: Ventral tegmental area, LHA: Lateral hypothalamic area, ARC: arcuate nucleus, VMH: ventromedial hypothalamus, PVN: paraventricular nucleus, OFC: orbitofrontal cortex, NAc: nucleus accumbens, DS: dorsal striatum. From Tulloch et al. (20).

1.1.1.2 Hedonic control of feeding

The homeostatic-metabolic processes to drive appetite and food intake described above are integrated with signals from a neural system implicated in hedonic control of food intake that attributes rewarding properties to food items (Figure 1.2) (21). This complex, extensive neural network was first described in 1954 (22) and is involved in the motivational, reinforcing, emotional as well as learning aspects of (food) reward. It includes midbrain regions such as the ventral tegmental area (VTA) and substantia nigra, together with their (dopaminergic) projections to the nucleus accumbens (ventral striatum), putamen and caudate (dorsal striatum), pallidum, amygdala, (p)ACC, OFC, and (anterior) insula (20, 23). The OFC and amygdala are involved in the encoding of information related to the reward value of food and moreover, the amygdala is the primary brain area regulating appetite in response to emotions (24, 25). The insula processes information related to the taste of food and consequently, its hedonic evaluation. The nucleus accumbens and dorsal striatum are responsible for the encoding motivational and incentive properties of food (24). Integration of interoceptive homeostatic signals with oral (taste) as well as exteroceptive sensory input such as sight and smell, and ongoing cognitive-affective processes ultimately determines the reward value of food, including its motivational (incentive salience, 'wanting') and hedonic (subjective pleasure, 'liking') properties, which profoundly impact on appetite and food intake (26).

Normally, the connection between homeostatic and hedonic control of eating is in balance, but it is clear that hedonic processes can easily overrule the homeostatic control of energy balance and stimulate eating in the absence of nutritional or caloric deficiency, thereby (in the long term) driving weight gain and obesity. Our Western environment, inundated by food cues and actual foods that are readily and cheaply available, amplifies facilitating this innate desire to eat (6).

1.1.1.3 Gut peptides

Changes in gut-brain signalling follow the hunger and satiety and feeding cycles. As mentioned earlier, these gut-brain signals include production and secretion of several (an-)orexigenic gut peptides by EEC found in the mucosal layer of the gut (16).

Ghrelin

Ghrelin, a 28-amino acid peptide also known as the "hunger hormone", is the main orexigenic peptide of the body and is an endogenous ligand for the growth hormone secretagogue receptor 1a (GHSR) (16, 27). The most abundant source of circulating ghrelin are X/A-cells of the oxyntic glands of the gastric fundus (27). Ghrelin is also synthesised lower in the GI tract, but to a lesser extent compared to the production in the stomach, with a diminished amount of ghrelin produced with increasing distance from the pylorus (28). Ghrelin is produced during fasting, resulting in peak levels before meals, which fall rapidly after nutrient ingestion, where the magnitude of ghrelin reduction is proportional to the amount of calories ingested (29). Those preprandial peaks are associated with meal initiation. Such increased appetite has not been observed for other gut peptides (16, 19, 28).

To obtain its biologically active characteristics, ghrelin requires post-translational modification where a fatty acid side chain (preferably C8 or C10, derived from ingested dietary lipids) is covalently linked to its serine-3 residue by ghrelin O-acyltransferase (GOAT) (29, 30). This acylation is indispensable for ghrelin to bind to the GHSR1a and cross the blood-brain barrier (28). GHSR are expressed on the vagal nerve and within the CNS, among others in the ARC of the hypothalamus where activation of the receptor by binding of ghrelin stimulates the secretion of NPY and AgRP (19).

Recently, the classic view of ghrelin's function as "hunger hormone" has been expanded. GHSR are also localised within the VTA, part of the mesolimbic dopaminergic pathway. By virtue of its projections to the nucleus accumbens, among others, it has also been shown to be important for motivation-related behaviours, including motivational aspects of feeding (28, 29).

Motilin

Motilin is a 22-amino acid hormone synthesised during fasting and after food intake by endocrine M-cells in the upper part of small intestinal, mostly duodenal and jejunal, mucosa. Smaller amounts are present in other GI tract regions, including the gastric antrum (31). Release of motilin coincides with phase III of the migrating motor complex (MMC), a cyclic motor pattern in the GI tract that occurs during the interdigestive state, with a cycle duration of 90-120 minutes (32). This pattern is divided in three phases, of which phase III is the most active, with bursts of contractions originating in the antrum or duodenum and migrating distally. The role of this phase is to clear residual food particles and to prepare the stomach for a next meal. Phase III is characterised by "hunger contractions"; the rumbling noise of the stomach during the fasted state associated with hunger feelings (33).

Although previous studies have shown that motilin receptors are expressed in the brain, their exact role remains unexplored (34).

Cholecystokinin

CCK was the first gut hormone demonstrated to have an effect on food intake (35). CCK is composed of varying numbers of amino acids, ranging from 8 to 83 amino acids, depending on post-translational modification of its 115-amino acid precursor preprocholecystokinin (36).

After food intake, especially of lipids and proteins, CCK is rapidly released by I cells, located primarily in the upper small intestine. Plasma CCK levels peak within 15 minutes after the meal, but its effects are short-term, given its half-life of 1 to 2 minutes (19, 37).

Two receptor types have been identified: CCK1R, which is predominantly expressed in the GI tract, whereas CCK2R predominates in the brain. The former is involved in the anorexigenic effect of CCK, as has been shown by pharmacological and genetic experiments. The CCK1R is expressed on vagal afferents and within the CNS in the hypothalamus and brainstem. Peripheral administration of CCK increases neuronal activation in brainstem areas and modulates vagal afferent firing, which then relays satiety signals to the brainstem. This finding indicated a critical signalling pathway between the vagal nerve and the brainstem, involved in CCK-induced satiation (38, 39). Additionally, CCK1R are found on peripheral tissues including the gallbladder and pancreas, regulating bile excretion and release of pancreatic peptides. Its postprandial release initiates an intestinal feedback loop to control GI functions, such as a short-term inhibition of gastric emptying and acid secretion, through CCK1R on vagal afferent terminals that run in the lamina propria, adjacent to the mucosal epithelium (37).

Glucagon-like peptide 1

GLP-1 is a 30-amino acid circulating hormone released from enteroendocrine L-cells, predominantly located in the mucosa of the ileum and large bowel (40). It is formed by cleavage of the pre-pro-glucagon gene by prohormone convertase 1 and is further processed after release to its equipotent biological active forms GLP-1(7-37) and GLP-1(7-36), the latter being the major circulating form in humans (19, 38).

GLP-1 levels are low in the fasted state and rise after a meal. The main trigger of GLP-1 release is the intraluminal presence of nutrients, specifically carbohydrates and fat, after which the hormone is released into the systemic circulation, in proportion to the ingested caloric load (13, 37, 40). Its mechanism of action occurs via augmentation of a glucose-dependent post-prandial insulin release, inhibition of glucagon release and a delay in gastric emptying. This integrative role in post-prandial digestion is accompanied by an inhibitory effect on food intake (19, 39).

GLP-1 receptors are centrally expressed within the NTS and hypothalamus, as well as peripherally on vagal afferents (37, 38). The latter is regarded as the main route of action for peripheral GLP-1 to induce anorexigenic effects, as vagotomy abolishes the decrease in food intake caused by GLP-1. Moreover, as GLP-1 is rapidly broken down by dipeptidyl peptidase IV (DPP4), which results in a short half-life of 1 to 2 minutes, it is questionable whether peripheral GLP1 can activate central receptors through humoral routes (19, 38).

Peptide YY

PYY is a 36-amino acid peptide and is a member of the PP-fold family, which also includes NPY (19). Two main endogenous forms have been identified: PYY(1-36) and PYY(3-36). The latter is formed by cleavage of the N-terminal tyrosine-proline residues from PYY(1-36) by DPP4 and is the major postprandially circulating, biologically active form (6, 40, 41). Release of PYY occurs within 15 minutes after food intake (with a greater release after lipid ingestion compared to carbohydrates and proteins (19)) from mucosal enteroendocrine L-cells, in proportion to the energy load of the meal, and reaches a plateau 1 to 2 hours after meal consumption. Circulating levels remain elevated for up to 6 hours (37, 39, 41).

PYY is a selective substrate of the Y2R, highly expressed in NPY neurons within the ARC. Activation of Y2R results in inhibition of NPY and AgRP release along with disinhibition of POMC/CART neurons, which in turn results in the inhibition of (homeostatic) food intake (41). Furthermore, Y2R is also expressed in the nodose ganglion of the vagus nerve and the NTS, both providing ascending

information to the hypothalamus, indicating that PYY(3-36) can also influence the ARC neurons indirectly via vagal-brainstem pathways (13, 41).

As for ghrelin, the role of PYY outside of homeostatic control has gained interest over the past decade. In addition to a positive correlation between brain activity in the hypothalamus and PYY levels, brain activity in regions relevant for decision-making and reward processing (including the left OFC, ACC and ventral striatum), has been shown to increase with increasing plasma levels of PYY (40, 42).

An overview of the pathways by which gut hormones regulate energy homeostasis is found in Figure 1.3.

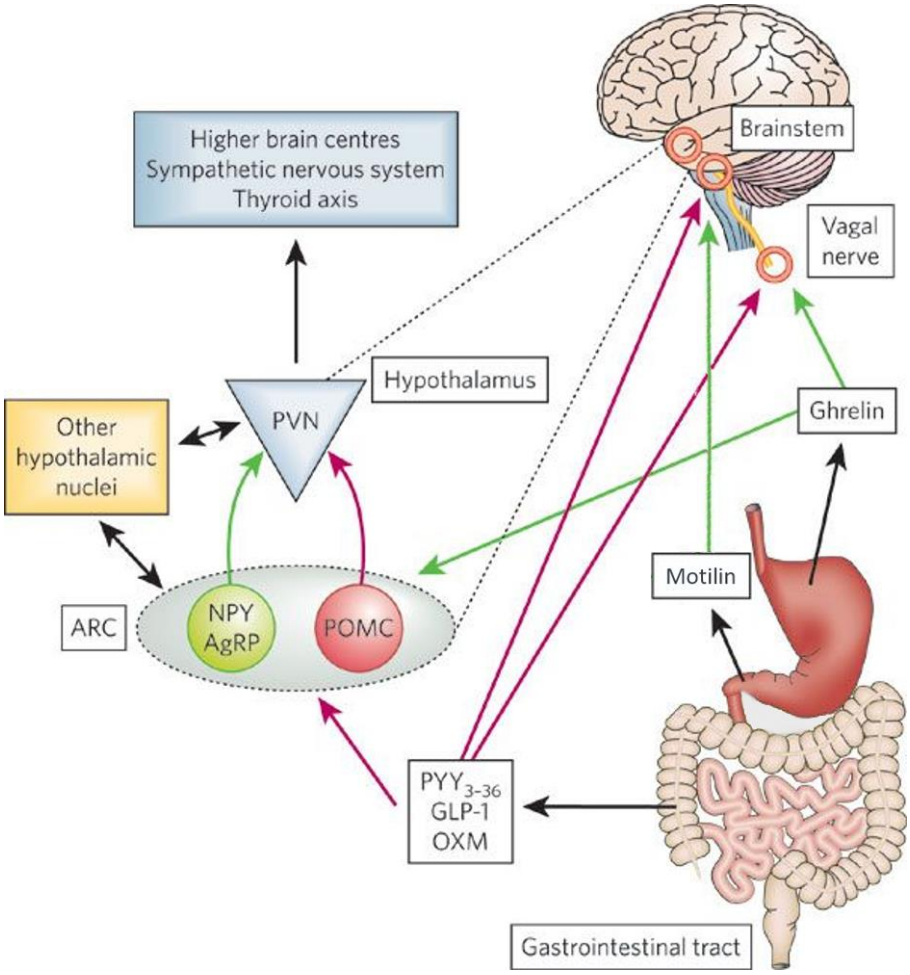

Figure 1.3 Pathways of gut hormones to regulate energy homeostasis
PYY and GLP-1 are postprandially released from L-cells in the lower gastrointestinal tract. Ghrelin is released from X/A-cells in the stomach during fasting. Motilin is released from M-cells in the duodenum and jejunum in cyclic phases, following the hunger cycle. Gut hormones signal directly to the hypothalamus and brainstem or through vagal afferents to stimulate or inhibit food intake. NPY/AgRP and POMC neurons signal to the PVN and other hypothalamic nuclei to increase or decrease appetite, respectively. Green arrows indicate orexigenic signals and red arrows indicate anorexigenic signals. PYY: peptide YY, GLP-1: glucagon-like peptide 1, NPY: neuropeptide Y, AgRP: agouti-related peptide, POMC: pro-opiomelanocortin, PVN: paraventricular nucleus, ARC: arcuate nucleus. Adapted from Murphy et al. (16).

1.2 The gut can taste what we eat

The activation of EEC and the consequent release of the abovementioned gut peptides, representing key metabolic gut-brain signals, is not a consequence of caloric properties of ingested food only (38). Recently, it has been shown that tastant molecules, without caloric properties, can also induce satiation (43). It has been discovered that intestinal EEC express taste receptors, similar to those present on lingual taste buds, on their luminal membrane. Activation of intestinal taste receptors activates the same post-receptor chemosensory signalling pathway, previously thought to be unique to taste buds (Figure 1.4) (43, 44). Several EEC throughout the gut wall express G-protein coupled receptors (GPCR) for sweet taste (TAS1R2/3), bitter taste (TAS2R) and the taste-specific G-protein Gα-gustducin. Stimulation of taste receptors leads to the activation of α-gustducin, which is coupled to the GPCR, resulting in a signalling cascade via phospholipase C that ultimately results in increased intracellular Ca^{2+} concentration and depolarisation of the cell. This causes the release of neurotransmitters or gut peptides, which will then activate sensory nerves (44, 45).

Out of the five taste qualities (bitter, sweet, salty, sour and umami) humans can sense, bitter tastants and bitter taste receptors of the TAS2R family are of particular interest for this doctoral thesis. The human genome encodes 25 different TAS2Rs (44). Within the GI tract, taste-signalling molecules are present in solitary cells in the epithelial layer and gustducin is co-localised with GLP-1 and PYY in L cells and with ghrelin in X/A cells within the stomach. Furthermore, TAS2R are found in an EEC cell line (STC-1 cells) resulting in the release of CCK (46). The influence of bitter TRC activation on gut peptide release can be considered an additional defence mechanism to the lingual bitter taste detection, to limit further ingestion of (toxic) food (47).

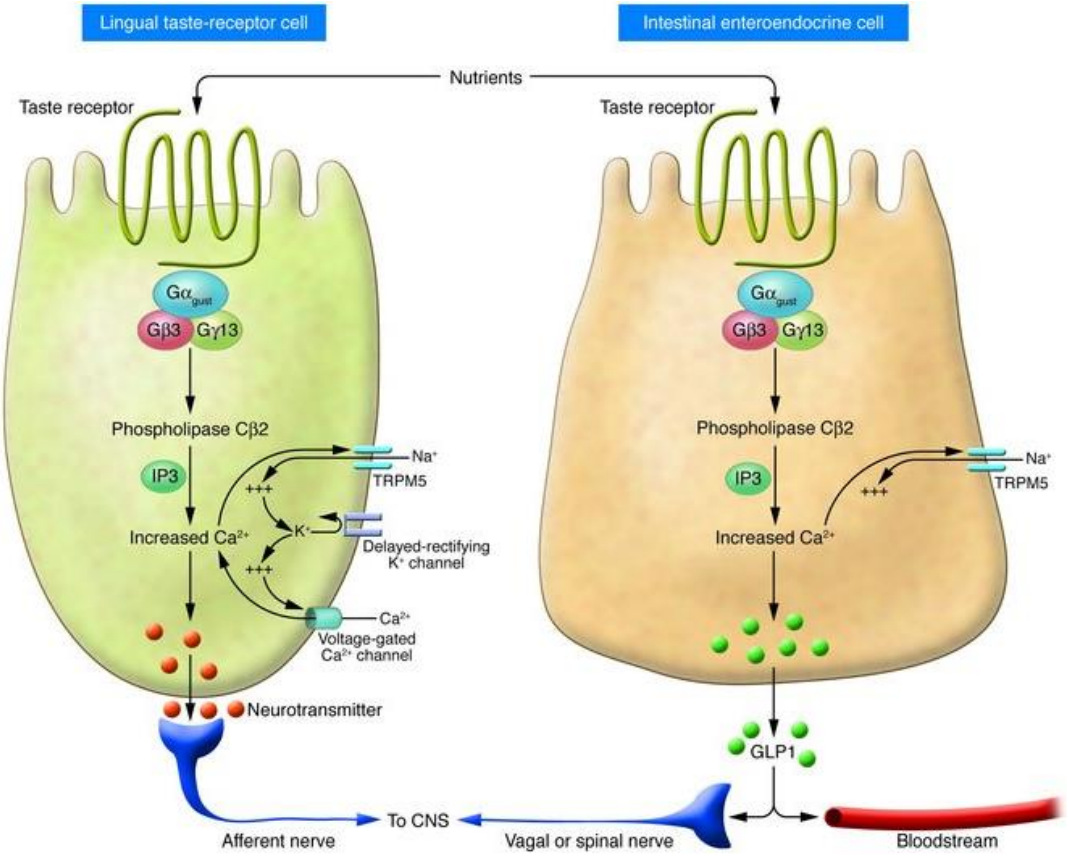

Figure 1.4 Comparison of lingual taste-receptor cells with intestinal enteroendocrine cells
The taste transduction signalling pathway is not only present on lingual cells but is also expressed on enteroendocrine cells of the gastrointestinal tract. Activation of the taste receptor on the apical side of both cell types, leads to a cascade of transduction mediators, which will eventually lead to an increased intracellular calcium concentration. In lingual cells, this leads to the release of neurotransmitters into synapses with afferent nerve fibres that relay information to the CNS. In enteroendocrine cells, this leads to the release of gut peptides (e.g. GLP-1), which enters the circulation or interact with nearby afferent nerve terminals (e.g. from vagal or spinal neurons). CNS: central nervous system, GLP-1: glucagon-like peptide 1. From Cummings et al. (38).

1.3 Interoceptive (nutrient)-induced gut-brain signals

Neuroimaging techniques such as functional magnetic resonance imaging (fMRI) have greatly facilitated the investigation of the impact of nutrient-induced gut-brain signals on the abovementioned homeostatic and hedonic neural circuits regulating appetite and feeding behaviour in humans. More specifically, researchers recently started using pharmacological MRI (phMRI) paradigms to assess homeostatic and reward system responses to infusions of nutrients or hormones over time (48-50). The first neuroimaging studies investigating brain responses to intake of a meal or isolated nutrients (e.g. glucose) in healthy humans used oral ingestion, but this does not allow to disentangle the effects of exteroceptive sensory stimulation from those of interoceptive gut-brain signals. Intragastric administration, on the contrary, allows the assessment of purely interoceptive responses and limits

swallowing-induced head movements (50, 51). Recently, human studies shifted toward assessing brain responses to subliminal (i.e. not consciously perceived) nutrient administration, including intragastric and intravenous infusions, that bypass exteroceptive (sight, smell) and oral (taste) sensory stimulation.

1.3.1 Brain responses to (subliminal) nutrient administration

Lassman et al. were the first to study brain responses to intragastrically administered nutrients in healthy humans using phMRI. They observed responses in key homeostatic areas such as hypothalamus, medulla (including the NTS), and other brainstem areas after subliminal intragastric infusion of 250 ml of the fatty acid dodecanoic acid (C12, 0.05M) (49). However, brain responses were not limited to homeostatic regions, but also included the ACC and dorsal striatum, two key reward regions (49). Furthermore, they showed that these brain responses were mediated by CCK responses to the fatty acid administration as they were abolished by dexloxiglumide, a CCK receptor antagonist (49). The effect of fatty acids on reward system function was confirmed by a fMRI study using oral intake of short chain fatty acids, which reduced responses to food pictures in reward regions, including the caudate, insula, amygdala and nucleus accumbens, accompanied by decreased wanting ratings of high caloric food items and reduced *ad libitum* food intake. These findings hint towards a potential role for short chain fatty acids in the prevention of reward-driven food intake and potentially overeating (52).

The first study to investigate carbohydrate-induced gut-brain signals used an intragastric infusion of 45 g of glucose dissolved in 250 ml water. The investigators showed a glucose-induced blood oxygenation level-dependent (BOLD) signal decrease within 5 minutes in the hypothalamus, brainstem, thalamus and insula/putamen (50). These brain regions are largely similar to those responding to intragastric fatty acid administration (49), albeit with a response in the opposite direction. The time course of the decreases in BOLD response was negatively correlated with the rise in blood glucose and insulin levels and was not CCK-dependent, as the responses to glucose were not blocked by dexloxiglumide, in contrast to the abovementioned effects of fatty infusion (49).

These findings are in line with an earlier study that used a stepped hyperinsulinemic euglycemic-hypoglycaemic clamp to induce hypoglycaemia or hyperglycaemia. They showed that circulating levels of glucose interact with processing of external food cues to modulate reward-related brain activity. More specifically, under normal blood glucose levels, responses to both high- and low-calorie food cues were found in the mPFC/pACC, accompanied by lower wanting ratings. In contrast, under lower levels of circulating glucose, responses were found in the insula, putamen, nucleus accumbens, hypothalamus and caudate. This response was shown to be food type-dependent, as this was only observed when exposed to high-calorie food cues (and not low-calorie), thereby driving a greater

desire and craving for these foods (53). These findings were confirmed in a fMRI study during which subjects performed a food/non-food discrimination task, using low-calorie and high-calorie food as well as neutral images, during a fasted baseline as well as after intravenous administration of 10 g glucose. Stronger VTA activation was observed after exposure to high-versus low-calorie food items during baseline; this effect was reversed after glucose infusion. In a control group that did not receive glucose, VTA activation remained higher for high-calorie food items during the entire duration of the task. These findings indicate that glucose modulates salience encoding in the VTA (54).

Besides glucose, recent studies investigated neural responses to fructose administration, which is less satiating and produces a weaker gut hormone response compared to glucose (55, 56). An arterial spin labelling (ASL) MRI study showed that regional cerebral blood flow in the hypothalamus, thalamus, insula, ACC and striatum decreased following oral glucose ingestion (75 g), but not after ingestion of 75 g fructose, except in the thalamus. However, only the hypothalamic response was significantly different upon direct comparison of both carbohydrates, so threshold effects cannot be ruled out for the other regions. Moreover, the observed decrease in regional blood flow in the striatum was correlated with changes in plasma insulin levels. Thus, anorexigenic hormones such as insulin impact on reward system function. Furthermore, glucose ingestion increased functional connectivity between the hypothalamus, thalamus and striatum, whereas fructose only increased connectivity between the hypothalamus and thalamus (57). In a more recent study, Wölnerhanssen et al. used fMRI to investigate dissociable effects of intragastric infusion of 25 g fructose and 75 g glucose on resting state functional connectivity (rsFC) of the "basal ganglia/limbic network", including most of the reward system. Glucose increased rsFC of the left caudate and putamen more than fructose, whereas fructose increased rsFC of the left amygdala, left hippocampus, right parahippocampus, and OFC more than glucose. Moreover, the increased rsFC after glucose positively correlated with the glucose-induced insulin increase (58). In sum, although limited to only a few studies, these data indicate that there are nutrient-specific effects on the human brain, with differential responses to lipids (short chain fatty acids) and carbohydrates (glucose, fructose), both in terms of direction and spatial extent.

1.3.2 Gut hormones: more than just hunger and satiety signals

The GI tract is the largest endocrine organ in the human body and releases more than 20 different peptide hormones that can affect, amongst others, smooth muscle cells and visceral afferent nerves, and have important sensing and signalling roles in appetite and feeding regulation. The majority of these hormones are sensitive to gut nutrient content and are released in response to the presence (or absence) of nutrients within the GI tract, as outlined previously in paragraph 1.1.1 (16).

Besides their effect on the homeostatic-metabolic control of food intake in the hypothalamus, gut hormones also affect hedonic brain regions. For example, ghrelin not only plays a role in homeostatic feeding regulation by activating the hypothalamic AgRP/NPY orexigenic pathway, but also influences striatal food-cue responses (59). Malik et al. demonstrated that intravenous ghrelin administration increases neural responses to high-calorie food images in several reward-related areas, including the OFC, anterior insula, striatum and amygdala (60). Further, Kroemer and colleagues demonstrated that fasting ghrelin levels covaried with brain responses to palatable food cues in the hypothalamus and midbrain, as well as with appetite ratings (61). These studies indicate that ghrelin can drive food consumption by reinforcing hedonic neural responses to food cues, thereby increasing subjective craving when exposed to such cues. In addition, Jones and colleagues showed that intravenous ghrelin infusion can also inhibit nutrient-induced activation of the hypothalamus and brainstem. Postprandially, when endogenous ghrelin levels are low, exogenous ghrelin deactivated homeostatic regions, including the hypothalamus, medulla and pons, in addition to reward-related regions such as the amygdala and anterior insula, compared to saline infusion. When ghrelin was infused 30 minutes before intragastric fatty acid (C12) administration, a small increase in BOLD signal was observed in the medulla, midbrain, hypothalamus, insula, amygdala and hippocampus. These findings were in line with the study of Malik et al., who showed increased reward responses (to exteroceptive food cues) in similar regions (48, 60). Further, the intragastric C12 administration increased neural responses in the hypothalamus and brainstem, which were abolished when the C12 administration was preceded by intravenous infusion of ghrelin. Taken together, this study suggests that ghrelin is able to reverse the neural response to nutrients in hypothalamic and brainstem regions involved in the control of food intake and autonomic reflexes controlling digestion (48). These findings together with the findings by Lassman et al., indicate that the effect of lipids is mediated by CCK, and that this is potentially due to a suppression by ghrelin of the CCK-induced activation of vagal afferent neurons (49).

Besides the orexigenic hormone ghrelin, anorexigenic hormones such as PYY have also been shown to modulate reward system function and food reward responses in healthy subjects. Batterham and colleagues demonstrated that PYY causes a switch in in the regulation of food intake from homeostatic to hedonic areas. They used a phMRI design to show that intravenous administration of PYY, mimicking physiological postprandial PYY levels, activated the lateral OFC, insula and ACC as well as hypothalamus, whereas placebo infusion only activated the hypothalamus. Further, the OFC response predicted magnitude of food intake under conditions of high plasma PYY. In contrast, in the absence of PYY (mimicking a fasted state), activity in the hypothalamus predicted food intake. These findings indicate that the postprandial satiety signal PYY, in the absence of any other food-related sensory signal, causes a switch to more hedonic control of food intake, suggesting that the rewarding aspects

of food can potentially be diminished by PYY-induced modulation of left OFC activity (42). These effects of PYY on the reward system were further elaborated by De Silva et al. They showed that the responses to visual food cues in six reward system regions (including the amygdala, insula, nucleus accumbens, OFC and putamen) following PYY infusion was similar to that after feeding, and significantly lower than in the fasted state. However, since gut hormones are released simultaneously in physiological conditions, they also investigated the cumulative effect of PYY and GLP-1 infusion on human brain activation in the same six regions of interest (ROIs), and found a reduction of the brain response similar to the cumulative decrease after infusion of each hormone separately (62). Van Bloemendaal and colleagues have further investigated the effect of GLP-1 administration alone. Intravenous administration of the GLP-1 receptor agonist exenatide decreased anticipatory food reward responses in the OFC in lean healthy subjects, which was accompanied by increased caudate activation after receipt of chocolate milkshake (compared to a tasteless solution), followed by decreased *ad libitum* food intake (63). These effects were mediated by GLP-1 receptors as they were blocked by the GLP-1 receptor antagonist exendin. In summary, this study suggests that GLP-1 activation could potentially reduce food craving by reducing anticipatory and increasing consummatory food reward responses, which may serve as preventive mechanism for overeating.

1.3.3 There is more than meets the eye: beyond caloric content

Increasing evidence suggests that the reward value of food not only results from the caloric content of ingested nutrients (as outlined above), but also from non-caloric properties of tastants in food (43). Given the potential important clinical implications for the prevention of obesity and associated disorders, recent studies have started focusing on various low- or non-caloric sweeteners (NCS), which provide sweet taste without calories. However, until now, human research has focused solely on reward system responses to oral NCS administration, with evidence on the effect of subliminal (intragastric) administration being limited to animals. For example, Frank and colleagues compared the neural response to sips of sucrose versus its non-caloric counterpart, sucralose. They showed that both activated the primary taste pathway, which comprises the frontal operculum and the anterior insula. However, sucrose provoked a stronger response in key reward system regions including the anterior insula, ventral striatum, midbrain and ACC. Furthermore, only sucrose stimulated the insula and VTA proportionally to pleasantness ratings. This study indicates that sugar activates more food reward-related areas in comparison to NCS, without subjects being able to consciously distinguish them (64). Later, Smeets et al. showed that the amygdala, involved in the processing of sensory cues related to reward, deactivates in response to a drink sweetened with sucrose compared to a drink sweetened with NCS. Further, they also found that the ventral striatum responded differently depending on the caloric content in that before ingestion, the caloric beverage evoked stronger striatal

responses than the non-caloric drink, whereas this activation diminished after caloric consumption. This suggests that the ventral striatal taste response may be modulated by caloric content (65).

1.4 When gut-brain signalling goes wrong

1.4.1 Functional gastrointestinal disorders

Functional gastrointestinal disorders (FGID) are a spectrum of symptom-based, heterogeneous disorders, generally characterised by chronic pain and other abdominal symptoms attributed to various parts of the GI tract, for which no structural basis is found to explain the clinical features (66). FGID, including the most prominent ones, irritable bowel syndrome (IBS) and functional dyspepsia, are extremely common and account for a substantial percentage of primary care as well as gastroenterology specialist visits (67). For example, the prevalence of IBS is 11.2% worldwide and risk factors include female gender, age below 50 years, chronic stress, prior gastroenteritis and food intolerances (68). Comorbidity with depressive and anxiety disorders in IBS is around 30% and 30% - 50% respectively (69). Moreover, higher levels of anxiety and depressive symptoms constitute a risk factor for developing FGID later and conversely, patients with FGID demonstrate higher levels of depressive and anxiety symptoms at follow-up (70). Therefore, FGID are currently conceptualised as disorders of gut-brain interactions and these interactions constitute the physiological basis for the idea that a combination of psychological, environmental and biological factors results in the genesis and clinical expression of FGID symptoms (71).

1.4.2 Can food intake be the culprit for GI symptoms in FGID?

Food processing is the primary function of the GI tract and food ingestion causes major changes in GI sensorimotor and secretory function, which may contribute to symptom generation (72). Food intolerances are a potentially important contributor to (the risk of developing) FGIDs. Postprandial worsening of GI symptoms and adverse reactions to one or more food items is a common feature in IBS, as reported by more than 60% of the patients. As a result, many patients avoid specific foods to cope with their complaints (72, 73). Common self-reported triggers leading to bloating, abdominal pain and diarrhoea include food items containing wheat, foods rich in incompletely absorbed carbohydrates or food items with a high fat content (73). Until recently, diet has largely played an adjunctive rather than a primary role in the management of FGID patients, but recently, our understanding of how food relates to GI symptoms has increased (74). There is cumulative recognition that luminal food components may be responsible for symptom induction via enhanced gut-brain signalling because of an altered intestinal barrier function and/or exaggerated neuroimmune responses, including increased numbers of mast cells, lymphocytes and/or eosinophils (9, 74).

One group of dietary components known to trigger GI complaints in IBS are FODMAPs: Fermentable oligo-, di-, and mono-saccharides and polyols, which include fructose (in excess of glucose, e.g. in honey, apples and pears), lactose (in dairy products), fructans (e.g. in onions, asparagus, cereals) and galacto-oligosaccharides (e.g. in pulses) (67, 75). These short-chain carbohydrates are poorly absorbed in the small intestine and are osmotically active (i.e. they increase the fluid of luminal contents and subsequently alter gut motility). Moreover, FODMAPs serve as a substrate for colonic bacterial fermentation, leading to rapid production of gas and subsequently, luminal distension (76, 77). A low-FODMAP diet has been shown to reduce symptoms in IBS (67).

Besides the individual FODMAPs such as fructose and lactose, or all FODMAPS grouped together, proteins may also play a role in the development of GI symptoms. Specifically the major dietary protein gluten, a complex mixture of proteins from wheat, rye and barley, has been implicated (78). It is causally associated with coeliac disease, defined as an immune reaction to gluten characterised by immunological changes and structural abnormalities in the bowel, resulting in GI symptoms and/or dysfunctions (79). There is strong belief that gluten can also generate symptoms in other diseases, including functional bowel disorders such as IBS. An increased colonic transit is observed in diarrhoea-predominant IBS patients who express the human leukocyte antigen, HLA-DQ2/8 genes, which are predisposing genes for coeliac disease. Furthermore, a gluten-free diet reduced diarrhoea in this patient group as well as complaints of abdominal pain and bloating (79).

A controversial entity has gained attention over the last decade and has been described as ‘the no man’s land of gluten sensitivity’ between coeliac disease and irritable bowel syndrome (80). Non-coeliac gluten sensitivity (NCGS) is an emerging clinical problem and is defined as those without coeliac disease but whose GI symptoms improve on a gluten-free diet (81). Besides GI symptoms, these patients also present with extraintestinal symptoms such as a ‘foggy mind’, fatigue, joint and muscle pain (82). However, despite increasing interest in the effects of a potential beneficial effect of the gluten-free diet outside of coeliac disease, the definition and diagnosis of NCGS remains in debate, and mechanisms generating symptoms are poorly understood.

CHAPTER 2

Research objectives

2 Research objectives

This doctoral project generally aimed to elucidate the influence of different (subliminal) nutrient- and tastant-induced gut-brain signals on GI symptoms, appetite-related sensations, and food intake, but also more broadly on emotional state. This aim was pursued by combining neurophysiological, psychological, behavioural and biochemical methods, thereby allowing exploration into the integration between central and peripheral mechanisms along the brain-gut axis.

More specifically, our research objectives were to investigate:

1. The effect of intragastric administration of the bitter tastant quinine-hydrochloride (QHCl) in healthy volunteers on
    a. brain activity in homeostatic and hedonic brain regions, appetite-related sensations, and food intake;
    b. subjective, behavioural, and neural anticipatory reward system responses during presentation of high- and low-calorie food cues and during a food incentive delay task;

2. The effect of intragastric administration of fructose on subjective and neural responses to an experimentally induced negative emotional state in healthy volunteers;

3. The effect of acute and sub-acute administration of gluten on emotional state and GI symptom levels in healthy volunteers and a NCGS patient group.

2.1 Effects of intragastric bitter tastants

2.1a. Effect of intragastric bitter tastant administration on brain activity in homeostatic and hedonic brain regions, appetite-related sensations and food intake

This study aimed to investigate the gut-brain signalling and brain mechanisms underlying the putative effect of intragastric bitter tastant administration on appetite-related sensations and (hedonic) food intake. We hypothesised that intragastric QHCl infusion would decrease appetite and hedonic food intake compared to placebo by altering activity in homeostatic and hedonic brain regions, and that these effects would be mediated by inhibition of the release of orexigenic gut peptides.

This research aim is addressed in chapter 3 of this doctoral thesis.

2.1b Effect of intragastric bitter tastant administration on subjective, behavioural, and neural anticipatory reward system responses

The aim of this study was to expand the current knowledge about the brain mechanisms underlying the effect of bitter tastants on appetite-related sensations and food intake, generated by the first part of this research objective. For this purpose, we aimed to study the effect of intragastric administration of QHCl on anticipatory food reward responses. We hypothesised that intragastric infusion of QHCl would decrease the reward system response to high caloric food cues as well as during the anticipatory and receipt phase of the food incentive delay (FID) task.

This research aim is addressed in chapter 4 of this doctoral thesis.

2.2 Effect of fructose on subjective and neural responses to experimentally induced negative emotion

The aim of this study was to investigate whether an intragastrically administered dose of fructose influences subjective (i.e. self-reported emotional state) and neural responses to experimentally induced negative emotions, as has been previously shown for fatty acids, and to examine the potential mediating role of gut peptide release. Based on our previous research, we expected an attenuating effect of fructose compared to placebo on the induced negative emotional state.

This research aim is addressed in chapter 5 of this doctoral thesis.

2.3 Effect of acute and sub-acute gluten administration on emotional state and GI symptom levels

For this study, we investigated the effects of acute and sub-acute administration of gluten on emotional state (extraintestinal symptoms) and gastrointestinal symptoms in healthy volunteers and a NCGS patient population. Besides, we investigated intestinal permeability, cortisol levels and inflammation as potential mediators of this effect. We hypothesised that gluten administration would be associated with a decrease in emotional state and an increase of GI symptoms, compared to placebo, in the NCGS patient group, but not in the healthy control group.

This research aim is addressed in chapter 6 of this doctoral thesis.

CHAPTER 3

Intragastric quinine administration decreases hedonic eating in healthy women through peptide-mediated gut-brain signalling mechanisms

This chapter has been published as the following scientific publication: **Iven J***, Biesiekierski JR*, Zhao D, Deloose E, O'Daly OG, Depoortere I, Tack J, Van Oudenhove L. Intragastric quinine administration decreases hedonic eating in healthy women through peptide-mediated gut-brain signaling mechanisms. *Nutr Neurosci* 2018:1-13. doi: 10.1080/1028415x.2018.1457841.
* These authors contributed equally to this work as first authors.

3 Intragastric quinine administration decreases hedonic eating in healthy women through peptide-mediated gut-brain signalling mechanisms

3.1 Summary

Objectives: Intragastric bitter tastants may decrease appetite and food intake. We aimed to investigate the gut-brain signaling and brain mechanisms underlying these effects.

Methods: Brain responses to intragastric quinine-hydrochloride (QHCl, 10 µmol/kg) or placebo infusion were recorded using functional magnetic resonance imaging in 15 healthy women. Appetite-related sensations, plasma levels of gastrointestinal hormones and hedonic food intake (*ad libitum* drink test) were assessed.

Results: Lower octanoylated ghrelin ($P < 0.04$), total ghrelin ($P < 0.01$), and motilin ($P < 0.01$) plasma levels were found after QHCl administration, along with lower prospective food consumption ratings ($P < 0.02$) and hedonic food intake ($P < 0.05$). QHCl increased neural activity in the hypothalamus and hedonic (anterior insula, putamen, caudate, pallidum, amygdala, anterior cingulate cortex, orbitofrontal cortex, midbrain) regions, but decreased activity in the homeostatic medulla (all $p_{FWE\text{-}corrected} < 0.05$). Differential brain responses to QHCl versus placebo covaried with subjective and hormonal responses and predicted differences in hedonic food intake.

Discussion: Intragastric QHCl decreases prospective and actual food intake in healthy women by interfering with homeostatic and hedonic brain circuits in a ghrelin- and motilin-mediated fashion. These findings suggest a potential of bitter tastants to reduce appetite and food intake, through the gut-brain axis.

3.2 Introduction

Distinguishing bitter taste allows detection of toxic compounds in food (83). However, some people have a preference for bitter taste, depending on their sensitivity to bitter compounds, which is sex-dependent, with women on average being more sensitive (83, 84). Bitter tastants (i.e. chemicals stimulating the sense of taste) are sensed via taste receptors of the taste 2 receptor family (TAS2R) class of G-protein coupled receptors, located on taste receptor cells (TRC) in lingual taste buds. However, TAS2Rs are also present on enteroendocrine cells (EEC) throughout the gastrointestinal (GI) tract (85). Activation of taste receptors on EECs occurs via a chemosensory signalling pathway and results in altered secretion of GI peptide hormones involved in the regulation of food intake (86). More specifically, TAS2Rs are present on ghrelin-producing X/A-like cells in the gastric fundus, among others. Ghrelin, a 28-amino acid peptide, is the key orexigenic GI hormone as its plasma levels peak pre-

prandially and decrease rapidly with food ingestion (87). Motilin, a polypeptide hormone secreted by EECs in the duodenum, jejenum and neurons of the myenteric plexus, is the regulator of the migrating motor complex (MMC), a cyclic secretomotor pattern during the fasted state that originates in the stomach and small bowel. Plasma motilin levels fluctuate with the phases of the MMC, and motilin-induced gastric phase III contractions coincide with increases in fasting hunger ratings, pointing towards an orexigenic effect of motilin (32, 33).

These gut peptides constitute an important part of the afferent limb of the gut-brain axis, the bidirectional, neurohumoral communication system between the GI tract and the brain. Gut peptides signal to the brain both indirectly (i.e. neurally, through receptors on vagal afferents) and directly (i.e. humorally, by acting on brain regions that control energy homeostasis including hypothalamic (e.g. the arcuate nucleus) and brainstem nuclei (e.g. the nucleus of the solitary tract (NTS)) (38). Activation of these regions by peripheral endocrine and metabolic signals, including gut peptides, regulates energy intake and expenditure in line with the body's energy status (6, 23). However, gut peptides also impact on neural circuits involved in hedonic control of feeding, which closely interact with the homeostatic system (60). This reward network comprises midbrain regions such as the ventral tegmental area (VTA) and substantia nigra, together with their dopaminergic projections to the nucleus accumbens (ventral striatum), putamen and caudate (dorsal striatum), pallidum, amygdala, anterior cingulate cortex (ACC), orbitofrontal cortex (OFC) and insula. A previous functional magnetic resonance imaging (fMRI) study demonstrated an increased neural response to high-calorie food images in these reward system regions, particularly the striatum and the amygdala, after intravenous ghrelin administration, thereby indicating that metabolic signals, including ghrelin, can drive food consumption by reinforcing hedonic responses to food cues (60). In another study, we found that a motilin agonist, erythromycin, increases hunger by interfering with neurocircuits involved in homeostatic and hedonic control of appetite and feeding (88). The integration of homeostatic and hedonic signals is indispensable to determine the reward value of food, thereby driving appetite and eating behaviour (6).

Recently, it has been shown that intragastric administration of the bitter tastant denatonium benzoate (DB) suppressed hunger and prolonged satiety after a standard meal in healthy female volunteers, without altering gastric emptying. Moreover, DB blocked the occurrence of gastric phase III contractions of the MMC, and decreased motilin, but not ghrelin plasma levels. However, *ad libitum* food intake was not affected (84). Further, Andreozzi et al. showed that intraduodenal administration of the bitter tastant, quinine-hydrochloride (QHCl), reduced food intake (89), and Deloose et al. demonstrated that intragastric QHCl decreased motilin and total ghrelin plasma levels (90). These preliminary findings point towards an anorexigenic effect of intragastric bitter tastant administration, but findings are mixed and the mechanisms underlying this effect are incompletely understood.

3.3 Materials and Methods

Subjects

Sixteen non-obese (BMI 21.9 ± 0.4 kg/m²), healthy, right-handed women (age range 24.5 ± 1.6 years) were included in this study. One participant dropped out after the first test visit. This sample size was calculated using GLIMMPSE, a freely available online tool for power calculations for mixed model analysis developed at the University of Colorado Denver (91). In the absence of previous brain imaging data on intragastric QHCl administration, sample size was calculated based on motilin responses to intragastric administration of QHCl versus placebo from Deloose et al. (for the time-by-condition interaction) (90). This calculation indicated that a sample size of n = 14 was needed to achieve 80% power to detect a similar effect at alpha = 0.05, and we included two extra subjects to increase power and anticipate on potential drop-out. The brain imaging analysis has arguably higher power given the higher number of repeated measures over time in each condition (25 for brain imaging data versus 6 for hormone data). The recruitment of only women was based on our previous study in which a significant effect of intragastric DB administration on hunger scores and motilin levels was found in women only, which may be due to their higher sensitivity to bitter tastants (84). This phenomenon was already described for the lingual system, where it is linked to the density of fungiform papillae on the tongue and to polymorphisms of the TASR38 gene (92, 93), and has been extended to gastric stimuli in the study by Deloose et al. (84, 90). Because of this a priori knowledge that the endocrine and behavioural effects of intragastric bitter administration are sex-specific (i.e. only present in women), studying a mixed sex sample would have led to confounding sex effects, for which the study would not be powered, and which we wished to avoid. Subjects were recruited via local advertisements in the KU Leuven University buildings and via a database of previous participants of experiments in our research group. All candidates had normal or corrected to normal vision and were screened via an online system (Limesurvey) beforehand using self-report questionnaires (Patient Health Questionnaire, short form of the Rome III criteria questionnaire for functional GI disorders, Eating Disorders Inventory, Dutch Eating Behaviour Questionnaire, Anxiety Sensitivity Index and Power of Food Scale). Exclusion criteria included: history of GI, cardiovascular, neurological, endocrine or psychiatric disorders, abdominal surgery (except appendectomy), abnormal eating behaviour and eating disorders, use of medication on a regular basis (except oral contraceptives) and MRI-specific exclusion criteria (ferromagnetic implantations, claustrophobia), smoking, pregnancy and breast-feeding. The screening included a food preference questionnaire to assess liking and wanting of different savoury and sweet food items, including chocolate, ice cream and milkshake. Subjects who scored higher than 6 on a scale ranging from 0 to 10 for liking of milkshake, chocolate and ice cream, attended a separate taste session to ensure liking of the milkshake. Only subjects who rated the milkshake with a score between 60 and 90

on a 100 mm visual analogue scale (VAS) for liking, were included in the study (mean: 76.9 ± 2.2). All subjects were asked to refrain from caffeine and alcohol at least 12 hours prior to the study. All subjects gave written informed consent in compliance with the Declaration of Helsinki and received a compensation for their participation.

Experimental design

The study used a randomised, placebo-controlled, single-blind, cross-over design. The protocol was approved by the Ethics Committee of the Leuven University Hospital, Belgium and registered at ClinicalTrials.gov (NCT02946970). Subjects were studied at 08:00 after an overnight fast on two separate occasions. The study protocol is summarised in Figure 3.1. A nasogastric feeding tube and an intravenous cannula were placed at least 30 min before subjects entered the scanner room. The fMRI scan lasted for 49 min, including a baseline scan of 10 min. After the baseline scan, one of the two test solutions was intragastrically infused (t = 0). Appetite-related sensations and emotional state ratings were collected at t = -10, 0, 10, 20, 30, 40 and 50. Blood samples were collected at t = -10, 10, 20, 30, 40, 50 and 60. After scanning, hedonic food intake was assessed using an *ad libitum* drinking test with a rewarding chocolate milkshake.

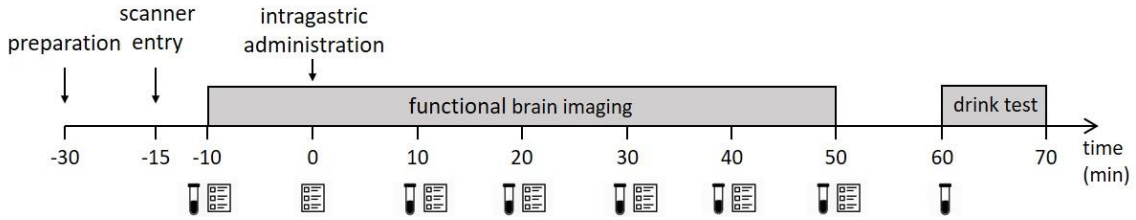

Figure 3.1 Schematic overview of one study visit
Preparation: placement of nasogastric feeding tube and intravenous catheter. Drink test: *ad libitum* drink test with rewarding chocolate milkshake, as measurement of hedonic food intake. Both study visits of the cross-over study were identical, except for the nature of the intragastric administration. Blood collection for total ghrelin, octanoylated ghrelin and motilin. Questionnaires: visual analogue scales for appetite-related sensations (including hunger, fullness, satiety, prospective food consumption and nausea) and the self-assessment manikin for arousal and valence.

Intragastric quinine-hydrochloride administration

A nasogastric feeding tube (RT8/801, Medicina, Massachusetts, United States) was positioned with its tip in the proximal stomach, and attached to the subject's face. To confirm the position of the probe, gastric fluids were aspirated and the pH was measured using pH strips (Merck Millipore, Billerica, USA). After an adaptation period of 15 min, subjects received either QHCl (Fragon, Rotterdam, The Netherlands) (10 µmol/kg bodyweight) dissolved in Milli-Q water (Millipore Corporation, Billerica, USA) or Milli-Q water only (t = 0) in a single-blind fashion. Dosage was based on a previous study (90).

The order of conditions was randomised and counterbalanced. The randomisation code was automatically generated by a computer program. The time window between two study visits was at least one week, to avoid any potential carryover effects (mean amount of days between two visits = 34.2 ± 7.7 days, interquartile range = 11 to 50 days).

Appetite-related sensations and emotional ratings

Every 10 min during scanning, subjects rated subjective sensations of hunger, prospective food consumption, fullness, satiety and nausea, responding to the questions: "How hungry do you feel?", "How much do you think you can eat?", "How full do you feel?", "How satisfied do you feel?" and "How nauseous are you?" on a computer-based 100 mm VAS ranging from "not at all" (0 mm) to "very much" (100 mm). At the same time points, they completed the Self-Assessment Manikin (SAM), using pictograms to assess emotional state on the dimensions valence and arousal (94). Ratings were given by moving a tick mark along a line segment, back-projected onto a screen in the scanner, using a response box in their right hand. The order in which ratings were presented was randomised. The tick mark was reset to the middle of the line at the start of each VAS. Ratings were programmed using Affect 4.0 software (95).

Gut hormone measurements

Motilin and ghrelin levels were determined using radioimmunoassays as previously described. Ghrelin blood samples were collected in standard EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 500 kIU/ml aprotinin (Roche Applied Science, Penzberg, Germany) and phenylmethanesulfonyl fluoride (PMSF, 57 µM) (Sigma-Aldrich, Steinheim, Germany). Plasma was acidified (10%) with 1N HCl before extraction on Sep-pak C18 columns (Waters Corporation, Milford, Massachusetts, USA) and vacuum-dried. Motilin blood samples were collected in lithium heparin tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 500 kIU/ml aprotinin. All blood samples were kept on ice immediately after collection. Plasma was separated by centrifugation at 4°C for 10 min at 3000 xg and stored at -80°C until analysis. Plasma total ghrelin levels were measured with an in-house developed radioimmunoassay (RIA) using ^{125}I [Tyr^{24}] human ghrelin [1-23] as the tracer and a rabbit antibody against human ghrelin [14-28], which recognises both octanoylated and desoctanoylated ghrelin. Plasma octanoylated ghrelin was measured using a rabbit antibody against human ghrelin [1-8], which does not cross-react with desoctanoylated ghrelin. Motilin levels were measured using ^{125}I [Nle^{13}] human motilin as the tracer and rabbit anti-human Nle^{13} motilin antibody (87, 96).

Hedonic food intake assessment

Hedonic food intake was measured by an *ad libitum* drinking test at the end of each test visit using a rewarding chocolate milkshake, containing 4 scoops of vanilla ice cream (Ijsboerke, Tielen, Belgium), 355 ml of 2% milk (Lactel, Brussels, Belgium) and 2 tablespoons of Imperial's chocolate topping (Continental foods, Puurs, Belgium) per serving. This was based on work by Stice et al. and adapted to Belgian Brands (97). Subjects were presented with a 200 ml glass that was repeatedly refilled as soon as they had emptied it and were instructed to cease drinking as soon as they felt fully, but comfortably, satiated.

MRI data acquisition

FMRI data were acquired on a 3.0 Tesla MR System (Philips Medical Systems, Best, The Netherlands) at the Radiology Department of the University Hospitals Leuven, Belgium. A total of 1416 functional volumes was acquired for an examination period of 59 min, including a baseline scan of 10 min. A T1-weighted structural scan was acquired for each subject at the end of each test visit to co-register with the functional images and to exclude anatomical abnormalities. No abnormalities were reported. Functional, T2-weighted volumes were acquired with a gradient echo planar imaging (EPI) sequence with blood oxygenation level-dependent (BOLD) contrast (parameters: TR = 2500 msec, TE = 30 msec, 46 slices of 2.70 mm thick, slice gap = 0.3 mm, flip angle = 90°, voxel size of 2.40 x 2.39 x 2.70 mm^3), covering the whole brain including the cerebellum. A 32 channel head-coil was used for radio frequency transmission and reception. The T1-weighted structural scan was acquired with following parameters: 46 slices of 3 mm thick, slice gap = 0.3 mm, TR = 9.6 msec, TE = 4.6 msec, flip angle = 90°, voxel size of 0.98 x 0.98 x 1.20 mm^3.

Statistical analysis

Behavioural data and gut hormone level analysis

Data were analysed using SAS 9.4 (SAS Institute, Cary, NC, USA). All data are shown as mean ± SEM. Significance level was set at a *p*-value ≤ 0.05. Data were logarithmically transformed to fulfil the assumption of normally distributed residuals in mixed model analysis. Data shown in the results section are estimates of transformed values for each specific outcome measurement, as these are used in the statistical analysis. Raw observed values of these outcomes are graphically presented in the corresponding figures. Condition (QHCl, placebo) and time (7 time points in total) were entered as categorical within-subject variables, controlling for potential visit effects. The condition-by-time interaction effect represented the main effect of interest. We tested our hypotheses (i.e. lower hunger and prospective food consumption, and higher satiety and fullness ratings after QHCl compared to placebo) using planned contrast analysis comparing the average change from baseline, calculated as the average of time points -10 and 0, between the two conditions. Since both time points -10 and 0

represent measurements before any intervention was performed, both can reliably serve as baseline measurements. The *a priori* hypothesis that orexigenic gut hormone levels would be lower after QHCl compared to placebo was tested using similar planned contrast analyses. The hypothesis that hedonic food intake would be lower after QHCl compared to placebo was tested using a paired Student's *t*-test. As we had specific hypotheses about the direction of all these effects based on previous research (84, 89) one-tailed p-values are reported for these tests. The effect of QHCl compared to placebo administration on emotional state ratings was explored using similar planned contrasts, but as we did not have a specific hypothesis about the direction of this putative effect, two-tailed *p*-values are reported.

Pharmacological MRI data analysis

Data were analysed using Statistical Parametric Mapping (SPM12, Wellcome Trust Centre for Neuroimaging, UCL, London, UK) implemented in MATLAB R2014b (The MathWorks Inc., Natick, MA). All individual images were pre-processed using the standard procedures implemented in SPM12, including spatial realignment as correction for small movements during the scan and co-registration of each functional image to the structural image of each subject and segmentation of the structural image. The structural image was used for each participant as reference for the spatial normalisation to the EPI template image supplied with SPM12, based on information obtained during the segmentation step. Spatial smoothing using a 8 x 8 x 8 mm^3 Gaussian smoothing kernel was applied to the normalised images to improve the signal-to-noise ratio.

First-level (subject level) analysis was performed according to a previously described pharmacological MRI analysis method (98). For each condition and each subject, post-infusion volumes were divided into 24 time bins of 2 minutes (48 volumes) each, and 1 time bin of 1 minute (T1 - T25), reflecting the change in brain activity compared to baseline. Cerebrospinal fluid (CSF) and white matter (WM) were defined based on the segmentation step of the pre-processing procedure. Global time series of CSF and WM activity were extracted. To correct for potential differences in low-frequency scanner drift between conditions, these time series were included as nuisance regressors of no interest in the design matrix. Six realignment parameters (translation in the x-, y- and z-direction and rotations: pitch, roll, yaw) were included to correct for residual motion artefacts. For each of the 25 time bins post-infusion within the general linear model framework, a t-contrast was calculated to compare brain responses (relative to pre-infusion baseline period) between the QHCl and placebo conditions. This resulted in 25 first level contrast images per subject, corresponding to the difference in BOLD signal after QHCl compared to placebo infusion in each time bin.

Second-level (group level) analysis was performed by applying a one-way within-subject ANOVA on the 25 individual first-level contrast images, with the condition-by-time bin interaction effect being the main effect of interest. Voxel-level threshold was set at $p_{FWE\text{-}corrected} < 0.05$. Voxel-based analysis was performed within a single mask consisting of regions of interest (ROI) selected based on previous human functional neuroimaging studies on hedonic and homeostatic control of appetite and food intake (49, 99). These ROIs are all anatomically defined atlas structures, including key homeostatic (medulla (NTS), hypothalamus) and hedonic (midbrain (VTA), dorsal (caudate, putamen) and ventral (nucleus accumbens) striatum, pallidum, pregenual anterior cingulate cortex (pACC), medial and lateral rectus of the orbitofrontal cortex, amygdala, anterior insula) brain regions involved in the regulation of appetite and food intake (49, 99). To investigate if changes are specific to our *a priori* ROI, the same second level analysis was performed in a priori irrelevant regions, such as the occipital lobe (visual cortex, Brodmann Area 17, 18 and 19) and the primary motor cortex (Brodmann Area 4). For each time bin, contrast estimates reflecting the difference in brain response (relative to pre-infusion) between QHCl and placebo administration were extracted from the entire cluster (averaged across all voxels) in representative ROIs with the MarsBar toolbox for SPM12. These estimates were used to plot the time course of the differential effect of the intragastric infusions on the BOLD signal.

To test whether the difference between conditions in appetite-related sensations, hormone levels and hedonic food intake was related to the difference in brain response, the differences in appetite-related sensations and hormone levels (both relative to pre-infusion baseline) and hedonic food intake were used to weigh first-level contrasts. For this purpose, appetite-related sensation ratings and hormonal measurements were linearly interpolated to match the number of time bins in the *p*hMRI analysis and each time bin was weighed with the value at the corresponding time point. For hedonic food intake, the difference in milkshake intake between QHCl and placebo condition was used as weight across all time bins. All second level analysis were performed in the same mask of a priori defined ROIs mentioned earlier at the same voxel-level threshold of $p_{FWE\text{-}corrected} < 0.05$.

3.4 Results

Intragastric QHCl suppresses orexigenic gut hormone levels

As hypothesised, the decrease in total ghrelin after QHCl infusion was significantly stronger than after placebo, where an increase was found compared to baseline (delta log10 total ghrelin -0.046 $\pm$ 0.016 vs. 0.013 $\pm$ 0.021 pg/ml in QHCl versus placebo, respectively, $p < 0.01$) (Figure 3.2A). Similarly, the decrease in octanoylated ghrelin after QHCl infusion was significantly stronger than after placebo, where an increase was found compared to baseline (delta log10 octanoylated ghrelin -0.020 $\pm$ 0.014 vs. 0.008 $\pm$ 0.011 pg/ml in QHCl versus placebo, respectively, $p < 0.04$) (Figure 3.2B). Further, the

decrease in motilin was significantly stronger after QHCl compared to placebo (delta log10 motilin -0.056 ± 0.011 vs. -0.021 ± 0.012 pg/ml, $p < 0.01$) (Figure 3.2C).

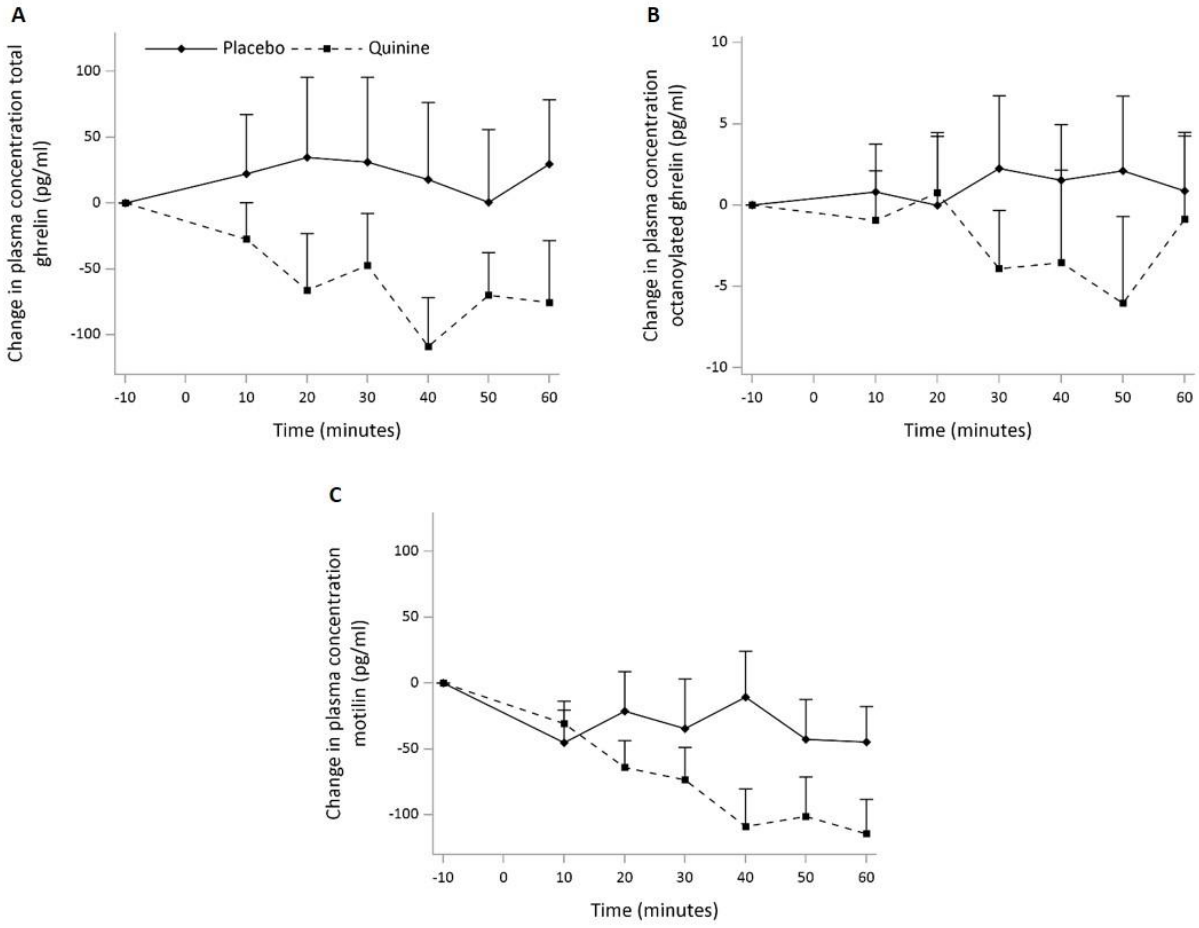

Figure 3.2 Effect of intragastric administration of quinine versus placebo on plasma levels of orexigenic gut hormones.
A Total ghrelin. The decrease in total ghrelin after quinine infusion was significantly stronger than after placebo ($p < 0.01$), **B** Octanoylated ghrelin. The decrease in octanoylated ghrelin after quinine infusion was significantly stronger than after placebo ($p < 0.04$), **C** Motilin. The decrease in motilin was significantly stronger after quinine compared to placebo ($p < 0.01$). Time point 0 indicates the intragastric administration. Data are represented as mean ± SEM. VAS: visual analogue scale.

Intragastric QHCl alters brain activity in homeostatic and hedonic regions

A significant condition-by-time interaction effect was observed on the BOLD signal, driven by a significantly stronger increase in activity, relative to pre-infusion baseline, after QHCl compared to placebo in most pre-hypothesised ROI, including the anterior insula, ACC, amygdala, putamen, nucleus accumbens, pallidum, caudate head and caudate body, medial and lateral OFC, hypothalamus and midbrain. In the brainstem/medulla, a key homeostatic region, activity decreased after QHCl compared to placebo administration (Figure 3.3, Table 3.1). No significant differences were observed in the

occipital lobe or primary motor cortex (data not shown). The time course of the response in selected regions is shown in Figure 3.4.

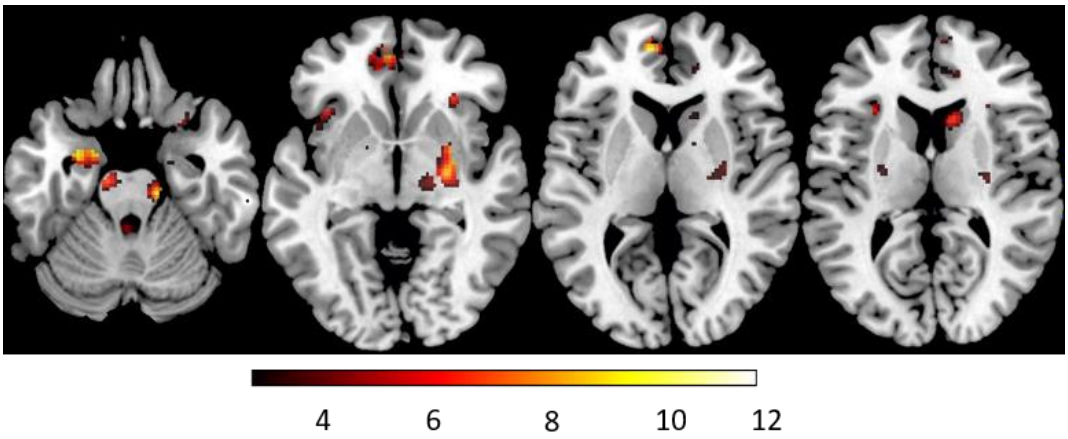

Figure 3.3 Brain regions in which a stronger increase of activity (relative to baseline) was found after intragastric quinine compared to placebo administration
Voxel-level threshold: $p_{FWE\text{-}corrected} < 0.05$. Colour scale reflects F-values of the functional activity.

Table 3.1 Brain regions in which different responses to intragastric quinine compared to placebo administration were found.

region	sub region	side	peak coordinates (mm)			cluster volume (voxels)	F-value
			x	y	z		
brainstem	medulla	right	2	-44	-47	13	4.656
	midbrain	left	-12	-16	-23	44	7.541
		right	14	-22	-23	24	9.614
hypothalamus		right	4	-2	-19	6	3.719
caudate	head	left	-14	22	1	15	7.718
		right	12	22	-1	78	4.419
	body	left	-10	12	19	7	6.870
		right	12	18	11	78	7.072
putamen		left	-18	6	1	15	6.443
		right	28	0	-3	373	8.512
nucleus accumbens		right	12	18	-13	6	4.348
anterior cingulate		left	-8	54	3	155	14.923
		right	2	48	-7	29	5.209
middle frontal gyrus	orbital part	right	2	54	-1	29	3.466
lateral frontal gyrus	orbital part	left	-46	48	-5	2	5.227
		right	28	16	-23	9	5.027
anterior insula		left	-40	16	-9	136	18.880
		right	30	28	-5	33	7.691
amygdala		left	-20	0	-21	99	10.827
		right	22	-6	-21	9	4.920
pallidum		right	26	-12	-1	373	10.403
		left	-26	-4	-25	99	10.606

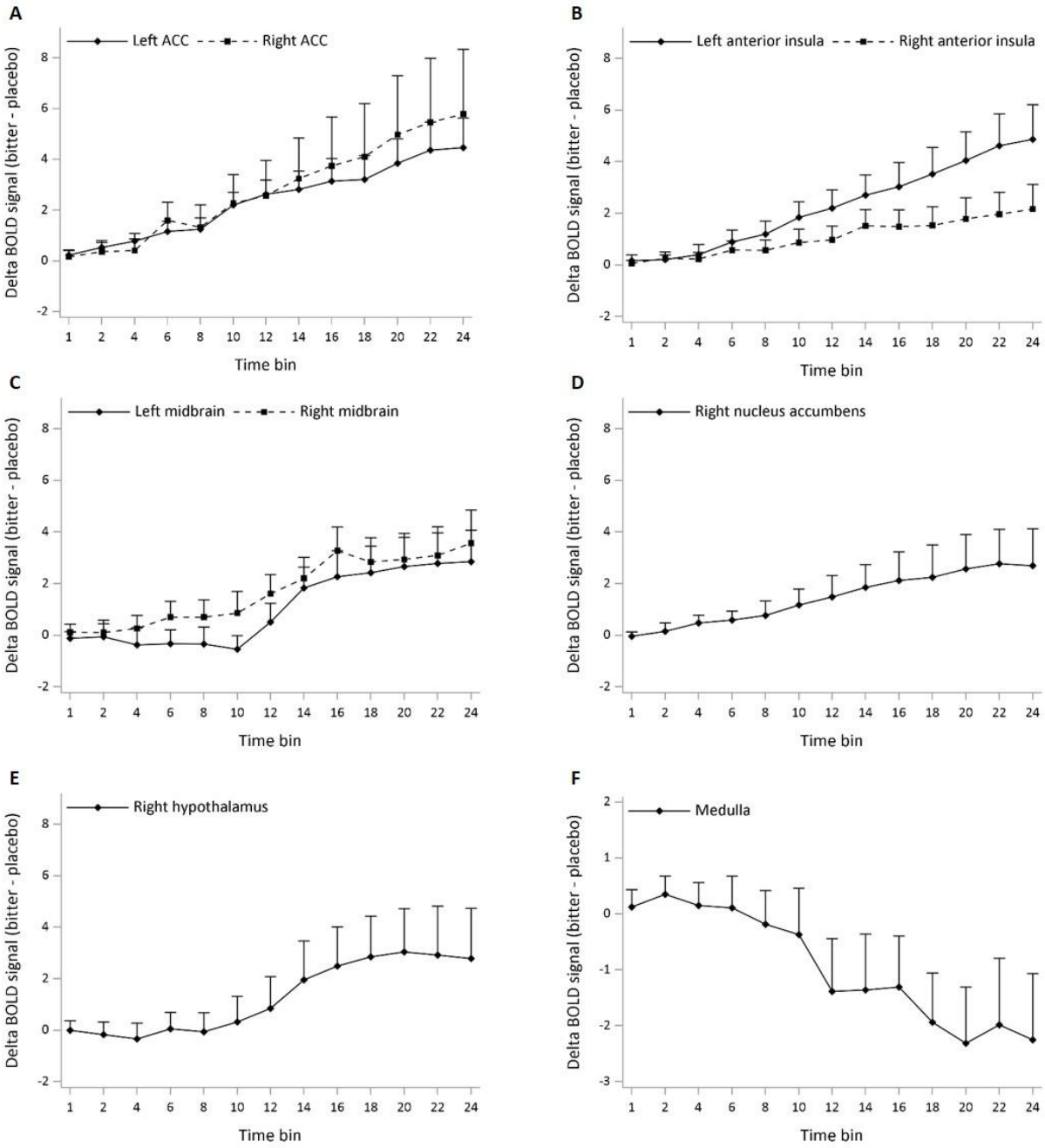

Figure 3.4 Time course of the brain response after intragastric quinine compared to placebo infusion
Brain responses increased after quinine compared to placebo administration in hedonic regions: ACC (**A**), anterior insula (**B**), midbrain (**C**) and nucleus accumbens (**D**) and in the hypothalamus (**E**). Brain response decreased after quinine compared to placebo administration in the homeostatic medulla (**F**). Data are represented as mean ± SEM. BOLD: blood oxygen level dependent, ACC: anterior cingulate cortex.

Intragastric QHCl alters appetite-related sensations, but not emotional state

Compared to baseline, hunger scores increased after placebo and decreased after QHCl, but the difference between conditions was not significant (delta log10 hunger -0.048 ± 0.055 vs. 0.033 ± 0.055

in QHCl versus placebo, respectively, $p > 0.10$) (Figure 3.5A). The change from baseline in prospective food consumption ratings was significantly different between conditions, in the hypothesised direction with an increase after placebo and a decrease after QHCl (delta log10 prospective food consumption -0.082 $\pm$ 0.044 vs. 0.059 $\pm$ 0.044 in QHCl versus placebo, respectively, $p < 0.02$) (Figure 3.5B). The increase in satiety scores post-infusion compared to baseline was significantly stronger after QHCl administration compared to placebo, confirming our hypothesis (delta log10 satiety 0.293 $\pm$ 0.104 vs. 0.034 $\pm$ 0.084 in QHCl versus placebo, $p < 0.02$) (Figure 3.5C). As for satiety and as hypothesised, the increase in fullness scores after QHCl administration was significantly stronger than after placebo administration (delta log10 fullness 0.32 $\pm$ 0.074 vs. 0.061 $\pm$ 0.074 in QHCl versus placebo, $p < 0.01$) (Figure 3.5D). Minimal nausea scores were reported (strongly zero-inflated distributions with very limited amount of variability, not permitting formal statistical analysis), indicating good tolerance of both infusions. No effects were found on emotional state ratings (valence and arousal), based on planned contrasts analysis (data not shown).

Intragastric QHCl decreased hedonic food intake

Hedonic food intake was significantly lower after QHCl compared to placebo (mean milkshake intake 346.3 $\pm$ 36.9 g vs. 413.9 $\pm$ 45.7 g, $p < 0.05$, Cohen's d = 0.50).

Gut peptide responses to intragastric QHCl covary with brain responses

The difference in brain response between both conditions covaried with total and octanoylated ghrelin plasma levels in most ROIs including the amygdala, putamen, anterior insula, ACC, caudate, hypothalamus, medial and lateral OFC and brainstem. Brain responses in the pallidum covaried with circulating octanoylated ghrelin levels only. The differences in brain responses between QHCl and placebo covaried with differences in motilin levels in the amygdala, basal ganglia (caudate, putamen, pallidum), ACC, medial and lateral OFC, anterior insula, hypothalamus and brainstem (Table 3.2).

Brain responses to intragastric QHCl covary with appetite-related sensations

ROIs where the difference in brain responses between both conditions covaried with the difference in prospective food consumption scores included the amygdala, putamen, pallidum, anterior insula, ACC, medial and lateral OFC and brainstem (Table 3.2).

Brain responses to intragastric QHCl predict differences in hedonic food intake

Differences in hedonic food intake after QHCl versus placebo were predicted by differential brain responses in the basal ganglia (including the pallidum, putamen and caudate), amygdala, ACC, medial and lateral OFC, anterior insula, hypothalamus and brainstem (Table 3.2).

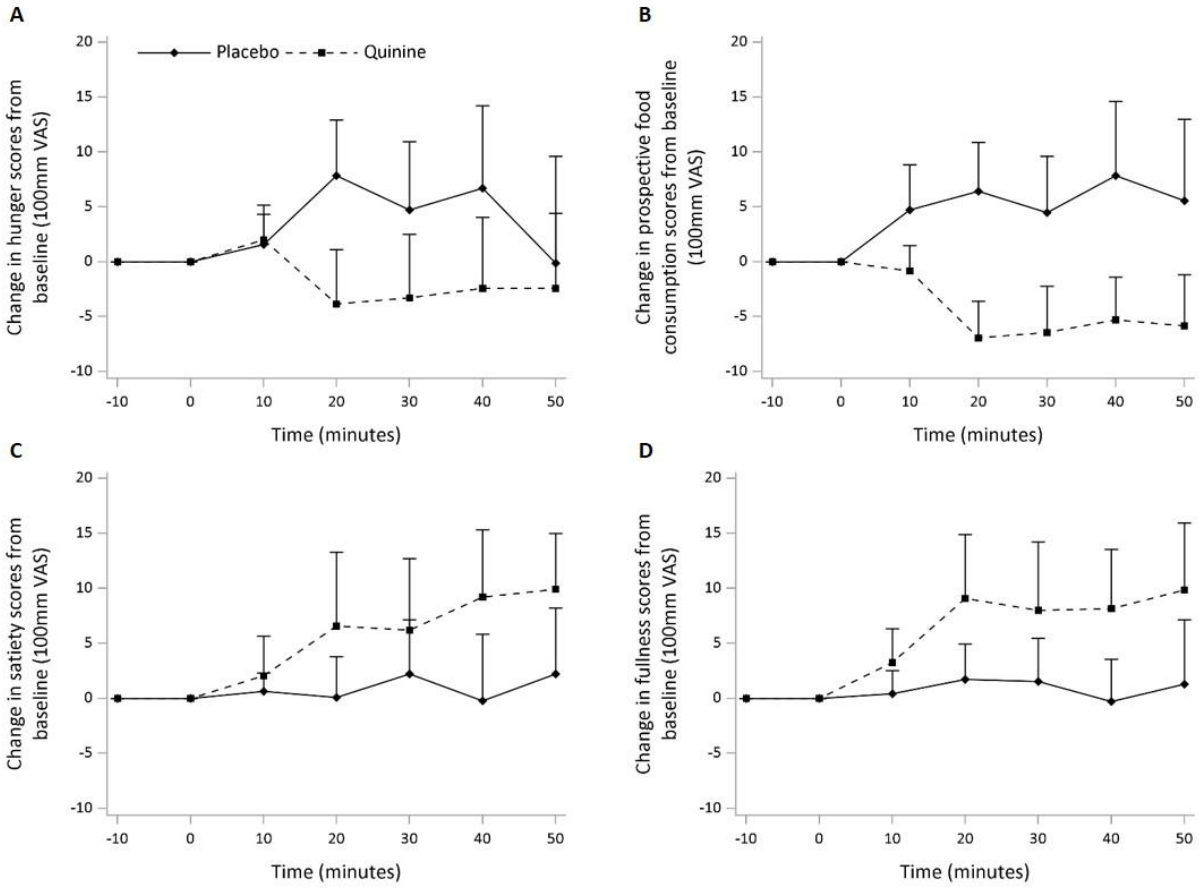

Figure 3.5 Effect of intragastric administration of quinine versus placebo on appetite-related sensations

A Hunger. Compared to baseline, hunger scores increased after placebo and decreased after quinine, but the difference between conditions was not significant ($p > 0.1$). **B** Prospective food consumption. The change from baseline in prospective food consumption ratings was significantly different between conditions, with an increase after placebo and a decrease after quinine ($p < 0.02$). **C** Satiety. The increase in satiety scores post-infusion compared to baseline was significantly stronger after quinine administration compared to placebo ($p < 0.02$). **D** Fullness. The increase in fullness scores after quinine administration was significantly stronger than after placebo administration ($p < 0.01$). Time point 0 indicates the intragastric administration. Data are represented as mean ± SEM. VAS: visual analogue scale.

Table 3.2 Overview of regions in which brain responses to quinine versus placebo covaried with behavioral and hormonal responses

region	sub region	side	total ghrelin	octanoylated ghrelin	motilin	prospective food consumption	hedonic food intake
brainstem	medulla	left	(-6, -46, -43)				(-6, -36, -45)
		right			(4, -46, -47)	(6, -34, -45)	(6, -34, -45)
	midbrain	left	(-12, -12, -7)	(-14, -16 -19)	(-20, -24, 5)	(8, -12, -23)	(-6, -30, -19)
		right	(8, -28, -1)	(6, -16, -7)	(0, -18, -23)	(-10, -20, -5)	(2, -42, -21)
hypothalamus		left	(-2, -10, -9)				
		right		(-8, -4, -7)			(6, 0, -15)
caudate	head	left			(-10, 22, 1)		(-6, 16, 7)
		right	(16, 10, 11)		(8, 18, 9)		(14, 22, 9)
	body	left	(-16, -18, 25)	(-16,-14,23)	(-6, 10, 15)		(-12, -12, 23)
		right	(8, 4, 19)	(10, -2, 19)	(14, -2, 15)		
putamen		left		(-20, 18, -7)	(-28, -12, 15)	(32, -16, -5)	(-20, 18, -5)
		right	(20, 0, 9)	(22, 4, 13)		(-22, -2, -7)	(24, -8, 11)
anterior cingulate		left	(-4, 38, 13)	(-4, 34, 9)	(-2, 28, -7)	(-8, 48, 7)	(-6, 54, 1)
		right	(8, 40 ,13)	(10, 58, 11)	(2, 28, -7)	(12, 54, 9)	(2, 34, -3)
middle frontal gyrus	orbital part	left	(-18, 4 ,-17)		(-10, 12, -15)	(-18, 6, -17)	(-2, 40, -15)
		right		(8, 8, -15)	(6, 18, -11)		(10, 50, -11)
lateral frontal gyrus	orbital part	left	(-42, 38, -17)	(-30, 38, -15)	(-44, 50, -3)	(22, 48, -13)	(-48, 34, -15)
		right	(30, 26, -21)	(28, 42, -15)	(38, 46, -13)	(-22, 10, -23)	(28, 38, -9)
anterior insula		left	(-28, 14, -19)	(-32, 16, -1)	(-32, 28, -3)		(-34, 14, -13)
		right	(36, 24, 3)	(46, 12, -5)	(30, 20, -17)	(44, 18, -3)	(34, 24, -3)
amygdala		left	(-26, -2, -15)	(-20, -8 ,-15)		(-26, -4, -27)	(-18, -2, -17)
		right	(22, -2, -17)		(20,-2,-17)	(26, 2 -17)	(24, -2, -13)
pallidum		left		(-16, 0 -5)		(-16, 0, 7)	
		right			(20, -2 , 11)		(24, -4, -5)

3.5 Discussion

This study is, to the best of our knowledge, the first to demonstrate that the bitter tastant QHCl alters brain activity in homeostatic and hedonic regions in healthy women, even when administered intragastrically to bypass effects of taste and smell. In addition, we show that these brain responses to QHCl covary with quinine-induced decreases in circulating levels of orexigenic gut hormones. Furthermore, these differential brain responses covaried with decreased prospective food consumption scores after QHCl and predicted the magnitude of the decrease in hedonic food intake observed after QHCl versus placebo. In summary, these results indicate that intragastric QHCl administration decreases prospective as well as actual hedonic food intake in healthy women by interfering with homeostatic and hedonic brain circuits, and that this effect may be mediated by a quinine-induced reduction in levels of orexigenic hormones. This is summarised in Figure 3.6.

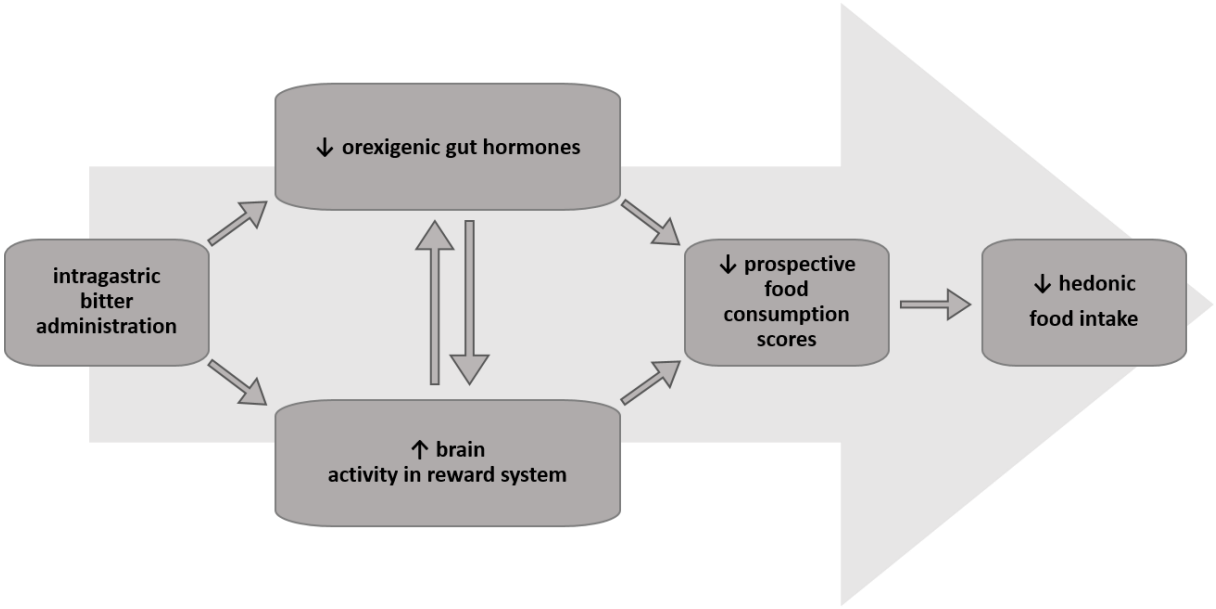

Figure 3.6 Schematic representation of the effect of intragastric administration of quinine on brain activity and hedonic food intake
Proposed mechanism of action to indicate that intragastric QHCl administration decreases prospective as well as actual hedonic food intake in healthy women by interfering with homeostatic and hedonic brain circuits, and that this effect may be mediated by a quinine-induced reduction in levels of orexigenic hormones.

First, we observed a significant decrease in motilin, total ghrelin and octanoylated ghrelin (the biologically active form, representing less than 20% of circulating ghrelin) levels after QHCl compared to placebo administration. As the regulator of the MMC, motilin levels fluctuate with gastric phase III contractions, which have in turn been associated with increases in fasted hunger ratings, thereby identifying motilin as a potential orexigenic hormone in humans (33). Previous work indicates that

intragastric administration of both DB and QHCl decreases motilin levels (84, 90). The effect of QHCl on motilin levels in the present study is in line with these earlier findings. Whether the effect of bitter agonists on motilin levels is direct (i.e. through direct activation of bitter TAS2Rs on motilin-producing cells in the duodenum) or indirect (e.g. through the release of other gut peptides), remains to be explored, as to date, the taste receptor expression of motilin cells has not been reported (87). These findings are partially in line with a previous study in healthy women, in which circulating levels of total, but not octanoylated ghrelin, decreased after administration of QHCl (90). The reasons for this partial discrepancy for octanoylated ghrelin remains unclear. Although the smaller sample size and hence lower statistical power of the previous study may provide an explanation, further research is needed to confirm our current findings. Another study failed to find an effect of intraduodenal QHCl administration on total as well as octanoylated ghrelin levels (89). This can be explained by the lower dosage used and the different route of administration, as intraduodenal administration bypasses the stomach, where most ghrelin-secreting cells are located.

Second, responses to quinine administration were found in most ROIs involved in homeostatic and hedonic control of appetite and food intake, including the basal ganglia, anterior insula, hypothalamus, lateral and medial OFC, ACC and nucleus accumbens. In other regions, which are not relevant in the regulation of food intake, no differences were observed after QHCl versus placebo administration. This indicates that our findings are specific to our a priori defined homeostatic and hedonic brain areas. In most of these regions, an increased activity in response to QHCl versus placebo was found. We can speculate that this increase in resting activity may “saturate” the reward system and decrease its responsiveness to rewarding food stimuli (e.g. smell, sight or taste of food). However, this interpretation requires validation in future studies. In the medulla, an important part of the homeostatic system, activity decreased after QHCl compared to placebo administration, which may be mediated by the observed lower ghrelin and motilin levels. Although standard fMRI does not yield the spatial resolution to identify individual brainstem nuclei, this medulla cluster likely contains the NTS based on its location. The NTS may receive less vagal afferent input after QHCl due to the inhibitory effect of bitter tastants on the MMC, which is likely mediated by decreased motilin levels. The resulting decreased antral phasic contractile activity could induce a decrease in vagal afferent signalling, leading to lower activity in the NTS, the key afferent vagal nucleus (96, 100). A potential direct effect of motilin on vagal afferents (i.e. not mediated by an effect on antral contractions) remains another possibility as *in vitro* studies indicated that motilin increased gastric vagal afferent activity without affecting mesenteric nerve activity (101). Additionally, decreased circulating ghrelin levels might result in lower activation of vagal afferents and, hence, a lower activation of the NTS since ghrelin receptors are present in the nodose ganglion of the vagus nerve and activation of vagal afferents by circulating

ghrelin levels activates the NTS (102). Interpreting the different direction of the brain responses to QHCl administration in hedonic regions and hypothalamus (activations) and medulla (deactivations) is complicated because brain activity as measured by the fMRI BOLD signal could be driven by either inhibitory or excitatory neurons. The current fMRI data do not allow differentiation between these two interpretations, and the only way to do so *in vivo* in humans would be to quantify the release of key reward system neurotransmitters such as dopamine in response to intragastric bitter administration using positron emission tomography. A recent study observed significantly more activity in right precentral, medial frontal and somatosensory areas during execution of a working memory task, but not in working memory task performance after intragastric administration of 17mg of quinine (103). This was a task-based fMRI study quantifying the brain response to a working memory task, contrary to the present study that quantified the resting brain response to quinine in the context of appetite or food intake regulation. Further, dosage differed greatly between both studies (17mg vs. 4mg per kg bodyweight), further complicating comparison of both results.

Third, we demonstrated an anorexigenic effect of QHCl versus placebo, both on appetite-related sensation ratings and actual food intake. Few other studies to date have evaluated the effect of bitter tastants on food intake in humans, even though a delimiting effect on food intake of oral quinine was already found in rats in 1982 (104), and the results of these human studies have been mixed. Van Avesaat et al. tested the effect of intraduodenal administration of 75 mg quinine dissolved in 120 ml tap water in humans and did not observe significant differences on food intake (105). Both female and male volunteers were enrolled, although it is known that men show a lower sensitivity towards bitter stimuli (84). However, another study reported significantly reduced food intake, without an effect on appetite-related sensations (satiety, fullness, desire to eat) or circulating ghrelin levels after intraduodenal administration by means of acid-resistant capsules containing 18 mg of quinine (89). The divergent results between our study and these two other studies are likely due to differences in dose, sample (especially sex distribution) and route of administration.

Finally, the observed differences in brain responses after QHCl compared to placebo covaried with differences in orexigenic gut hormone levels. These results indicate that the effect of QHCl on the brain is likely to be mediated by its effect on circulating ghrelin and motilin levels through indirect, neural (activation of vagal afferents) and/or direct, humoral (gut peptides activate their receptors in the brain) routes. However, TAS2Rs are expressed in various brain regions of the rat, and they are functional as an increase in intracellular Ca^{2+} is found after stimulation with DB and quinine (106). Therefore, since QHCl is absorbed through the GI system, it might be able to directly interact with TAS2R in brain areas of the homeostatic and reward system, independently of its effect on gut peptides. Despite the fact that QHCl does not freely move across the blood-brain-barrier, it does penetrate into cerebrospinal

fluid (CSF) with a CSF/plasma ratio of 7%, therefore a direct effect of QHCl on the brain cannot be excluded (107). The current findings on the covariance between ghrelin and brain responses to QHCl are in line with previous fMRI research showing that intravenously administered ghrelin enhances brain responses to high-calorie food images in the amygdala, anterior insula, OFC and the striatum (60). It is important to note the differences in study design between these studies, where the previous study used a task-based paradigm with exteroceptive food cues that activate anticipatory food reward, we modelled the brain responses to QHCL at rest without exteroceptively stimulating the reward system.

We would like to emphasise some limitations of the current study. First, it is hard to distinguish the direct from the indirect QHCl effects on brain responses. However, this limitation would be very hard, if not impossible, to overcome in *in vivo* human studies. As we wanted to investigate purely interoceptive gut-brain signals in order to avoid potentially confounding taste effects, we excluded the exteroceptive properties of the tastant by using intragastric administration. We acknowledge that this does not reflect normal meal ingestion by excluding oral and cephalic phases. However, adding relative high doses of QHCl directly to a meal is not a realistic option in clinical studies, as it would lead to aversive taste reactions, thereby compromising tolerance. We acknowledge the limited possibility of generalisation of our results to both sexes, as only healthy female volunteers have been included in this study. Although previous studies have demonstrated that the endocrine and behavioural effects of intragastric bitter administration are only present in women, further studies in larger samples including both sexes and measurements of potential sex-specific mediators are warranted. Lastly, although fMRI constitutes one of the most sensitive techniques to assess a brain activation matrix, the spatial resolution did limit the possibility to detect changes in specific nuclei (NTS, parabrachial nucleus) in the brainstem.

In summary, this study is, to the best of our knowledge, the first to demonstrate that the bitter tastant QHCl alters brain activity in homeostatic and hedonic regions in healthy women, even when administered intragastrically to exclude effects of aversive taste or smell. Furthermore, we show that these brain responses to QHCl covary with quinine-induced decreases in levels of the orexigenic gut hormones ghrelin and motilin. In addition, these differential brain responses covary with decreased prospective food consumption scores after QHCl. They also predict the magnitude of the decrease in hedonic food intake observed after QHCl versus placebo, measured using an *ad libitum* milkshake drinking test. Taken together, these results indicate that intragastric quinine administration decreases prospective as well as actual hedonic food intake in healthy women by interfering with homeostatic and hedonic brain circuits, and that this effect may be mediated by a quinine-induced reduction in orexigenic hormone levels. These findings do not only increase our fundamental knowledge of the

mechanisms by which the GI tract controls appetite and food intake, but may also hint towards a potential therapeutic effect of bitter tastants to reduce appetite-related sensations and actual food intake. However, further studies investigating whether similar effects are observed in patient populations with disorders of food intake and body weight, including obesity and eating disorders, are warranted, as well as studies investigating the effect of chronic administration of QHCl on appetite and food intake. Given the observed brain responses in reward-related regions, future studies investigating the effect of acute bitter tastant administration on anticipatory and consummatory food reward responses in healthy volunteers and in obese patients are equally warranted, since increased anticipatory food reward but decreased consummatory food reward has already been observed in this patient population (108).

CHAPTER 4

Intragastric quinine alters brain responses to the anticipation and receipt of reward in attentional and executive regions, but not in core reward circuitry

4 Intragastric quinine alters brain responses to the anticipation and receipt of reward in attentional and executive regions, but not in core reward circuitry

4.1 Summary

Background: Intragastric bitter tastants alter circulating levels of orexigenic gut peptides and brain activity in homeostatic and hedonic brain regions, indicating a potential role in the control of food intake.

Objectives: To investigate the effect of intragastric quinine-hydrochloride on anticipatory food reward responses.

Design: Brain responses to intragastric quinine-hydrochloride (QHCl, 10 µmol/kg) or placebo infusion (t = 0) were recorded using functional magnetic resonance imaging in 21 healthy women in a single-blind, cross-over design. Subjects were presented with pictures of high-calorie food and non-food items and performed a food incentive delay (FID) task. Appetite-related sensation ratings were collected at t = -5, 15, 25, 35 and 55 min. Blood samples for metabolic hormone measurements were collected at time points: t = -10, 10, 35 and 55 min. Brain responses to QHCl compared to placebo were analysed for different contrasts (high-calorie versus non-food items, anticipation and receipt of high versus no reward) using a whole brain approach, with a voxel level threshold of $p_{uncorrected} < 0.005$, with a cluster level extent threshold of $p_{FWE\text{-}corrected} < 0.05$.

Results: No significant differences in brain response to high-calorie compared to non-food items after QHCl compared to placebo administration were observed during the passive viewing task. During the FID task, a stronger response during the anticipation of high reward (in contrast to no reward) was found in the precuneus after QHCl compared to placebo administration. During the receipt of high reward (in contrast to no reward), a stronger brain response after QHCl compared to placebo administration was found in the middle frontal gyrus, including the dorsolateral prefrontal cortex and the superior parietal cortex. No significant differences in circulating levels of gut hormones were observed between both conditions. At t = 35, desire to eat ratings were lower after QHCl compared to placebo administration.

Conclusions: The anorexigenic effect of bitter tastants is most likely due to an increased brain response during the receipt of food reward, in brain regions involved in attentional control and cognitive inhibition, rather than responses in the core reward circuit.

4.2 Introduction

Taste perception is essential to avoid the ingestion of potential harmful foods. For example, bitter taste, sensed by 25 subtypes of the taste receptor type 2 (TAS2R) family of G-protein coupled receptors (GPCR), is aversive by nature and is often related to toxic compounds (83). Moreover, TAS2R are also present beyond the oral cavity and are, among others, expressed in the human gastrointestinal (GI) tract (85), thereby opening up the possibility for bitter tastants to influence GI physiology. Intragastric administration of denatonium benzoate (DB), a strong bitter tastant, has been shown to reduce hunger and prolong satiety after intragastric administration of a liquid meal (84). Another bitter tastant, quinine-hydrochloride (QHCl), decreased food intake when administered intraduodenally (89). Moreover, Deloose et al. demonstrated that intragastric QHCl decreased circulating motilin and total ghrelin plasma levels (90). Motilin, a polypeptide hormone secreted by enteroendocrine cells (EEC) in the duodenum and jejunum, and by neurons of the myenteric plexus, is the key regulator of the migrating motor complex (MMC), a cyclic secretomotor pattern during the fasted state associated with peaks in hunger ratings (33). Ghrelin, a 28-amino acid, is the only pure orexigenic GI hormone; its plasma levels peak pre-prandially and decrease with food ingestion (87). Gut peptides constitute an important part of the afferent limb of the gut-brain axis, the bidirectional, neurohumoral communication system between the GI tract and the brain (1).

Gut peptides signal to the brain both indirectly (i.e. neurally, through receptors on vagal afferents) and directly [i.e. humorally, by acting on brain regions that control energy homeostasis including hypothalamic (e.g. the arcuate nucleus) and brainstem nuclei (e.g. the nucleus of the solitary tract (NTS))] (38). Activation of these regions by peripheral endocrine and metabolic signals, including gut peptides, regulates energy intake and expenditure in line with the body's energy status (6, 23). However, gut peptides also impact on brain regions in the reward network, which closely interact with the homeostatic system (60). This network comprises midbrain regions such as the ventral tegmental area (VTA) and substantia nigra, together with their dopaminergic projections to the nucleus accumbens (ventral striatum), putamen and caudate (dorsal striatum), pallidum, amygdala, anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), and insula. The abovementioned research identified anorexigenic properties of intragastric bitter tastant administration, mediated by an effect on circulating levels of gut peptides. Therefore, we examined the neural mechanisms underlying this anorexigenic effect in our previous study. More specifically, we demonstrated that intragastric administration of the bitter tastant QHCl (bypassing orosensory effects) alters brain activity in homeostatic (hypothalamus, medulla) and hedonic brain regions (including the anterior insula, ACC, amygdala, putamen, nucleus accumbens, basal ganglia, …) in healthy women (as described in chapter 3 of this doctoral thesis). Additionally, QHCl decreased circulating levels of gut hormones, including

ghrelin and motilin, and the effect of QHCl on brain responses covaried with these quinine-induced decreases in circulating levels of orexigenic gut hormones. Furthermore, these differential brain responses covaried with decreased prospective food consumption scores after QHCl, and predicted the magnitude of the decrease in hedonic food intake after QHCl compared to placebo, measured by an *ad libitum* chocolate milkshake drink test, performed right after the functional magnetic resonance imaging (fMRI) scan (109).

To expand upon these results, studying the effect of bitter tastants on anticipatory food reward responses (i.e. affective responses to food-related stimuli, e.g. food images or cues announcing actual food intake (110)) would offer further insight in the gut-brain signalling mechanisms underlying the abovementioned effects of bitter tastants on food intake regulation. Results from previous fMRI studies showed activation in reward-related brain areas, including the dopaminergic midbrain (VTA), insula, amygdala, striatum (nucleus accumbens, caudate, putamen), ACC and anterior hippocampus during exposure to food cues, reflecting anticipatory food reward responses. These responses are accompanied by activation in the OFC and ventromedial prefrontal cortex to assign incentive motivation to the presented food stimuli, which in turn leads to feeding behaviour (23, 97, 111). Another fMRI study demonstrated an increased neural response to high-calorie food images in reward system regions, particularly the striatum and the amygdala, after intravenous ghrelin administration, thereby indicating that metabolic signals, including ghrelin, can drive food consumption by increasing hedonic responses to food cues (60).

The current study aimed to investigate the effect of intragastrically administered QHCl, as used in our previous study (chapter 3), on neural anticipatory food reward responses to visual palatable food cues and to a food incentive delay task (to explicitly disentangle the anticipation and actual receipt of reward), and the potential role of metabolic gut hormones herein. We hypothesised to observe decreased anticipatory food reward responses to high-calorie food items as well as during the anticipation as well as receipt phase of a food incentive delay task after QHCl compared to placebo administration.

4.3 Materials and Methods

Subjects

Twenty-three healthy, female subjects (mean age: 24.05 ± 0.79 years) were recruited for this study. Because of the lack of previous studies assessing the effect of a bitter tastant on food reward responses, the expected effect size cannot be estimated. A sample size of 21 provides 80% power to detect a medium-sized effect (Cohen's d = 0.65) for the paired *t*-test (QHCl versus placebo) of the second level fMRI analyses described below, at the 95% confidence level. Two additional subjects were

recruited to anticipate potential dropouts. Data from 1 subject was excluded from analysis due to substantial movement artefacts (> 5 mm overall displacement). Further, data from 1 subject was excluded, as a functional abnormality around the anterior lobe of the pituitary gland was observed on the anatomical scan. The enrolment of only female subjects was based on our previous study in which a significant effect of intragastric DB administration on hunger scores and motilin levels was found in women only, which might be due to their higher sensitivity to bitter tastants (84). Subjects were recruited via advertisements in the buildings of the Catholic University of Leuven, and from a database of volunteers who previously participated in studies within our research group. All subjects were required to have a normal body mass index (mean BMI: 21.50 ± 0.39 kg/m^3) and to have maintained a stable body weight during the previous 3 months before the start of the study without ever having undertaken behavioural, therapeutic or surgical treatment to lose weight. All subjects were right-handed and had normal or corrected to normal vision. All potential candidates were screened beforehand using an online electronic system (Limesurvey) using the following validated self-report questionnaires: Patient Health Questionnaire, short form of the Rome III criteria questionnaire for functional GI disorders, Eating Disorders Inventory, Dutch Eating Behaviour Questionnaire, Anxiety Sensitivity Index and Power of Food Scale. Exclusion criteria included: history of GI, cardiovascular, neurological, endocrine or psychiatric disorders, abdominal surgery (except appendectomy), abnormal eating behaviour and eating disorders, use of medication on a regular basis (except oral contraceptives) and MRI-specific exclusion criteria (ferromagnetic implantations, claustrophobia), smoking, pregnancy and breast-feeding. The screening included a food preference questionnaire to separately assess liking ("How pleasant would you find the taste of this food right now") and wanting ("How much would you want some of this food right now") of 10 different savoury and sweet food items, including breakfast items (butter and chocolate croissants, waffles, muffins), using a 10-point Likert scale ranging from "not at all" to "very much". In order to avoid subjects to whom the items used during the food incentive delay task were not appealing and/or rewarding, only subjects who classified at least half of the breakfast items as palatable (a score above 6 on the Likert scale) were considered eligible for inclusion. Subjects were asked to refrain from caffeine and alcohol at least 12 hours prior to each study visit. All subjects gave written informed consent in compliance with the Declaration of Helsinki and received compensation for their participation. This study was approved by the Medical Ethics Committee of the Leuven University Hospital, Belgium with reference number S59779.

Experimental design

The study used a randomised, single-blind, cross-over design. Subjects came to the Radiology Department of the University Hospitals Gasthuisberg, Leuven, Belgium after an overnight fast on two

separate occasions. For all subjects, both study days began at 08:00 in light of circadian rhythms of central and peripheral (an)orexigenic signals. Subjects were familiarised with the study procedures and a training session of the tasks was performed to minimise novelty and learning effects. A nasogastric feeding tube was placed and an intravenous cannula was inserted in a forearm vein of the non-dominant arm at least 30 min before subjects entered the scanner room. Subjects were positioned in the scanner with their head fixated on the scanner bed using foam cushions to minimise head movements. After positioning, a structural scan was acquired. Afterwards, one of the two test solutions was intragastrically infused (t = 0). After 15 min, the fMRI scan, which consisted of 2 runs of 9 min and 2 runs of 9.5 min, was performed. The former consisted of a passive viewing task during which subjects were presented with a set of food and non-food images, the latter of a food incentive delay (FID) task during which subjects were able to win snack points, which they could exchange for breakfast items immediately after scanning. The order of the tasks was identical for all study visits, to avoid differences between or within subjects when the tasks were performed on different time points within the time course of the effect of QHCl on brain responses. Appetite-related sensation ratings were collected at t = -5, 15, 25, 35 and 55 min. Blood samples for metabolic hormone measurements were collected at time points: t = -10, 10, 35 and 55 min. Immediately after the last run, subjects left the scanner and the nasogastric tube and cannula were removed. Subjects traded their gained "snack points" (SP) for pastries, presented as a small buffet, and consumed them in a separate room. Subjects were only allowed to leave 2 hours after the end of the scan to avoid a lack of motivation during the FID task (e.g., if they would have the opportunity to buy food immediately after the study). The study procedures are summarised in Figure 4.1.

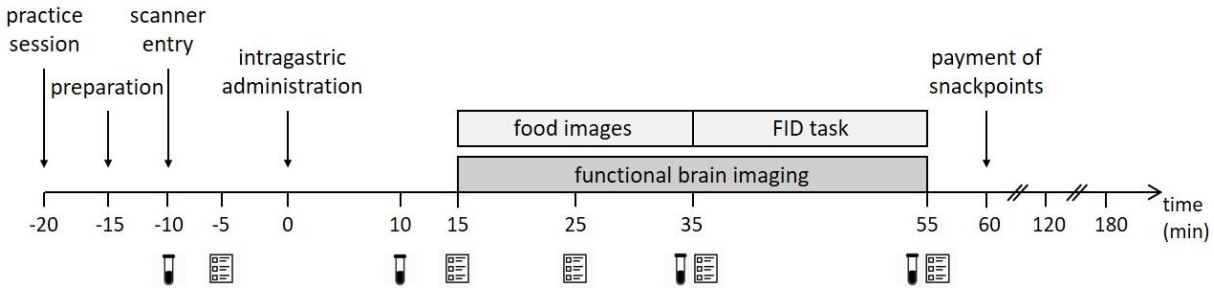

Figure 4.1 Overview of the study protocol.
Preparation: placement of nasogastric feeding tube and intravenous cannula. Both study visits of the cross-over study were identical, except for the nature of the intragastric administration (QHCl, 10 µmol/kg bodyweight or Milli-Q water as the placebo condition). Blood collection for total ghrelin, octanoylated ghrelin and motilin. Questionnaires: visual analogue scales for appetite-related sensations (including hunger, fullness, satiety, prospective food consumption, desire to eat) and nausea. Food images: passive viewing task with low-calorie, high-calorie and non-food items. FID: food incentive delay task, QHCl: quinine-hydrochloride.

Intragastric quinine-hydrochloride administration

Either QHCl (Fragon, Rotterdam, The Netherlands) (10 µmol/kg bodyweight) dissolved in Milli-Q water (Millipore Corporation, Billerica, USA) or Milli-Q water only as the placebo condition was intragastrically infused at room temperature in a single-blind, randomised, and counterbalanced fashion (t = 0). Dosage was based on our previous studies (90, 109). To this end, a clear nasogastric feeding tube (RT6/80, Medicina, Massachusetts, United States) was inserted with its tip in the proximal stomach and attached to the subject's face using medical tape. To confirm the position of the probe, gastric fluids were aspirated and the pH was measured using pH strips (Merck Millipore, Billerica, USA). This was followed by an adaptation period of at least 15 min prior to positioning in the scanner. The time window between two study visits was at least one week to restrict potential carryover effects (although unlikely due to acute administration in the first place).

Appetite-related sensation ratings

Computer-based 100 mm VAS ratings (ranging from 0 – "not at all" to 100 – "very much") of appetite-related sensations (hunger, prospective food consumption, fullness, satiety and desire to eat) and nausea were collected at baseline, before, between and after the food image paradigm blocks, and at the end of the scan (t = -5, 15, 25, 35 and 55). VAS were projected on a screen in the scanner at the end of the scanner bed and could be seen by subjects using a twin mirror system, mounted on the head coil. Ratings were given by moving a tick mark along a line segment, using a MR-compatible response box in their right hand, responding to the questions: "How hungry do you feel?", "How much do you think you can eat?", "How full do you feel?", "How satisfied do you feel?", "How pleasant would it be to eat something right now?" and "How nauseous are you?". The order in which the questions were presented was randomised. Ratings were programmed using Affect 4.0 software (95).

Food image paradigm

During the first 20 min of scanning, a passive viewing task was performed where subjects were shown high-calorie (HC) or low-calorie (LC) food images alternated with neutral, non-food (NF) items. This was done using a block design with two imaging runs in a counterbalanced order, based on previous research (112). Each run consisted of 4 epochs, each starting with a resting period of 24 sec during which a blank screen was shown, followed by 3 image-viewing blocks (HC, LC and NF). These blocks were pseudo-randomly presented in a counterbalanced order. Within each 24 sec images block, 8 individual images were presented for 3 sec each. In total, 32 images were used per run, resulting in 64 unique images for each category. At the end of each block two 100 mm VAS questions ranging from 0 ("not at all") to 100 ("very much") were presented, to assess linking ("How pleasant were the images?") and wanting (only in case of the food images, "How much would you like to eat one of the presented items right now?"). Images were acquired from an image database, and only food items that were

appealing in the morning were selected to avoid an adverse repelling effect on the reward system (113). Low-calorie food images included low-fat items (fruits, vegetables, crackers, ...). High-calorie food images consisted of sweet and savoury high-fat, high-sugar items (croissants, waffles, cake, donuts, …). Control images were non-food related household and office items. Before the first run, in between run 1 and 2 and after the second run, appetite-related sensations were assessed as described above. The food image paradigm is summarised in Figure 4.2.

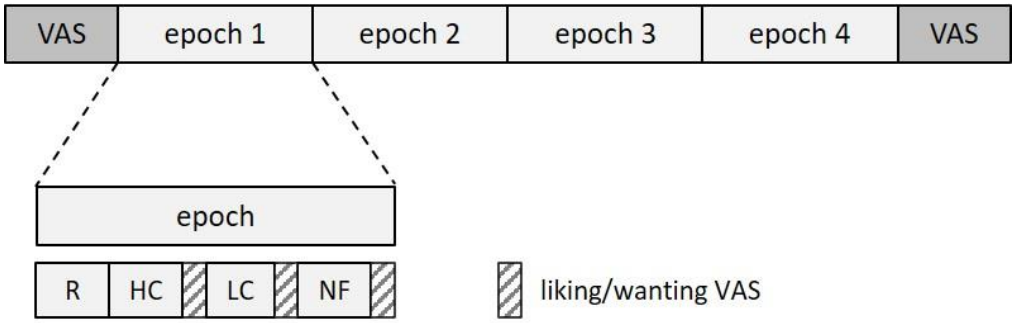

Figure 4.2 Schematic representation of 1 run of the food image paradigm.
This paradigm was build up out of two consequent runs. Each run consisted of four different epochs. Each epoch started with a 24 sec rest period, followed by 3 image-viewing blocks: high-calorie images (HC), low-calorie images (LC) or non-food images (NF), pseudo-randomly presented in a counterbalanced way. Within each block, 8 images were presented for 3 sec each. At the end of each block, liking and wanting (only in case of HC and LC), were assessed using VAS questions. At the beginning and the end of each run, appetite-related sensations (hunger, fullness, satiety, prospective food consumption, desire to eat) and nausea were assessed using VAS. VAS: visual analogue scale.

Food incentive delay task

During the food incentive delay task, subjects were able to win snack points (SP), which they could exchange for pastries immediately after the scan. This is a modification of the 'monetary incentive delay task', which allows an efficient probing of both anticipation and consumption of reward (114). The task consisted of 2 identical blocks, divided over 2 imaging runs. Each block consisted of 55 trials each. During each trial, SP could be gained. The degree of potential reward varied between 3 levels as indicated via graphical cues. Each trial started with the presentation of a symbol ('cue') for 750 msec, indicating the amount of SPs subjects could win during this trial. Cues consisted of an empty circle (0 SP), a circle with one line (2 SP) or a circle containing two lines (10 SP). After an anticipation period ('delay') of 3000 msec, subjects had to correctly react to one out of two different symbols ('target', i.e. a triangle inclined to the right or to the left). Using a MR compatible button box, subjects answered with a left or right button press, corresponding to the direction of the target within a fixed interval of 1000 msec. To ensure a steady rate of reward and non-reward throughout all subjects, a probabilistic reward pattern was applied, i.e. reward was not paid out in 15 predefined trials out of 55 trials.

Immediately after target presentation, feedback appeared ('feedback') for 1500 msec, notifying subjects about the amount of SPs they won during the trial and about their cumulative total. For this purpose, images of either an empty basket (0 SP won), one croissant (2 SP won) or a collection of croissants (10 SP won) were shown. Intertrial intervals (ITI) were jittered ranging from 1 to 8 sec (mean ITI = 3.5 sec). An incorrect button press resulted in a zero pay-out. If a button press was missed, a penalty of -10 SP was applied, in order to ensure that subjects paid attention to every trial. The maximum of snack points that could be won during 1 run was 150 SP, with every pastry being worth 50 SP. At the start of the study visit, before placement of the nasogastric tube, subjects performed a try-out version of the task for 3 min, to avoid learning effects while the task was performed during scanning. They did not receive any SP during this practice session. This protocol is slightly adapted from previous research and summarised in Figure 4.3 (115).

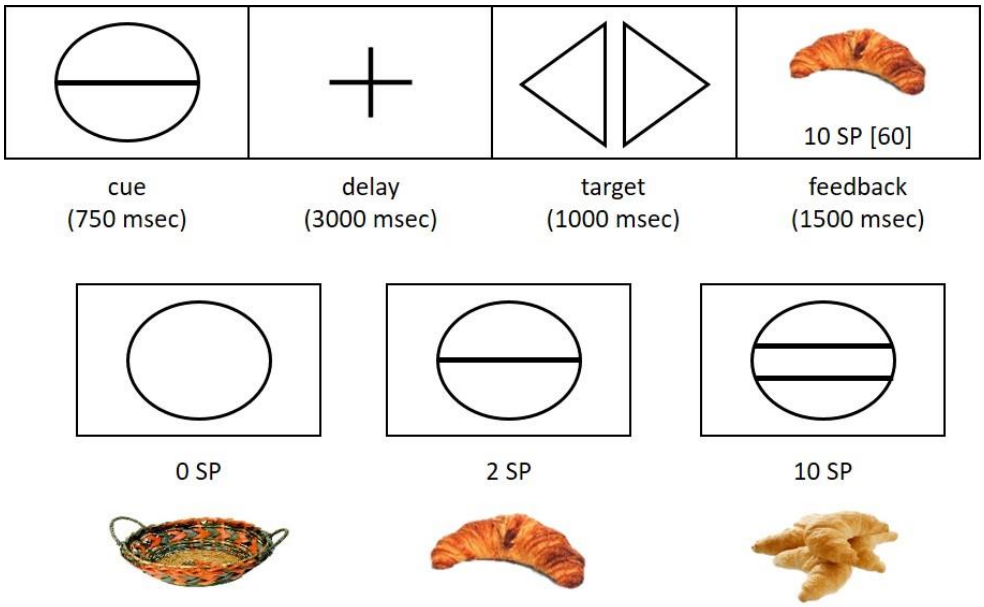

Figure 4.3 Overview of one trial of the food incentive delay task
Each trial started with the representation of a cue, representing different rewarding outcomes (0 snack points (SP), 2 SP or 10 SP), followed by a delay period. The amount of SP indicated by the cue was won if they responded with a right button box press to the direction of the arrow ('target'). Feedback about the amount of SP won during the trial was displayed immediately, together with their cumulative amount between square brackets. The entire task consisted of 2 imaging runs, each containing 55 trials. Figure adapted from Simon et al. (115).

Gut hormone measurements

At various time points during the scan (t = 10, 35 and 55), blood samples were collected for quantification of plasma octanoylated and total ghrelin, and motilin. A baseline sample was taken right before positioning of the subjects (t = -10). Ghrelin blood samples were collected in standard EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 500 kIU/ml aprotinin

(Roche Applied Science, Penzberg, Germany) and phenylmethanesulfonyl fluoride (PMSF, 57 µM) (Sigma-Aldrich, Steinheim, Germany). Plasma was acidified (10%) with 1N HCl before extraction on Sep-pak C18 columns (Waters Corporation, Milford, Massachusetts, USA) and vacuum-dried. Motilin blood samples were collected in lithium heparin tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 500 kIU/ml aprotinin. All blood samples were kept on ice immediately after collection. Plasma was separated by centrifugation at 4℃ for 10 min at 3000 xg and stored at -80℃ until analysis. Plasma total ghrelin levels were measured with an in-house developed radioimmunoassay (RIA) using ^{125}I [Tyr^{24}] human ghrelin [1-23] as the tracer and a rabbit antibody against human ghrelin [14-28], which recognises both octanoylated and desoctanoylated ghrelin. Plasma octanoylated ghrelin was measured using a rabbit antibody against human ghrelin [1-8], which does not cross-react with desoctanoylated ghrelin. Motilin levels will be measured using ^{125}I [Nle^{13}] human motilin as the tracer and rabbit anti-human Nle^{13} motilin antibody (87, 96). However, due to technical reasons data on motilin levels will not be reported in this doctoral thesis.

Functional magnetic resonance imaging acquisition

Images were collected using a 3.0 Tesla MR System (Philips Medical Systems, Best, The Netherlands) equipped with a standard 32-channel head coil for radiofrequency transmission and reception. The entire protocol consisted of 4 runs, the first two runs lasted for 9 min with 216 volumes each, the last two runs lasted for 9.5 min and contained 228 volumes each.

A high resolution T1 weighted anatomical image was acquired right after intragastric infusion on each study visit for anatomical reference and to exclude anatomical abnormalities (parameters: 182 slices of 3 mm thick, slice gap = 0.3 mm, TR = 9.6 msec, TE = 4.6 msec, flip angle = 8°, voxel size of 0.98 x 0.98 x 1.20 mm³). Starting 15 min after intragastric infusion, functional, T2-weighted volumes were acquired with a gradient echo planar imaging (EPI) sequence, covering the whole brain including the cerebellum, with blood oxygenation level-dependent (BOLD) contrast and the following parameters: TR = 2500 msec, TE = 30 msec, 46 slices of 2.70 mm thick, slice gap = 0.3 mm, flip angle = 90°, voxel size of 2.40 x 2.38 x 2.70 mm³. Timing was chosen to ensure the peak of the effect of QHCl on brain activity, which was found in our previous study, was within the time range of the tasks (109).

Statistical analysis

Visual analogue scale ratings and gut hormone level analysis

Data were analysed using SAS 9.4 (SAS Institute, Cary, NC, USA). All data are shown as mean ± SEM. Significance level was set at a *p*-value ≤ 0.05. Data were logarithmically transformed to fulfil the assumption of normally distributed residuals in mixed model analysis. All subjects were included in the analysis, but data on hormone levels were missing for 1 subject, due to technical problems with blood

sampling. Data shown in the results section are estimates of transformed values for each specific outcome measurement, as these were used in the statistical analysis. Raw observed values of these outcomes are graphically presented in the corresponding figures. Condition (QHCl, placebo) and time (amount of time points, depending on the outcome variable) were entered as categorical within-subject variables. All models were controlled for potential visit effects. For appetite-related sensations and gut hormone levels, the main effect of condition and the condition-by-time interaction effect represented the principal effect of interest. In case of a trend ($p < 0.10$) for a condition or condition-by-time interaction effect, planned contrast analyses were performed to compare the difference in appetite-related sensations ratings or gut hormone levels between conditions at the different time points. In our previous study, differences in most appetite-related sensations were observed after 20 min post-infusion, except for prospective food consumption where differences were observed after 10 min. Ratings on appetite-related sensations were collected until 50 min post-infusion. In that study, differences in octanoylated ghrelin and motilin levels were observed starting 20 min post-infusion up to 60 min post-infusion (109) (chapter 3). Given these results, we had specific hypotheses about the timing of the effect of QHCl in the present study for VAS ratings of appetite and gut hormone levels. One-tailed (given the *a priori* hypothesis about the direction of the effect) *p*-values are reported for the planned contrast analyses at 35 min and 55 min post-infusion for hormonal levels and for the planned contrast analyses at 15 min, 25 min, and 35 min post-infusion for ratings of appetite. For liking and wanting scores during the passive viewing task, the main effect of condition was the effect of interest. Appetite-related sensations were analysed as change from baseline (t = -5). Gut hormone levels were analysed as percentage change from baseline (t = -10). The variance-covariance structure providing the best fit was chosen based on the minimum value of Akaike's information criterion (AIC).

Functional MRI data analysis

Data were analysed using Statistical Parametric Mapping (SPM12, Wellcome Trust Centre for Neuroimaging, UCL, London, UK) implemented in MATLAB R2014b (The MathWorks Inc., Natick, MA). All individual images were pre-processed using the standard procedures implemented in SPM12, including spatial realignment as correction for small movements during the scan and co-registration of each functional image to the structural image of each subject and segmentation of the structural image. The structural image was used for each subject as reference for the spatial normalisation to a standard T1 template brain (Montreal Neurological Institute (MNI)) spacing using the EPI template image supplied with SPM12, based on information obtained during the segmentation step. Pre-processing of the functional scans also included slice time correction to correct for image acquisition time. Spatial smoothing using a 8-mm full width at half maximum (FWHM) Gaussian smoothing kernel was applied to the normalised images to improve the signal-to-noise ratio. To account for magnetic

field equilibration, four dummy scans from the start of each functional run were disregarded for analysis.

For first (subject) level analyses, pre-processed data of the passive image viewing task and the FID task were analysed separately for each condition (QHCl, placebo) in the context of the general linear model (GLM) approach. For both tasks, 6 realignment parameters (translation in the x-, y- and z-direction and rotations: pitch, roll, yaw) were included to correct for residual motion artefacts. Individual contrast images corresponding to the effects of interest were calculated as follows:

Food image paradigm

Separate statistical contrasts were generated at first level between regional BOLD responses to HC versus NF images.

FID task

Regressors were modelled separately for the three different anticipation phases (0 SP, 2 SP, 10 SP) and the 5 different outcome phases during the feedback period of one trial (payment of 10 SP after correct response, no payment of 10 SP after correct response during unpaid trial, payment of 2 SP, no payment of 2SP after correct response during unpaid trial and neutral outcome of 0 SP). Trials in which subjects responded incorrectly or trials in which subjects' button press was not within the time window of the target (1000 msec), were not modelled as there were too few instances for these conditions. For the anticipation phase, the anticipation of high reward (10 SP) was contrasted with the anticipation of no reward (0 SP) (cue 10 SP > cue 0 SP). To analyse the effect of receiving the anticipated reward, the receipt of a high reward (10 SP) was contrasted with the receipt of no reward (0 SP) (payment 10 SP > feedback 0 SP).

For both tasks separately, these individual contrast images of all subjects for both conditions, were entered into a second (group) level analysis. A paired sample *t*-test was performed to compare the within-group difference between QHCl and placebo administration using the contrasts QHCl > placebo and placebo > QHCl. We used a whole brain approach, using a voxel level threshold of $p_{uncorrected} < 0.005$, with a cluster level extent threshold of $p_{FWE\text{-}corrected} < 0.05$. A cluster level extent threshold of $0.05 < p_{FWE\text{-}corrected} < 0.10$ was considered a non-significant trend. Results were masked using a grey matter mask, created by averaging the gray matter images of all subjects generated during the segmentation step of the pre-processing pipeline, using the ImCalc function in SPM12. Additionally, voxel-based analyses were also performed within a single mask of regions of interest (ROI) acquired from the brain results observed in our first study, investigating the effect of QHCl on brain responses in homeostatic and hedonic brain regions using phMRI (109) (chapter 3). These ROIs included key

homeostatic and hedonic brain regions as mentioned above. This analysis was performed at a voxel-level threshold of $p_{FWE\text{-}corrected} < 0.05$.

When significant differences between QHCl and placebo were observed in the present study, outside the ROI mask acquired from our first study, we used a ROI mask consisting of these clusters to reanalyse the data of our first study (109) (reported in chapter 3). This was done to test whether QHCl effects were present in these regions outside the core reward system in our first study, to interpret our current findings.

4.4 Results

Visual analogue scale ratings

Appetite-related sensations

Most appetite-related sensations did not differ after QHCl and placebo administration (main effect of condition; F (1, 135) = 0.36, p = 0.55 for hunger, F (1, 135) = 0.27, p = 0.61 for prospective food consumption, F (1, 135) = 0.05, p = 0.83 for satiety, F (1, 135) = 0.08, p = 0.78 for fullness) (Figure 4.4A till D). A non-significant trend for lower desire to eat scores after QHCl compared to placebo was observed (main effect of condition, F (1, 135) = 2.88, p = 0.092). Planned contrast analysis showed a significantly stronger decrease in desire to eat scores after QHCl compared to placebo administration 35 min post-infusion (F (1, 135) = 3.54, p = 0.031) (Figure 4.4E). No significant condition-by-time interaction effect was observed (F (3, 135 = 0.76, p = 0.52 for hunger, F (3, 135) = 0.57, p = 0.64 for prospective food consumption, F (3, 135) = 0.11, p = 0.95 for satiety, F (3, 135) = 1.40, p = 0.25 for fullness, F (3, 135) = 0.39, p = 0.76 for desire to eat) (Figure 4.4).

Liking and wanting

For the high-calorie food images, a non-significant trend for higher liking scores after QHCl administration compared to placebo administration was observed (main effect of condition; F (1,20) = 3.49, p = 0.077) (Figure 4.5A). For wanting of high-calorie images, no significant differences were observed after QHCl compared to placebo administration (main effect of condition; F (1, 20) = 0.14, p = 0.72) (Figure 4.5B).

For low-calorie food images, no significant differences in liking and wanting scores were observed between QHCl and placebo administration (main effect of condition; F (1, 20) = 0.94, p = 0.34 for liking and F (1, 20) = 0.51, p = 0.48 for wanting) (Figure 4.5C and D).

Amount of snackpoints gained during the food incentive delay task

The amount of snackpoints won during the FID task was similar after QHCl or placebo administration (Wilcoxon signed rank test, p = 0.46).

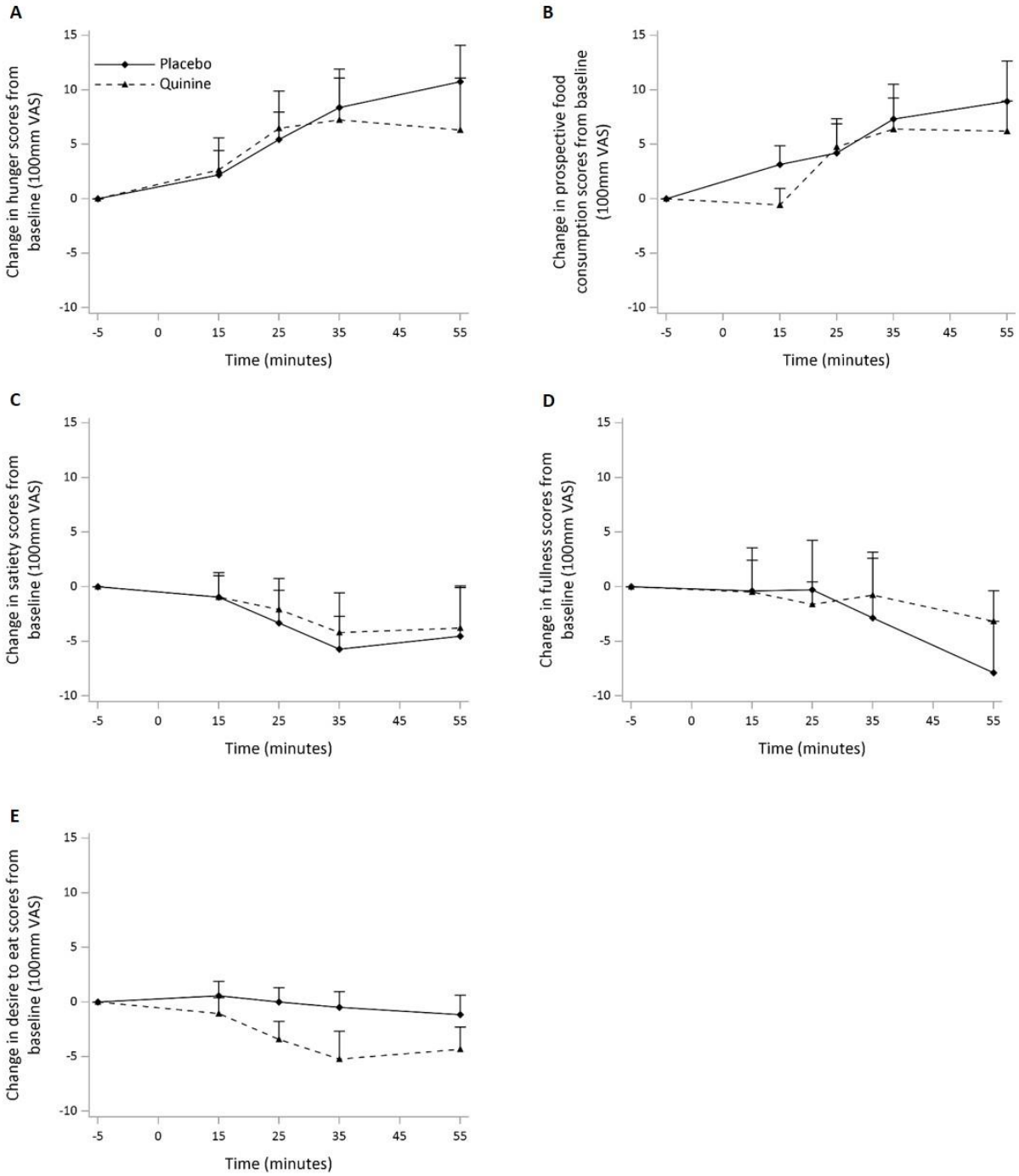

Figure 4.4 Effect of intragastric administration of quinine versus placebo on appetite-related sensations

A – **D:** No significant differences were observed for hunger, prospective food consumption, satiety or fullness scores after quinine compared to placebo administration **E:** Desire to eat. A stronger decrease in desire to eat scores was observed after quinine compared to placebo administration 35 min post-infusion (p = 0.031). Data are represented as mean ± SEM. Time point 0 indicates the time of intragastric administration. VAS: visual analogue scale.

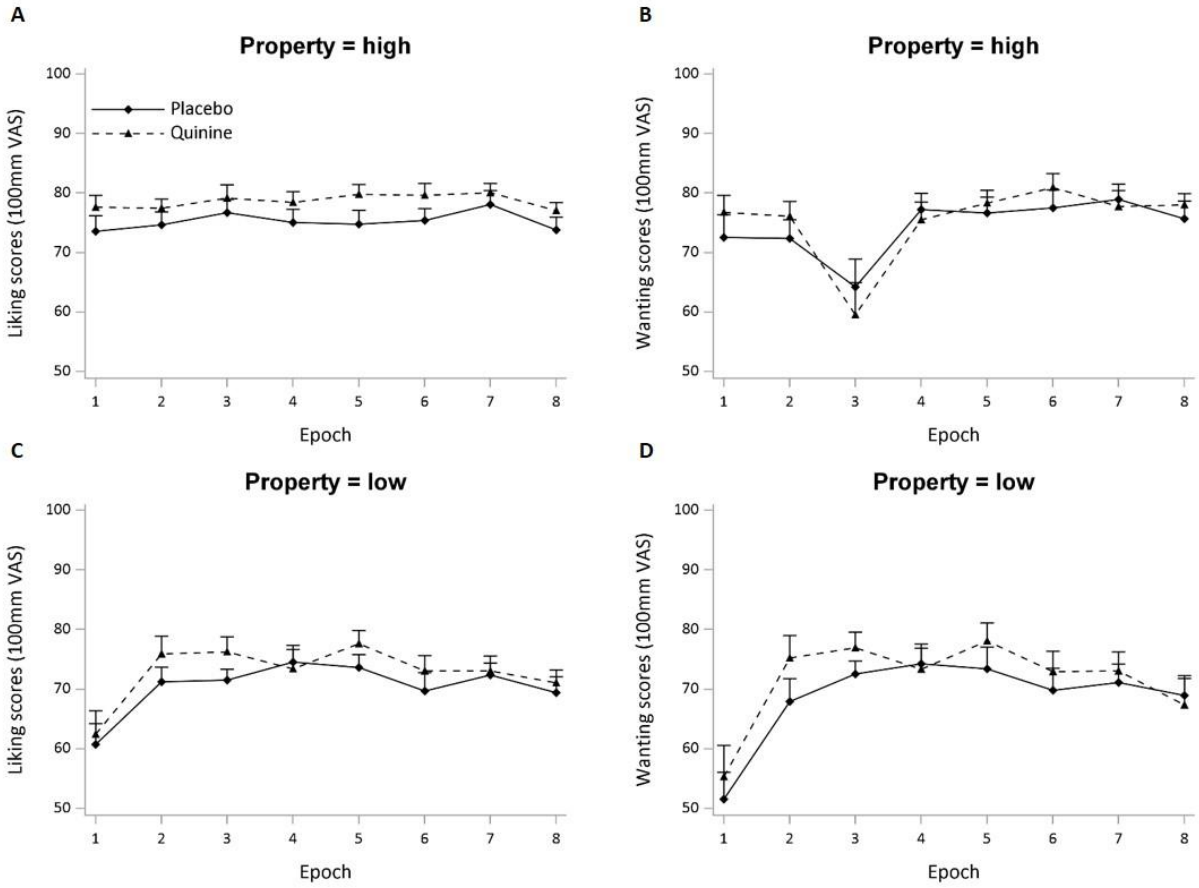

Figure 4.5 Effect of intragastric administration of quinine versus placebo on liking and wanting of high- and low-calorie food items
A-B: Liking and wanting scores for high-calorie food items. A non-significant trend for higher liking scores after quinine administration compared to placebo administration was observed (p = 0.077), but no difference in wanting scores was observed. **C-D**: Liking and wanting scores for low-calorie foods. No differences in liking and wanting scores of low-calorie food items was observed between quinine and placebo administration. Data are represented as mean ± SEM. VAS: visual analogue scale.

Gut peptide levels

No significant differences were observed after QCHl and placebo administration for circulating levels of octanoylated or total ghrelin (main effect of condition; F (1, 19) = 0.09, p = 0.76 for octanoylated ghrelin and F (1, 19) = 0.64, p = 0.43 for total ghrelin). No significant condition-by-time interaction effects were observed (F (2, 32) = 0.04, p = 0.96 for octanoylated ghrelin and F (2, 32) = 0.96, p = 0.39 for total ghrelin).

Brain responses

Food image paradigm

After placebo administration, BOLD responses to HC food items were observed in the insula, cuneus, putamen, caudate, amygdala, hippocampus, medial part of the OFC, precuneus, supplementary motor

area (SMA), the mid occipital lobe (Brodmann area (BA) 19), superior and medial frontal gyrus (BA 11, BA 10), frontal operculum, inferior temporal lobe (BA 37), lingual gyrus (BA 19), supramarginal gyrus (BA 40), ACC (BA 24, BA 32) and cerebellum (Figure 4.6, Table 4.1). Very similar BOLD responses were observed after QHCl administration (data not shown).

However, QHCl administration did not alter the BOLD response to HC food items compared to placebo, for both contrasts (QHCl > placebo and placebo > QHCl) at our applied threshold ($p_{uncorrected} < 0.005$ with a cluster level extent threshold of $p_{FWE\text{-}corrected} < 0.05$).

Analyses within a mask of regions acquired from our first study at a voxel-level threshold of $p_{FWE\text{-}corrected} < 0.05$, did not show significant differences between HC and NF images for the contrasts (QHCl > placebo or placebo > QHCl).

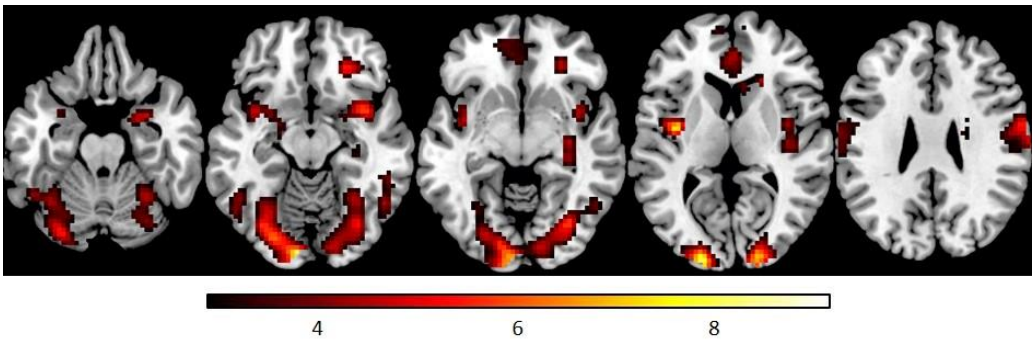

Figure 4.6 Brain regions in which a stronger BOLD response was observed after high-calorie food images compared to non-food items during the placebo condition
Voxel level threshold: $p_{uncorrected} < 0.005$ with with a cluster level extent threshold: $p_{FWE\text{-}corrected} < 0.05$. Colour scale reflects the T-value of the functional activity. BOLD: blood oxygenation level-dependent.

Food incentive delay task

Anticipation phase

After QHCl administration, a BOLD response during the anticipation of high reward (compared with anticipation of no reward, cue 10 SP > cue 0 SP) was found in the insula, caudate, precuneus, middle and superior occipital lobe (BA 17, BA 18, BA 19), ACC (BA 24, BA 32), anterior midcingulate cortex (BA 24), lingual gyrus (BA 18), superior frontal gyrus (BA 6 BA 32, BA 9), frontal operculum, superior and inferior parietal cortex (BA 7, BA 3) and the right SMA (Figure 4.7A, Table 4.1).

No significant BOLD responses to the anticipatory cue were observed after placebo administration.

After QHCl administration compared to placebo, a stronger response during the anticipation of high reward (in contrast with anticipation of no reward, cue 10 SP > cue 0 SP) was found in the precuneus (Figure 4.7B, Table 4.1).

In the same region, a stronger BOLD response after QHCl compared to placebo was also observed in our previous phMRI study, using the current cluster as a ROI mask (data not shown).

ROI analyses within a mask of regions acquired from our first study at a voxel-level threshold of $p_{FWE\text{-}corrected} < 0.05$, did not show significant differences between the anticipation of high versus no reward for the contrast (QHCl > placebo and placebo > QHCl).

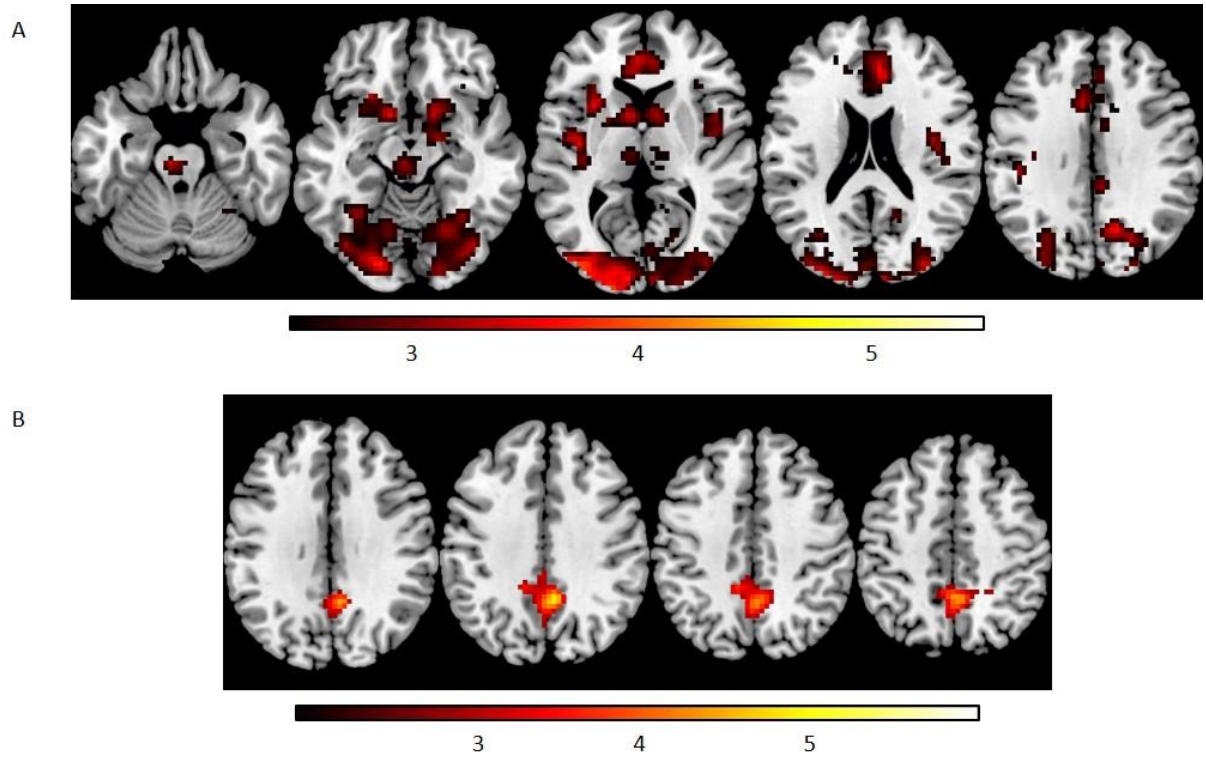

Figure 4.7 Brain regions in which a stronger BOLD response was observed after anticipation of high reward (cue 10 SP) compared to no reward (cue 0 SP) after quinine administration and quinine compared to placebo administration
A: Anticipation of high reward versus no reward within the quinine condition. **B**: Anticipation of high reward versus no reward within the contrast (quinine > placebo). Anticipation of reward was measured during a food incentive delay task. Voxel level threshold: $p_{uncorrected} < 0.005$ with a cluster level extent threshold: $p_{FWE\text{-}corrected} < 0.05$. Colour scale reflects the T-value of the functional activity. SP: snack points, BOLD: blood oxygenation level-dependent.

Receipt phase

After QHCl administration, a BOLD response during the receipt of high reward (in contrast with receipt of no reward, payment 10 SP > feedback 0 SP) was found in the caudate, precuneus, superior and inferior parietal cortex (BA 2, BA 7, BA 40), midfrontal cortex (BA 45), middle orbitofrontal cortex, SMA, putamen, ACC (BA 32), posterior midcingulate cortex (BA 23, BA 24), lingual gyrus (BA 18), fusiform gyrus (BA 18) and inferior and middle occipital gyrus (BA 19, BA 37). A trend was observed in the thalamus (Figure 4.8A, Table 4.1).

After placebo administration, a BOLD response during the receipt of high reward (in contrast with receipt of no reward, payment 10 SP > feedback 0 SP) was found in the occipital lobe (BA 18, BA 19), inferior temporal lobe (BA 19, BA 20, BA 37), lingual gyrus (BA 18), superior and inferior parietal cortex (BA 2, BA 40) (Figure 4.8B, Table 4.1).

During the receipt of high reward (in contrast to receipt of no reward, payment 10 SP > feedback 0 SP), a stronger BOLD response after QHCl compared to placebo administration was found in the middle frontal gyrus (BA 6, BA 45, BA 46) and the superior parietal cortex (BA 40, BA 7), including the angular gyrus. A trend for a stronger BOLD response was observed in the inferior and middle frontal gyrus (BA 45, BA 46, BA 47, BA 10) (Figure 4.8C, Table 4.1).

In the same regions, a stronger BOLD response after QHCl compared to placebo was also observed in our previous phMRI study, using the current clusters as a ROI mask (data not shown).

ROI analyses within a mask of regions acquired from our first study at a voxel-level threshold of $p_{FWE\text{-}corrected} < 0.05$, did not show significant differences between the receipt of high versus no reward for the contrast (QHCl > placebo and placebo > QHCl).

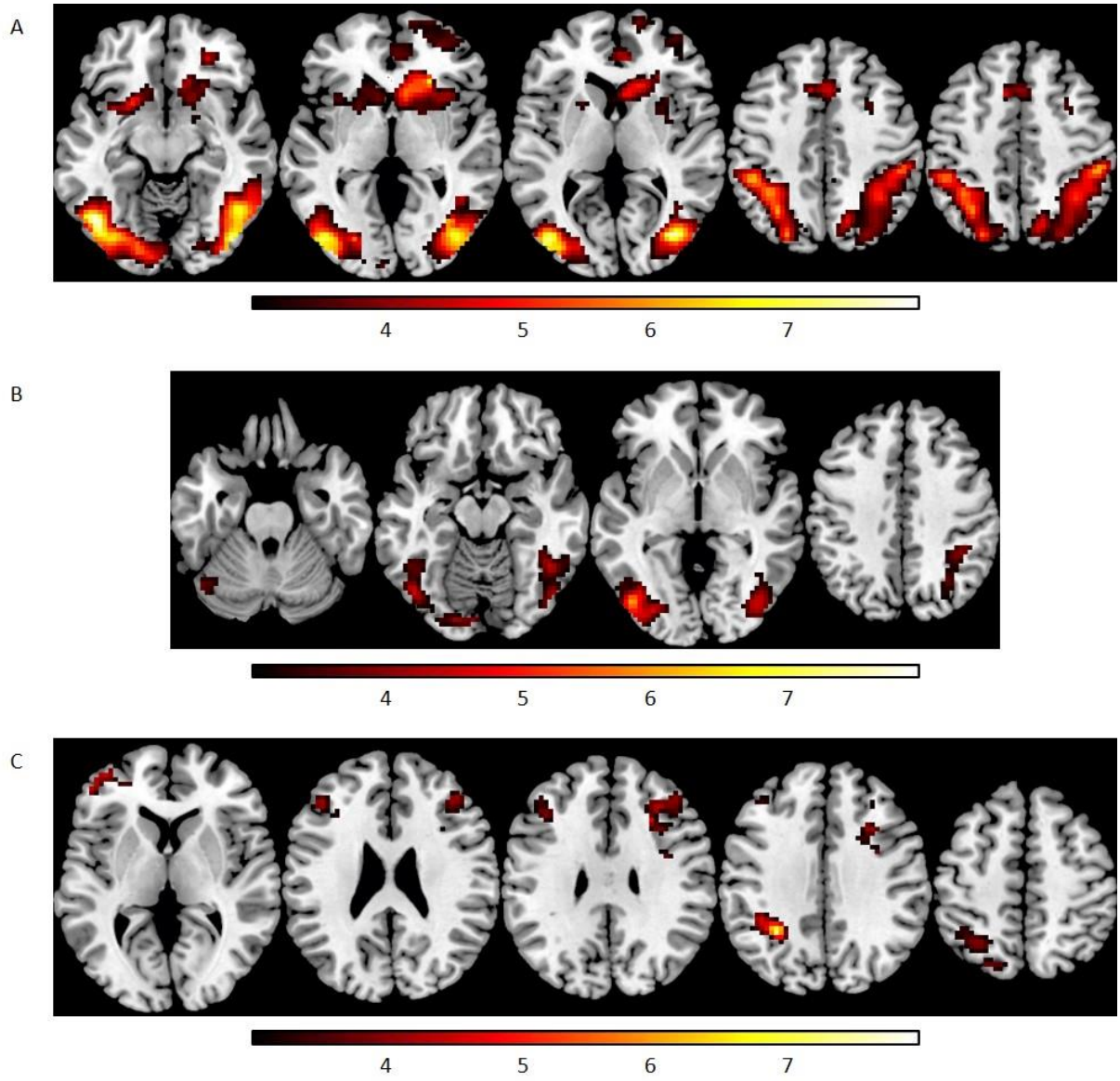

Figure 4.8 Brain regions in which a stronger BOLD response was observed after the receipt of high reward (payment 10 SP) compared to no reward (feedback 0 SP) after quinine or placebo administration and quinine compared to placebo administration

A: Receipt of high reward versus no reward within the quinine condition alone. **B**: Receipt of high reward versus no reward within the placebo condition alone **C**: Receipt of high reward versus no reward within the contrast (quinine > placebo). Receipt of reward was measured during a food incentive delay task. Voxel level threshold: $p_{uncorrected} < 0.005$ with with a cluster level extent threshold: $p_{FWE\text{-}corrected} < 0.05$. Colour scale reflects the T-value of the functional activity. SP: snack points, BOLD: blood oxygenation level-dependent.

Table 4.1 Whole brain results for the passive viewing task and the anticipation and receipt of reward phase of food incentive delay task

	region	sub region	side	peak coordinates (mm)			T-value	cluster volume (voxels)
				x	y	z		
food image paradigm								
HC > NF								
QHCl	occipital gyrus	middle	L	-15	-94	2	12.648	1294
			R	33	-82	14	3.176	1294
	parietal cortex	inferior	L	-39	-46	47	4.985	299
	cingulate cortex	anterior	L	-3	44	5	4.103	199
	insula		L	-36	-7	11	6.709	599
			R	39	8	-13	5.099	862
	putamen		L	-24	2	-7	5.615	599
			R	30	-16	5	3.842	862
	pallidum		L	-12	-4	-10	4.533	599
	amygdala		L	-27	2	-13	5.369	599
	caudate		L	-6	2	2	4.301	599
			R	15	14	-10	3.671	862
	cuneus		R	15	-94	2	9.463	1294
	hippocampus		L	-12	-13	-16	3.056	599
			R	9	-4	-10	4.501	862
	supramarginal gyrus		L	-60	-22	35	6.319	299
			R	54	-22	35	4.868	862
	fusiform gyrus		L	-30	-73	-16	7.444	1294
			R	30	-79	-13	5.871	1294
	cerebellum		R	27	-67	-19	4.222	1294
placebo	occipital gyrus	middle	L	-18	-97	5	8.967	1887
			R	33	-88	17	4.477	1887
	cingulate cortex	anterior	L	-3	32	20	3.570	762

			R	6	38	2	5.114	762
	temporal gyrus	inferior	L	-48	-55	-16	4.729	1887
			R	51	-58	-10	5.105	1887
	frontal gyrus	superior-orbital	L	-6	44	-7	3.752	762
			R	24	35	-13	5.363	762
		medial	L	-9	56	2	4.309	762
			R	9	62	5	3.480	762
	frontal opperculum		L	-39	11	17	3.062	277
	supramarginal gyrus		R	57	-22	32	3.250	193
	fusiform gyrus		L	-36	-58	-19	4.791	1887
			R	33	-73	-4	5.722	1887
	insula		L	-36	-7	11	7.911	277
			R	39	5	-13	5.760	376
	putamen		R	27	23	2	3.757	762
	caudate		R	21	26	5	5.125	762
	cuneus		R	18	-94	8	6.962	1887
	amygdala		L	-27	2	-16	4.878	277
	hippocampus		L	-21	-10	-13	3.411	277
			R	33	-16	-7	4.629	376
	precuneus		L	-12	-37	59	3.569	246
	SMA		R	9	-22	65	3.759	246
	lingual gyrus		L	-27	-82	-16	6.398	1887
	cerebellum			18	-88	-16	4.874	1887
food incentive delay task								
<u>cue 10 SP > cue 0 SP</u>								
QHCl > placebo	precuneus		L	-6	-46	44	5.296	254
			R	6	-52	35	3.753	
QHCl	insula		L	-30	20	2	8.677	5108

			R	30	26	-1	4.522	289
	occipital gyrus	middle	L	30	26	-1	4.522	289
			R	-18	-100	5	6.438	5108
		superior	L	-9	-94	8	5.345	5108
			R	24	-67	29	5.351	5108
	parietal cortex	superior	R	24	-49	50	3.865	280
		inferior	L	-42	-28	35	5.036	278
	frontal gyrus	superior	R	18	-13	65	4.827	1189
	cingulate cortex	middle	L	-6	14	32	4.560	1189
			R	12	5	38	4.525	1189
		anterior	L	-3	44	11	5.698	1189
			R	9	35	17	5.787	1189
	caudate		L	-9	11	-7	5.879	5108
			R	6	11	2	6.179	5108
	precuneus		R	15	-64	32	5.211	5108
	SMA		R	9	2	62	3.974	1189
	frontal opperculum		R	45	14	11	3.039	278
	lingual gyrus		L	-21	-85	-10	5.456	5108
payment 10 SP > feedback 0 SP								
QHCl > placebo	parietal cortex	superior	L	-30	-52	35	7.361	307
	frontal gyrus	middle	R	30	32	29	4.314	163
		inferior*	L	-42	35	23	4.394	137
QHCl	occipital gyrus	inferior	L	-45	-67	-10	7.969	2986
			R	42	-79	-10	7.234	2529
		middle	L	-42	-79	5	7.440	2986
			R	36	-76	5	7.642	2529
	temporal gyrus	inferior	R	45	-58	-13	7.292	2529
	parietal cortex	superior	L	-24	-76	44	5.910	2986

			R	33	-67	53	4.977	2529
		inferior	L	-45	-40	44	6.221	2986
			R	51	-34	47	6.113	2529
	frontal gyrus	middle	R	42	47	20	5.794	1840
		middle - orbital part	R	27	41	-10	4.599	1840
		inferior	R	54	11	20	3.591	268
	precuneus		R	15	-67	44	4.766	2529
	putamen		L	-30	8	-7	4.379	1840
			R	24	26	-1	6.848	1840
	caudate		R	9	20	-1	5.610	1840
	cingulate cortex	middle	R	3	-31	23	4.876	1840
		anterior	R	6	41	5	4.293	1840
	SMA		L	-9	17	47	4.592	1840
	lingual gyrus		L	-18	-85	-7	5.729	2986
	fusiform gyrus		L	-24	-76	-4	7.163	2986
			R	36	-40	-22	6.356	2529
	thalamus*		R	15	-7	14	4.586	149
placebo	occipital gyrus	inferior	L	-45	-76	-4	6.024	552
			R	39	-79	-7	4.475	417
		middle	L	-36	-85	-1	4.850	552
			R	33	-82	11	3.784	417
		superior	R	27	-67	35	4.604	345
	temporal gyrus	inferior	L	-45	-49	-19	3.966	552
			R	45	-52	-13	4.372	417
	parietal cortex	superior	R	42	-46	56	4.616	345
		inferior	R	48	-37	53	4.979	345
	lingual gyrus		L	-18	-88	-16	4.351	552
	cerebellum		R	-45	-67	-22	3.947	552

Results reported at voxel level threshold: $p_{uncorrected} < 0.005$ with a cluster level extent threshold: $p_{FWE\text{-}corrected} < 0.05$. *: voxel level threshold: $p_{uncorrected} < 0.005$ with a cluster level extent threshold: $p_{FWE\text{-}corrected} < 0.01$. SMA: supplementary motor area, QHCl: quinine-hydrochloride, L: left, R: right

4.5 Discussion

After the discovery of bitter taste receptors on cells other than lingual taste buds (38), the attenuating effect of bitter taste molecules, such as DB and QHCl, on hunger feelings and orexigenic gut hormones has been of interest (84, 87). The neural mechanism behind this effect has been elucidated in our previous study, where QHCl decreased circulating levels of orexigenic gut hormones and increased activity in the brain reward system, resulting in decreased prospective as well as actual food intake (109). However, the effect on anticipatory food reward responses had not been investigated.

To our knowledge, this is the first study to demonstrate that the bitter tastant QHCl alters neural anticipatory food reward responses during a food incentive delay task in healthy women after intragastric administration. The main findings included significantly increased BOLD responses in the precuneus after QHCl administration, compared to placebo, during the anticipation phase of this task. During the receipt phase of reward, BOLD responses increased after QHCl compared to placebo administration in the superior parietal cortex and the middle frontal gyrus, including the dorsolateral prefrontal cortex (dlPFC). The alteration in neural anticipatory food reward responses was not associated with a change in appetite-related sensations, except for decreased desire to eat ratings 35 min post-infusion, after QHCl compared to placebo administration. During a passive food picture viewing task, neither neural (alterations in the BOLD response), nor subjective (wanting scores of low- or high-calorie food items) anticipatory food reward responses, were different after QHCl compared to placebo administration. Against our expectations, no significant effect on brain responses in the core reward circuit was found after QHCl compared to placebo administration for neither of the tasks. Furthermore, no significant difference in circulating levels of the hunger hormone, ghrelin, was observed.

First, after QHCl administration, compared to placebo, we observed a BOLD response, i.e. increased brain activity, in the bilateral precuneus during the anticipation of high reward, compared to no reward (cue 10 SP > cue 0 SP). The precuneus is a region involved in attention, motivation and reward, and visual imagery (116). Although the precuneus is generally not considered part of the core reward system (20), a role in anticipatory food reward has been shown, as increased precuneus activity was observed in response to anticipated intake of a high-sugar, carbonated, palatable soft drink, compared to anticipation of a tasteless solution (117). This is replicated in the current study, as brain responses were observed in the precuneus during anticipation of palatable food. Moreover, we showed that this response is increased after QHCl administration, as we observed increased precuneus activity after QHCl administration compared to placebo during the anticipation of high reward, elicited by exposure to palatable breakfast items (cue 10 SP). Besides, activity in the precuneus is associated with attentional control, where deactivation in the precuneus is associated with increased attentional

demand (118). In the current study, this would imply that after QHCl administration, less attention is allocated to high food reward compared to no reward cues. This could lead to changes in food choice or food portions, as an additional explanation for the anorexigenic effect of bitter tastants. However, this hypothesis is based on reverse inference and future research looking at the effect of QHCl on the neural underpinnings of food choice is warranted. Decreased brain activity in the precuneus to high- versus low-calorie food has been described as the main finding in a previous study looking at anticipatory food reward using food cues of high- versus low energy food, where decreased precuneus activity was related to decreased food liking (119). However, in the current study we found no significant differences in food liking after QHCl compared to placebo administration, and we observed precuneus activity during the anticipation phase of a FID task, but not during anticipation elicited by high- and low-calorie food cues. The discrepancy between our two paradigms used, although both known to elicit anticipatory food reward responses, could be explained by the different natures of the anticipatory reward. Whilst subjects knew that no actual receipt of the food items presented would follow during the passive viewing task, they were aware of a future pay-out, resulting in actual consumption of the pastries used as visual cues during the FID task.

This pay-out is reflected during the contrast of the receipt of high reward (10 SP) versus no reward (0 SP), in which significant stronger BOLD responses were observed after QHCl compared to placebo administration in the superior parietal cortex, including the angular gyrus, and the dlPFC within the middle frontal gyrus. The superior parietal cortex is part of a top-down control system that also includes the precuneus, which is associated with attention, as described above. Our finding is in line with previous research, where increased superior parietal cortex activity has been found during reward anticipation (120). The prefrontal cortex (PFC), together with the posterior parietal cortex, constitutes the central executive network (121). This network is involved in the orienting attention as well as executive control (121). Moreover, a role for the dlPFC in the central regulation of eating behaviour is known as well, as the dlPFC sends inhibitory signals to orexigenic brain areas to suppress hunger and to terminate feeding episodes (122, 123). Furthermore, the dlPFC is primarily responsible for higher cognitive functioning, including reward evaluation and cognitive control over eating and emotions. An imbalance between reward and cognitive control in the dlPFC could lead to overeating in obesity (124). In the current study, increased dlPFC activity after QHCl administration, compared to placebo administration, was observed which may indicate that subjects showed higher executive control and inhibition, which could explain the observed anorexigenic effects observed in earlier studies (84, 109). Another recent study observed significantly more activity in right precentral and medial frontal areas during execution of a working memory task after intragastric administration of QHCl. Comparison between both studies is difficult, due to different dosage (17mg versus 4mg per kg bodyweight in our

study) and design (working memory task versus food incentive delay reward task), however it does confirm the influence of QHCl on central executive parietal and frontal regions (103). In our first phMRI study looking at neural responses to QHCl administration, we focussed on homeostatic and hedonic reward circuits only. No significant differences in the reward circuit were observed in the current study after QHCl compared to placebo administration during both anticipation and receipt of reward. If significant differences between QHCl and placebo were observed in the present study, outside the ROI mask acquired from our first study, for the anticipation and receipt phase of reward during the FID, a ROI mask consisting of these clusters was used to reanalyse the data of our first study (109). Also during our phMRI design, stronger brain activity after QHCl compared to placebo was observed in the precuneus, dlPFC and parietal cortex. This indicates that resting brain function within the reward circuitry is influenced by QHCl administration, however brain responses to food reward stimuli are not altered by QHCl. However, the cognitive inhibitory network is consistently activated compared to placebo, especially during the receipt phase of reward. This highlights a potential role for cognitive inhibitory regions as mediator of the orexigenic effect of bitter tastants.

Secondly, we also investigated the anticipation and receipt phase of the FID task within the QHCl and placebo condition separately as a manipulation check for our paradigms. After QHCl administration, activation in, amongst others, the anterior insula, middle and superior occipital cortex, frontal opperculum, caudate and ACC were observed for the contrast (cue 10 SP > cue 0 SP). This is in line with a previous study, which employed the same FID task to investigate food reward (115). The anterior insula and the frontal opperculum constitute the primary gustatory cortex and the anterior insula is involved in food craving (125, 126). The increased brain activity in the anterior insula, combined with the frontal operculum indicates that during the anticipation phase of this task, may therefore reflect an increased urge for food (118, 125). The caudate and anterior cingulate cortex are important parts of the dopaminergic circuit involved in rewarding aspects of food (23). These findings indicate that our paradigm was successful, as increased neural activity was elicited in key regions of the reward system. In addition, after QHCl administration alone, we observed increased activation in the SMA during both anticipation and receipt of high reward compared with no reward. The SMA is a region involved in movements of the body, however it also implies a reward expectancy signal (127), which may explain our finding of increased activity during the expectancy as well as receipt of high reward.

Anticipatory food reward was also investigated in the context of a passive viewing task using HC and LC food items compared to non-food items. For the contrast HC > NF no significant differences between QHCl and placebo administration were observed. Nonetheless, increased neural responses to high-calorie versus non-food items were observed in the insula, putamen, caudate, amygdala, hippocampus, and left ACC in both conditions. This increased brain activity in reward-related regions

in response to high-calorie food items, indicates, as observed for the FID task, that the paradigm is successful in eliciting anticipatory food reward responses. However, QHCl did not alter these responses compared to placebo administration, which is not in line with our hypotheses. As shown during the FID, QHCl impacted on brain areas involved in an attentional control network and cognitive inhibition. During a passive viewing task, without actual receipt of reward as used in the FID, no learning of probabilistic stimulus-reward associations is involved (99), as is during the FID. Besides, attention to the task stimuli and cognitive inhibition might be less important during a passive viewing task, which could explain the lack of a different effect on brain responses after QHCl administration compared to placebo. An additional possible explanation is the study design, where subjects were scanned after an overnight fast. In an earlier study, looking at neural responses using the same block design as applied in the current study, it was reported that in normal weight subjects, the biggest difference between responses was observed in the fed state compared with the fasted state (112).

Moreover, we did not observe significant differences in hunger, satiety, fullness or prospective food consumption scores after QHCl compared to placebo administration. This was not in line with our previous study, in which an anorexigenic effect of QHCl was observed on appetite-related sensation scores. However, when QHCl was administered in the duodenum by means of acid-resistant capsules, no bitter-specific effect on satiety, fullness and desire to eat scores was observed (105). It is important to note that during our previous study (chapter 3), appetite-related sensations were sampled more frequently (every 10 min) whereas in the current design, this was only the case during the passive viewing task, with scores only collected before and after the entire FID task. Besides, the current study design, including a passive viewing task and the FID, versus the phMRI design in the first study where subjects were not presented with any stimuli in between ratings, could have influenced the level attention towards interoceptive sensations, which in turn may have impacted on the VAS ratings. However, desire to eat scores showed a stronger decrease from baseline after QHCl compared to placebo administration, although this only reached statistical significance 35 min after the infusion. This indicates that without a decrease in hunger scores, subjects report decreased desire to eat levels, pointing towards a decreased intention to eat which could lead to a decreased food-seeking behaviour, contributing to the earlier described anorexigenic effect of bitter tastants. Furthermore, hunger is a feeling that reflects the internal state and survival need of the body (10) and might be more interoceptively driven compared to the desire to eat. The latter is more under cognitive control, as shown by a previous study using magnetoencephalography to assess neural activity. The authors reported that the dlPFC played a prominent role in the suppression of motivation to eat scores (128). This is in line with the neural findings of the current study, where increased dlPFC activity is observed in line with decreased desire to eat scores.

Lastly, no significant differences were observed in circulating levels of the hunger hormone, ghrelin, which is in line with the observation for hunger scores. Ghrelin is, besides its anorexigenic properties, associated with enhanced responses to food reward (60) and a previous study indicated that fasting ghrelin elevate the hedonic effects of food pictures (61). Moreover, QHCl-induced decreases in ghrelin levels covaried with brain responses in the reward circuitry, as observed in our previous study (109). Based on ghrelin's reward-related properties, the lack of a difference in circulating ghrelin levels in the present study could explain the absence of a difference in brain activity in the core reward circuit after QHCl compared to placebo, both during the passive viewing task and during the FID.

The main limitation of the current study is the lack of analyses of circulating motilin plasma levels for the scope of this doctoral thesis. As motilin levels have been shown to decrease after intragastric administration of both QHCl and DB (84, 109), motilin could be a potential mediator of the altered neural responses via an altered humoral gut-brain communication.

Taken together, our findings implicate that the anorexigenic effect of bitter tastants is most likely not due to an anticipatory response elicited by presentation of visual food cues but more likely to an effect of QHCl during the receipt of food reward. More specifically, an increased brain activity was observed in brain regions involved in attentional control and cognitive inhibition rather than in the core reward circuit, during the receipt phase of reward, after QHCl administration compared to placebo. Future studies evaluating the effect of intragastrically administered QHCl on consummatory food reward are warranted. Moreover, this study paradigm could be applied in an obese patient cohort. Impaired neural responses in the dlPFC are observed in obesity (123) and it would be of great interest to investigate if QHCl administration could restore responses in this inhibitory control network.

CHAPTER 5

Intragastric fructose administration interacts with emotional state in homeostatic and hedonic brain regions

5 Intragastric fructose administration interacts with emotional state in homeostatic and hedonic brain regions

5.1 Summary

Background: Not only exteroceptive, but also interoceptive properties of food may influence emotional state and its neural basis. This has been shown for fatty acids, but remains unstudied for carbohydrates.

Objectives: To study the effects of intragastric fructose and its interaction with sad emotion induction on brain activity in homeostatic and hedonic regions, and investigate whether gut hormone responses (ghrelin, CCK, GLP-1) can explain these effects.

Design: In 15 healthy subjects, brain activity before and until 40min after intragastric infusion of fructose (25g) or water was recorded using a cross-over pharmacological magnetic resonance imaging (phMRI) paradigm. Sad or neutral emotional states were induced by combining classical music and emotional facial expressions. Emotional state was assessed every 10min using the Self-Assessment Manikin. Blood samples to assess gut hormone levels, were taken at the same time points. Brain responses to fructose versus placebo, sad versus neutral emotion, and their interaction were analysed over time in a priori defined regions of interest at a voxel-level threshold of $p_{FWEcorrected} < 0.05$. Similar effects on emotional ratings and hormone levels were tested using linear mixed models.

Results: We did not observe significant main effects of fructose or emotion, nor a significant fructose-by-emotion interaction effect, on emotional ratings. However, significant main effects of fructose, emotion, and a significant fructose-by-emotion interaction effect were found on brain activity in the medulla, midbrain, hypothalamus, basal ganglia, anterior insula, orbitofrontal cortex, anterior cingulate cortex and amygdala. The increase in circulating levels of GLP-1 after fructose administration in neutral emotion was abolished during sad emotion (fructose-by-emotion-by-time, $p = 0.041$). Circulating ghrelin levels were higher in sad emotion (time-by-emotion, $p = 0.037$). CCK levels were higher after fructose administration (main effect of fructose, $p = 0.028$).

Conclusions: Emotional state interacts with brain and endocrine responses to intragastric infusion of 25g of fructose, however such an effect was not found at the behavioural level.

5.2 Introduction

Emotions, appetite-related sensations, and eating behaviour firmly interact with each other. In the beginning of the 21st century, Craig was the first to postulate the term 'homeostatic emotions', which defines emotions as a reflection of homeostatic interoceptive signals (e.g. hunger), and thus also of

the survival needs of the human body (3, 10). Interoceptive information is transferred to homeostatic brain regions, including hypothalamic (e.g. the arcuate nucleus) and brainstem nuclei [e.g. the nucleus of the solitary tract (NTS)]. This occurs via the afferent limb of the brain-gut axis, which is the bidirectional, neurohumoral communication system between the gut and the brain, including gut peptides like ghrelin, glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK) (129). Damasio demonstrated, using a Position Emission Tomography (PET) study, that the process of feeling emotions requires the activation of brain areas involved in the regulation of homeostasis, underlining the close connection between homeostasis and emotions (130).

Such homeostatic signals, besides maintaining the physiological state of the body within a healthy range, also strongly affect neural circuits involved in processing rewarding aspects of (food-related) stimuli. This reward network encompasses midbrain regions such as the ventral tegmental area (VTA), together with its dopaminergic projections to the nucleus accumbens (ventral striatum), putamen and caudate (dorsal striatum), pallidum, amygdala, anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), and (anterior) insula (6). Both Craig and Damasio state in their theories that an emotion is both a subjective feeling and a motivation, as the insular cortex (where emotional sensations are generated) and the ACC (which generates motivations) are co-activated in virtually all imaging studies regarding emotion (3). Gut peptides, including ghrelin, CCK and GLP-1 also impact on hedonic brain regions, including the insula and ACC, thereby influencing reward and motivational-emotional processes besides their homeostatic role (49, 60, 62).

The intrinsic reward value of food and its affective impact is defined by its exteroceptive properties such as sight, smell, taste, and texture (6). However, previous research indicated that also purely interoceptive, subliminal features of food can interact with exteroceptively generated emotions. More specifically, Van Oudenhove et al. showed that both subjective (i.e. emotional state ratings) and neural responses to experimentally induced sadness were attenuated after fatty acid infusion (131). However, studies investigating such effect for other nutrients, including carbohydrates, are lacking.

The increased consumption of simple sugars such as fructose in Western societies is thought to be a contributing factor to the obesity pandemic (132). Recent studies investigated the neural responses to fructose, which is less satiating and produces a weaker gut hormone response compared to glucose (55, 56). Wölnerhanssen et al. used functional magnetic resonance imaging (fMRI) to investigate potential dissociable effects of intragastric infusion of 25 g fructose and 75 g glucose on resting state functional connectivity (rsFC) of the “basal ganglia/limbic network”, which includes most of the reward system. Glucose increased rsFC of the left caudate and putamen more than fructose, whereas fructose increased rsFC of the left amygdala, left hippocampus, right parahippocampus, and orbitofrontal

cortex more than glucose (58). Any potential effect on (negative) emotions was not included in this study.

The primary aim of the present study was therefore to investigate a putative interaction between gut-brain signals induced by intragastric fructose infusion, and subjective and neural responses to sad emotion induction in healthy volunteers. Furthermore, we aimed to investigate the potential role of gut hormones herein. Secondly, we wanted to investigate the potential impact of this interaction on subsequent food intake. We hypothesised that an interaction between fructose and negative emotion would be observed, with attenuated responses to sad emotion induction after fructose compared to placebo administration, both at the subjective (emotional state ratings) and neural (responses in homeostatic and hedonic brain regions) level, based on the fatty acid study of Van Oudenhove et al. (131). Besides, as it has been shown that a negative emotional state increased subjective appetite and food intake (133, 134), we expected higher appetite-related sensation ratings and increased milkshake consumption after the sad emotion induction compared to induced neutral emotions. Moreover, as a mood-improving role for high-carbohydrate food has been shown (135), we expected that these increased appetite ratings and food intake during the sad state would be counteracted by fructose administration.

5.3 Materials and Methods

Subjects

Fifteen non-obese (mean BMI: 22.42 ± 0.33 BMI kg/m²), healthy, right-handed subjects (8 female, mean age: 22.60 ± 0.45 years) were recruited via local advertisements in the KU Leuven University buildings and via a database of previous participants of experiments in our research group. This sample size was based on the previous study by Van Oudenhove et al. and augmented to 15 to anticipate a potential smaller effect of fructose compared to fatty acids (131). All subjects were screened via an online system (Limesurvey) before inclusion. All candidates had normal or corrected to normal vision and were screened via an online system (Limesurvey) before inclusion using self-report questionnaires [Patient Health Questionnaire (PHQ), short form of the Rome III criteria questionnaire for functional GI disorders, Eating Disorders Inventory (EDI), Dutch Eating Behaviour Questionnaire (DEBQ), Anxiety Sensitivity Index (ASI) and Power of Food Scale (PFS)]. Exclusion criteria included: history of GI, cardiovascular, neurological, endocrine or psychiatric disorders, abdominal surgery (except appendectomy), abnormal eating behaviour and eating disorders, use of medication on a regular basis (except oral contraceptives), MRI-specific exclusion criteria (ferromagnetic implantations, claustrophobia), smoking, pregnancy, and breastfeeding. The screening included a food preference questionnaire to assess liking and wanting of different savoury and sweet food items, including chocolate, ice cream and milkshake. Subjects who scored higher than 6 on a scale ranging from 0 to 10

for liking of milkshake, chocolate and ice cream, attended a separate taste session to ensure liking of the milkshake. Only subjects who rated the milkshake with a score between 60 and 80 on a 100 mm VAS for liking were included in the study (mean: 71.73 ± 2.94). All subjects gave written informed consent in compliance with the Declaration of Helsinki.

Experimental design

This study used a randomised, placebo-controlled, single-blind, cross-over design. The protocol was approved by the Ethics Committee of the Leuven University Hospital, Belgium (Identification Number: S57996) and registered at ClinicalTrials.gov (NCT02946983). Subjects were studied at 8 am after an overnight fast (12 h) on four separate occasions. The study paradigm is summarised in Figure 5.1. A nasogastric feeding tube and an intravenous cannula were placed at least 15 min before subjects entered the scanner room. The fMRI scan lasted for 50 min, including a baseline scan of 10 min. After the baseline scan, one of the two test solutions was intragastrically infused (t = 0). Three minutes before the infusion, induction of sad or neutral emotional state started. Appetite-related sensations and emotional state ratings were collected at t = -10, 0, 10, 20, 30 and 40. Blood samples were collected at t = -10, 10, 20, 30, 40 and 50. After scanning, hedonic food intake was assessed using an *ad libitum* drinking test with a rewarding chocolate milkshake.

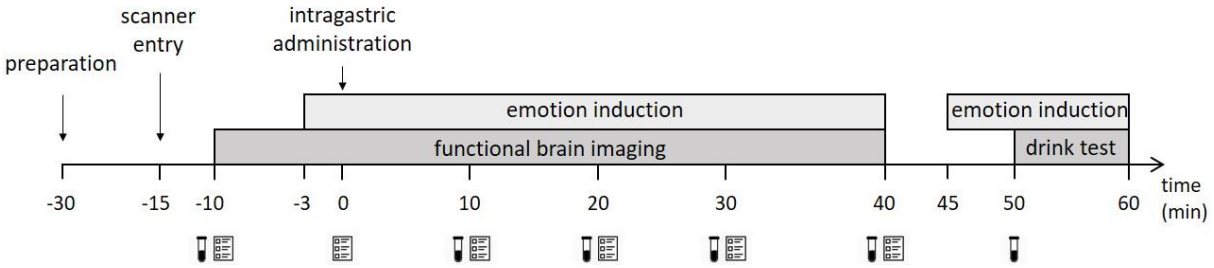

Figure 5.1 Schematic overview of one study visit
Preparation: placement of nasogastric feeding tube and intravenous catheter. Emotion induction: negative or neutral emotion was induced combining two validated methods (classical music and facial expressions). Drink test: *ad libitum* drink test with rewarding chocolate milkshake, as measurement of (hedonic) food intake. Both study visits of the cross-over study were identical, except for the nature of the intragastric administration. Blood collection for octanoylated ghrelin, GLP-1 and CCK. Questionnaires: visual analogue scales for appetite-related sensations (including hunger, fullness, satiety, prospective food consumption) and nausea and the self-assessment manikin for arousal and valence. GLP-1: glucagon-like peptide 1. CCK: cholecystokinin.

Intragastric fructose administration

A nasogastric feeding tube (RT8/801, Medicina, Massachusetts, United States) was positioned with its tip in the proximal stomach and attached to the subject's face using adhesive medical tape, to avoid awareness of the nature of the test solution by eliminating sensory or hedonic effects of oral sweet

tasting and to eliminate movement artefacts during drinking and swallowing. Gastric fluids were aspirated and the pH was measured using pH strips (Merck Millipore, Billerica, USA), to confirm the position of the probe. Afterwards, subjects were allowed an adaptation period of 15 min (9). At t = 0, subjects received either fructose (25 g, Prodia, Gent, Belgium) dissolved in 250 ml Milli-Q water (Millipore Corporation, Billerica, USA) or 250 ml Milli-Q water as the placebo condition. Infusions were administered at body temperature via the nasogastric tube, using a syringe, over a period of 2 min and flushed through with a small amount of Milli-Q water. Subjects were blinded to the nature of the test solutions. The order of conditions was randomised and counterbalanced. The randomisation code was automatically generated by a computer program. The interval between two consecutive study visits was at least one week to prevent potential carryover effects (although unlikely due to acute administration in the first place).

Emotion induction

Sad or neutral emotion was induced, combining two previously validated methods in order to increase the efficacy of the emotional modulation, as previously described (131). Eleven validated 1-minute pieces of sad or neutral classical music were played through headphones in a randomised order for 43 min, starting 3 min before the intragastric infusion (136). Simultaneously, validated facial expressions with the same emotional valence as the musical pieces were randomly back-projected onto a screen, visible for the participants by a mirror, for 5 sec each (137). Emotion induction was only interrupted briefly at t = 0, 10, 20 and 30 to give participants the opportunity to score their appetite-related sensations and emotional state (see below). After participants left the scanner room, emotion induction was restarted, in order not to lose the effect of the emotional modulation on hedonic food intake during the *ad libitum* milkshake drinking test (see below).

Appetite-related sensation and emotional ratings

At baseline (t = -10) and every 10 min during scanning (t = 0, 10, 20, 30 and 40), subjects rated their subjective sensations of hunger, prospective food consumption, fullness, satiety and nausea on computer-based 100 mm Visual Analog Scales (VAS) (0 mm: not at all, 100 mm: very much) in a randomised order (138). At the same time points, they completed the Self-Assessment Manikin (SAM), which uses pictograms to assess emotional state on the dimensions valence and arousal (94). Ratings were given by moving a tick mark along a line segment, back projected onto a screen in the scanner, using a response box in their right hand. Ratings were programmed using Affect 4.0 software (95).

Gut hormone measurements

At various time points during the scan (t = 10, 20, 30, 40 and 50), blood samples were collected for quantification of plasma octanoylated ghrelin, CCK and GLP-1. A baseline sample was taken at t = -10.

Ghrelin blood samples were collected in standard EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 500 kIU/ml aprotinin (Roche Applied Science, Penzberg, Germany) and phenylmethanesulfonyl fluoride (PMSF; 57 µM; Sigma-Aldrich, Steinheim, Germany). Plasma was acidified (10%) with 1N HCl before extraction on Sep-pak C18 columns (Waters Corporation, Milford, Massachusetts, USA) and vacuum-dried. CCK samples were collected in EDTA tubes and 1:10 diluted in ice-cold RAPID buffer containing 0.1 M ammonium acetate, 0.5 M NaCl and enzyme inhibitors diprotinin A, E-64-D, antipain, leupeptin, chymostatin (all 1 µg/ml; Peptide International, Louisville, KY). GLP-1 samples were collected in EDTA tubes containing 500 kIU/ml aprotinin and dipeptidyl peptidase IV inhibitor (DPP4, 10µl/ml blood; Merck Millipore, Billerica, USA). All blood samples were kept on ice immediately after the collection period. Plasma was separated by centrifugation at 4°C for 10 min at 3000 xg and stored at -80°C until analysis. Plasma concentration of octanoylated ghrelin levels, the biologically active form of the hormone, were determined using radioimmunoassays as fully described before (87, 96). GLP-1 levels were determined using Multi-spot assay system for human active GLP-1 (Meso Scale Discovery, Rockville, USA). CCK levels were determined using a commercially available radioimmunoassay (Eurodiagnostica, Malmö, Sweden).

Hedonic food intake

Hedonic food intake was measured using an *ad libitum* drinking test at the end of each test visit using a rewarding chocolate milkshake (4 scoops of vanilla ice cream (Ijsboerke, Tielen, Belgium), 355 ml of 2% milk (Lactel, Brussels, Belgium) and 2 tablespoons of Imperial's chocolate topping (Continental foods, Puurs, Belgium) per serving) that subjects had to drink until maximal satiation (97). The recipe used was adapted from work by Stice et al. to Belgian brands (97). The milkshake was presented in a 200 ml filled glass that was repeatedly refilled once emptied. Subjects were instructed to cease drinking as soon as they felt full but comfortably satiated. Glasses were weighed before and after the test to indicate how much they drank.

Functional magnetic resonance imaging acquisition

FMRI data were acquired on a 3.0 Tesla MR System (Philips Medical Systems, Best, The Netherlands) at the Radiology Department of the University Hospitals Leuven, Belgium. A total of 1200 functional volumes were acquired for an examination period of 50 min, including a baseline scan of 10 min. A T1-weighted structural scan was acquired for each subject at the end of each test visit, to co-register with the functional images and to exclude anatomical abnormalities. No abnormalities were reported. Functional, T2-weighted volumes were acquired with a gradient echo planar imaging sequence with blood oxygenation level-dependent (BOLD) contrast. Functional, T2-weighted volumes were acquired using the following parameters: TR = 2500 msec, TE = 30 msec, 46 slices of 2.70 mm thick, slice gap = 0.3 mm, flip angle = 90°, voxel size of 2.40 x 2.39 x 2.70 mm^3. A 32 channel head-coil was used for radio

frequency transmission and reception. The T1-weighted structural scan was acquired with following parameters: 182 slices, TE = 4.6 msec, flip angle = 8°, voxel size of 0.98 x 0.98 x 1.20 mm^3.

Statistical analysis

Ratings and gut hormone levels

Data were analysed using SAS 9.4 software (SAS Institute, Cary, NC, USA). If required, data were logarithmically transformed to fulfil assumption of normally distributed residuals in mixed model analysis. Data reported in the results section are estimates of transformed values for each specific outcome measurement, as these were used in the statistical analysis. Raw observed values of these outcomes are graphically presented in the corresponding figures. Appetite-related sensation and emotional state ratings, and hormone levels were analysed as change from baseline (t = -10). Nutrient (fructose, placebo), emotion (sad, neutral) and time (6 time points in total) were entered as categorical within-subject variables, controlling for potential visit effects. The nutrient-by-emotion interaction effect represented the principal effect of interest. In case of significant differences between baseline ratings between conditions and/or visits for a certain outcome variable, baseline ratings of that variable were added to the model as continuous covariates to control for these baseline differences. The variance-covariance structure providing the best model fit was chosen based on the minimum value of Akaike's information criterion (AIC). The nutrient-by-emotion-by-time 3-way interaction effect was also included in the models, but when its effect was not significant and its exclusion did not worsen model fit, the 3-way interaction was omitted from the model for reasons of parsimony. In case of significant main effects or interaction effects, *post hoc* tests were performed to compare both nutrients at each level of emotional state or post-infusion time point and to compare each emotional state at each level of nutrient or post-infusion time point. All data are shown as mean ± SEM.

Functional MRI data analysis

Data were analysed using Statistical Parametric Mapping 12 (SPM12, Wellcome Trust Centre for Neuroimaging, UCL, London, UK) implemented in MATLAB R2014b (The MathWorks Inc., Natick, MA). All individual images were preprocessed using the standard procedures implemented in SPM12, including spatial realignment as correction for small movements during the scan and co-registration of each functional image to the structural image of each subject and segmentation of the structural image. The structural image was used for each participant as reference for the spatial normalisation to the EPI template image supplied with SPM12, based on information obtained during the segmentation step. Spatial smoothing using a 8 x 8 x 8 mm^3 Gaussian smoothing kernel was applied to the normalised images to improve the signal-to-noise ratio.

First-level (i.e. subject level) analysis was performed according to a previously described pharmacological MRI analysis method (98, 131, 139). For each condition and each subject, post-infusion volumes were divided into 40 time bins of 1 min (24 volumes) each (T1 – T40), reflecting the change in brain activity compared to baseline. Global time series of cerebrospinal fluid and white matter activity were extracted. To correct for potential differences in low-frequency scanner drift between conditions, these time series were included as nuisance regressors in the design matrix, together with six realignment parameters (translation in the x-, y- and z-direction and rotation: pitch, roll, yaw) which were included to correct for residual motion artefacts. A *t*-contrast was calculated for each of the 40 time bins post-infusion within the general linear model framework to compare brain responses (relative to pre-infusion baseline period) between the fructose and placebo conditions and the sad and neutral emotion conditions. This resulted in 40 first level contrast images per subject, corresponding to the difference in BOLD signal after fructose compared to placebo infusion in each time bin and to the difference in BOLD signal after the sad compared to the neutral induced emotional state.

Second-level (i.e. group level) analysis was performed by applying a one-way within-subject ANOVA on the 40 individual first-level contrast images, with the condition-by-time bin interaction effect being the main effect of interest. Voxel-level threshold was set at $p_{FWE\text{-}corrected} < 0.05$. Voxel-based analysis was performed within a single mask consisting of regions-of-interest (ROI) selected based on previous research (49, 99). This mask contained all anatomically-defined atlas structures, including key homeostatic (medulla (nucleus tractus solitarii), hypothalamus) and hedonic (midbrain (VTA), dorsal (caudate, putamen) and ventral (nucleus accumbens) striatum, pallidum, pregenual anterior cingulate cortex (pACC), medial and lateral rectus of the orbitofrontal cortex, amygdala, anterior insula) brain regions involved in the regulation of appetite and food intake (49, 99).

For each time bin, contrast estimates reflecting the difference in brain response (relative to pre-infusion) between QHCl and placebo administration were extracted from the entire cluster (averaged across all voxels) in representative ROIs with the MarsBar toolbox for SPM12. These estimates were used to plot the time course of the differential effect of the intragastric infusions on the BOLD signal.

5.4 Results

Ratings

Appetite-related sensations

Fructose did not alter appetite-related sensation scores (main effect of nutrient, F (1, 14) = 0.00, $p = 0.96$) for hunger scores, F (1, 14) = 0.06, $p = 0.81$ for prospective food consumption, F (1, 14) = 0.09, $p = 0.77$ for satiety, F (1, 14) = 0.45, $p = 0.96$ for fullness). Further, appetite-related sensations did not

differ between both emotional states (main effect of emotion, F (1, 14) = 2.95, p = 0.11 for hunger, F (1, 14) = 0.05, p = 0.83 for prospective food consumption, F (1, 14) = 0.19, p = 0.67 for satiety, F (1, 14) = 0.45, p = 0.51 for fullness). Finally, the effect of fructose versus placebo did not differ between the two emotional states, as no significant nutrient-by-emotion interaction effects were found (hunger (F (1, 14) = 0.02, p = 0.89), prospective food consumption (F (1, 14) = 0.21, p = 0.65), satiety (F (1, 14) = 0.02, p = 0.89) and fullness (F (1, 14) = 1.73, p = 0.21) (data not shown).

Minimal nausea scores were reported (strongly zero-inflated distributions with very limited amount of variability, not permitting formal statistical analysis), indicating good tolerance of both infusions.

Emotional ratings

At the self-report level, fructose administration did not alter valence and arousal scores (main effect of nutrient, F (1, 14) = 0.21, p = 0.65 and F (1, 14) = 0.20, p = 0.67 respectively). Emotional ratings were also not different during neutral and sad emotion induction (main effect of emotion, F (1, 14) = 0.09, p = 0.77 for valence and F (1, 14) = 1.98, p = 0.18 for arousal). No significant nutrient-by-emotion interaction effect was observed, indicating that the effect of fructose versus placebo on emotional ratings was not different between both emotional states (F (1, 14) = 0.30, p = 0.59 for valence and F (1, 14) = 0.56, p = 0.47 for arousal) (data not shown). As no significant differences in emotional ratings were observed between neutral and sad emotion induction at the self-report level, we will further refer to 'emotional context', rather than 'emotional state'.

Food intake

No significant differences in amount of milkshake drank were observed between the four conditions (nutrient-by-emotion interaction effect, F (1, 14) = 0.07, p = 0.67). Neither were differences observed between both emotional states (main effect of emotion, F (1, 14) = 0.01, p = 0.95) or between fructose and placebo administration (main effect of nutrient, F (1, 14) = 0.01, p = 0.94).

Gut peptide levels

GLP-1

The increase in GLP-1 levels (versus pre-infusion baseline) was significantly stronger after fructose compared to placebo infusion (time-by-nutrient interaction effect, F (4, 47) = 3.19, p = 0.021), especially after 30 min (F (1, 47) = 5.76, p = 0.020) and, to a lesser extent, after 20 min (F (1, 47) = 3.71, p = 0.060) (Figure 5.2A). This effect was superseded by a significant 3-way (nutrient-by-emotion-by-time) interaction effect (F (4, 39) = 2.76, p = 0.041), driven by stronger circulating GLP-1 level increases at 20 and 30 minutes after fructose compared to placebo infusion during the neutral emotional context (F (1, 39) = 4.10, p = 0.050 and F (1, 39) = 8.27, p = 0.007, respectively). Such nutrient effect was not observed during the sad emotional context (F (1, 39) = 0.17, p = 0.68 and F (1, 39) = 0.02, p = 0.89,

respectively) (Figure 5.2B). In sum, these results indicate that the fructose-induced increases in GLP-1 during neutral emotional context is attenuated during sad emotional context.

Octanoylated ghrelin

Changes in circulating levels of octanoylated ghrelin were not significantly different after fructose compared to placebo infusion or after sad compared to neutral emotion induction, over the entire time course (main effect of nutrient, $F(1, 13) = 0.00$, $p = 0.99$ (Figure 5.2C) and main effect of emotion, $F(1, 14) = 2.23$, $p = 0.16$). However, octanoylated ghrelin levels decreased less in the sad emotional context compared to neutral (time-by-emotion interaction effect, $F(4, 56) = 2.75$, $p = 0.037$). This effect was driven by lower octanoylated ghrelin levels during the sad emotional context compared to neutral, 10 and 30 minutes post-infusion ($F(1, 56) = 4.96$, $p = 0.030$ at 10 min post-infusion and $F(1, 56) = 4.47$, $p = 0.039$ at 30 min post-infusion) (Figure 5.2D). In sum, these results indicate that sad emotional context causes a relative increase of ghrelin levels compared to neutral, regardless of nutrient infusion.

CCK

Circulating CCK levels increased significantly more after fructose infusion compared to placebo (main effect of nutrient, $F(1, 14) = 6.00$, $p = 0.028$) (Figure 5.2E). No significant differences were observed between both emotional contexts ($F(1, 14) = 0.01$, $p = 0.94$), nor was there a significant nutrient-by-emotion interaction effect ($F(1, 11) = 0.20$, $p = 0.67$) (Figure 5.2F).

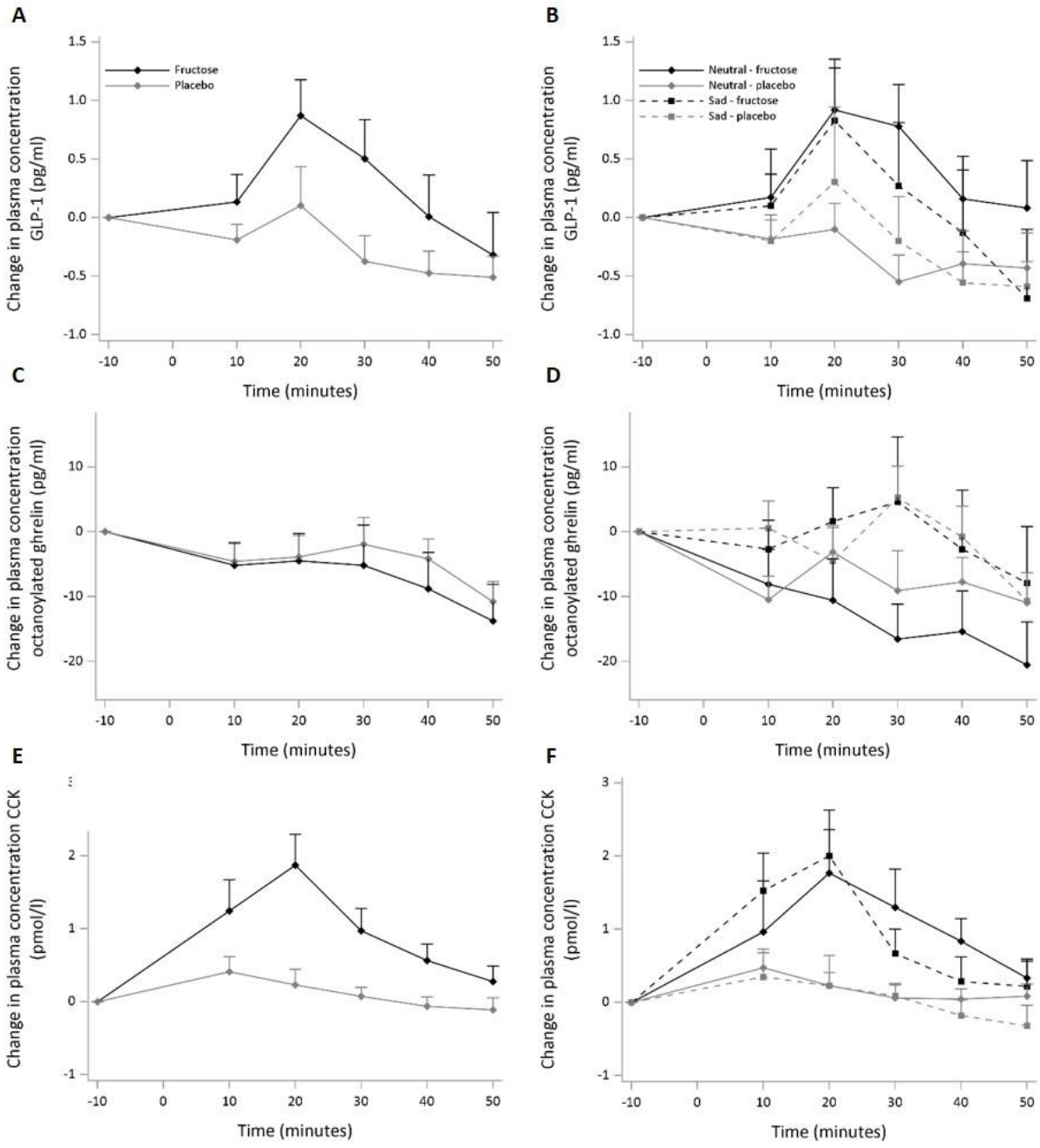

Figure 5.2 Effect of intragastric fructose administration on plasma levels of orexigenic gut hormones
A GLP-1 levels were significantly higher, compared to pre-infusion baseline, after fructose compared to placebo administration (p = 0.021). **B** GLP-1 levels were higher after fructose compared to placebo infusion during the neutral, but not sad, emotional context (nutrient-by-emotion-by-time interaction effect, p = 0.041). **C** No significant difference in octanoylated ghrelin levels was observed after fructose compared to placebo administration. **D** Circulating levels of octanoylated ghrelin decreased less in the sad emotional context, compared to neutral, this is more pronounced in the placebo condition compared to fructose (nutrient-by-emotion-by-time interaction effect, p = 0.037). **E** Circulating levels of CCK were higher after fructose compared to placebo (p = 0.028). **F** CCK levels were not significantly different between both emotional contexts after fructose or placebo administration. Time point 0 indicates the intragastric administration. Data are represented as mean ± SEM. GLP-1: glucagon-like peptide 1, CCK: cholecystokinin, VAS: visual analogue scale.

Brain responses

Effect of nutrient: fructose versus placebo

A significant nutrient-by-time interaction effect was observed on the BOLD signal, driven by a significantly stronger increase in activity (or less strong decrease in activity), relative to pre-infusion baseline, after fructose compared to placebo in most pre-hypothesised ROI, including brainstem (midbrain, medulla), hypothalamus, basal ganglia (putamen, pallidum, caudate), ACC, lateral and medial OFC, nucleus accumbens, anterior insula and amygdala (Figure 5.3, Table 1). The time course of the response in selected regions is shown in Figure 5.4.

Nutrient-by-emotion interaction effect

A significant nutrient-by-emotion-by-time interaction effect was observed on the BOLD signal in similar regions, including brainstem (medulla, midbrain), hypothalamus, caudate, putamen, ACC, medial and lateral OFC, anterior insula, amygdala and pallidum, indicating that the effect of fructose compared to placebo differed between the two emotional contexts (Figure 5.5, Table 2). In the hypothalamus, fructose attenuated the decrease in activity from baseline in the sad but not the neutral emotional context. In the medulla and the midbrain, a stronger fructose-induced decrease of the BOLD signal (from baseline) was observed during the sad compared to the neutral emotional context. Conversely, in the right anterior insula and left lateral OFC, the decrease in activity in response to fructose infusion compared to placebo during the neutral emotional context was attenuated during the sad emotional context. In the right ACC, a relative increase in activity in response to fructose was present during sad emotional context only. The time course of the response in selected regions is shown in Figure 5.6.

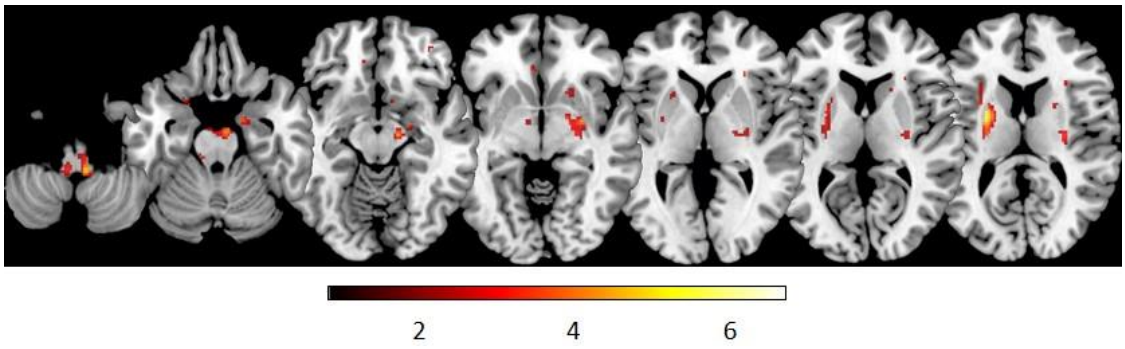

Figure 5.3 Brain regions in which a different response (relative to baseline) was found after intragastric fructose compared to placebo administration
Voxel-level threshold: $p_{FWE\text{-}corrected} < 0.05$. Colour scale reflects the F-value of the functional activity.

Table 5.1 Brain regions in which different responses to intragastric fructose compared to placebo administration were found

region	sub region	side	peak coordinates (mm)			cluster volume (voxels)	F-value
			x	y	z		
brainstem	medulla	left	-6	-44	-53	191	4.017
		right	8	-38	-43	191	6.850
	midbrain	left	-6	-32	1	16	3.521
		right	14	-14	-11	30	4.534
hypothalamus		left	-4	-6	-1	35	4.063
		right	6	2	-19	3	2.993
caudate	head	left	-16	12	19	4	2.680
		right	16	20	11	8	2.954
	body	left	-14	4	23	35	3.805
		right	18	-10	27	36	4.236
putamen		left	-26	0	13	203	5.876
		right	30	-4	-1	249	4.893
nucleus accumbens		right	10	10	-11	1	2.401
anterior cingulate		left	-10	34	-9	10	4.281
middle frontal gyrus	orbital part	left	-12	20	-15	3	2.894
		right	16	16	-17	4	3.002
lateral frontal gyrus	orbital part	left	-18	10	-21	12	4.127
		right	36	46	-11	3	2.567
anterior insula		left	-32	8	13	10	3.504
		right	28	30	3	4	3.372
amygdala		right	22	-4	-13	249	4.502
pallidum		right	20	-6	-9	249	6.780

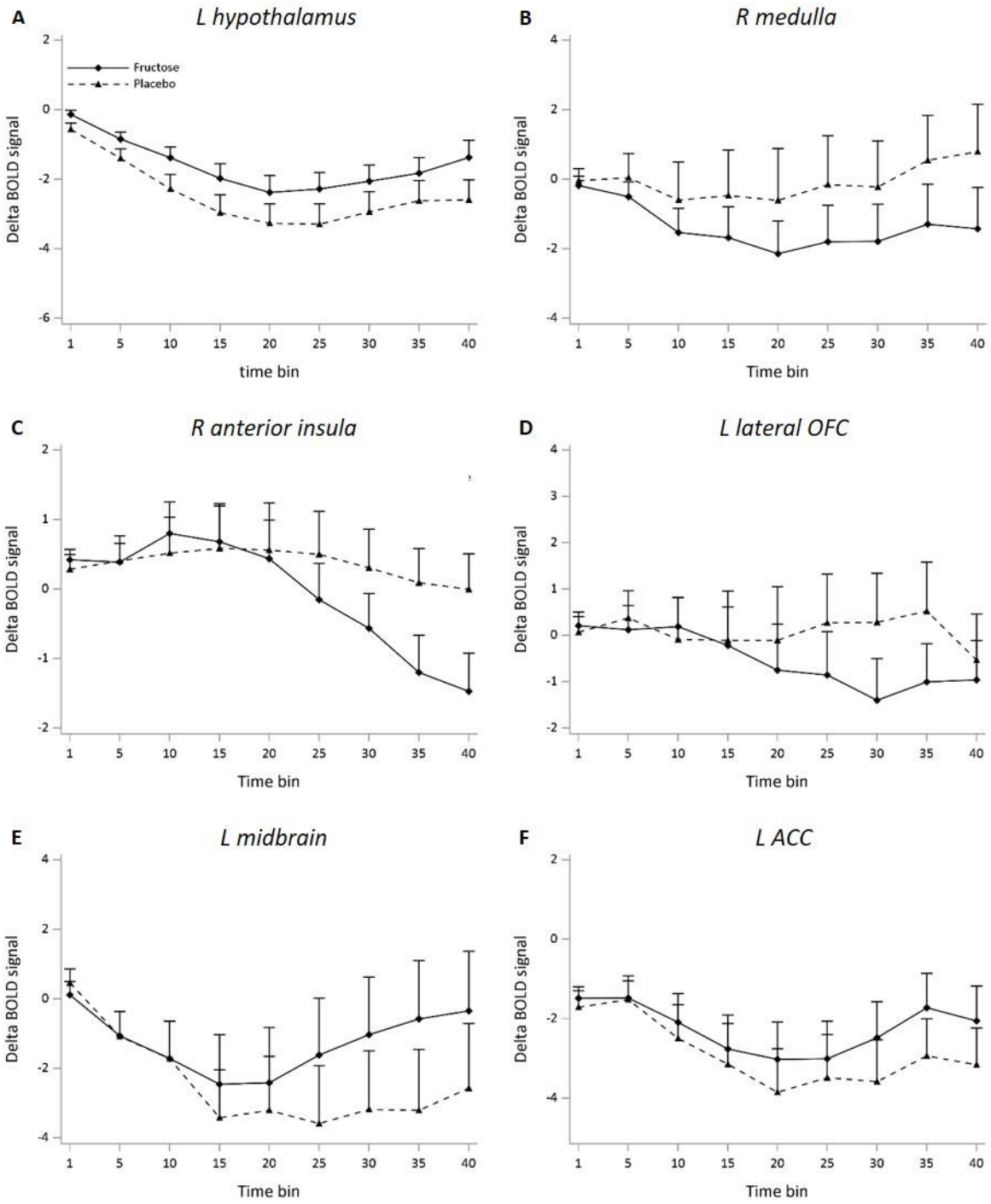

Figure 5.4 Time course of the brain response after intragastric fructose compared to placebo infusion
Time bin 1 reflects the first post-infusion 1-minute time bin. Data are represented as mean ± SEM. BOLD: blood oxygenation level-dependent. ACC: anterior cingulate cortex, OFC: orbitofrontal cortex, L: left, R: right.

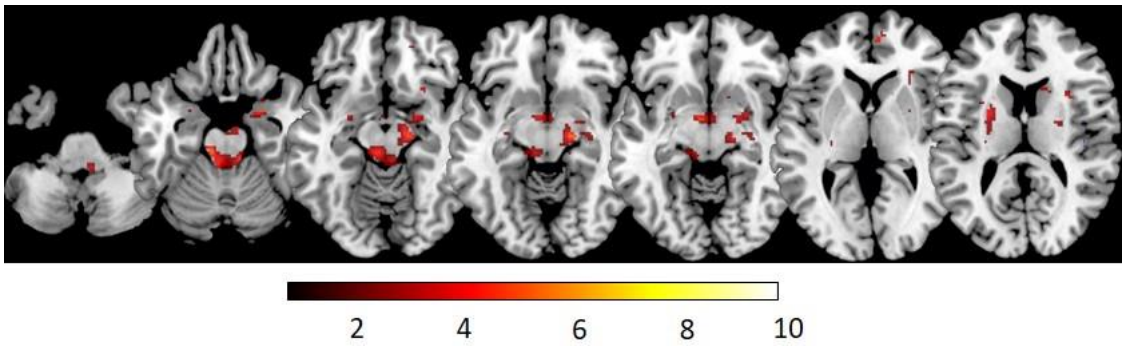

Figure 5.5 Brain regions in which a significant nutrient-by-emotion-by-time interaction effect was found

Voxel-level threshold: $p_{FWE\text{-}corrected} < 0.05$. Colour scale reflects the T-value of the functional activity.

Table 5.2 Brain regions in which a significant nutrient-by-emotion-by-time interaction effect was found

region	sub region	side	peak coordinates (mm)			cluster volume (voxels)	F-value
			x	y	z		
brainstem	medulla	right	6	-38	-43	18	3.620
	midbrain	left	-10	-28	-19	418	5.801
		right	16	16	-11	153	6.219
hypothalamus		left	-4	-4	-3	133	8.552
		right	6	-2	-19	133	3.710
caudate	head	left	-8	16	15	2	2.691
		right	14	22	11	31	5.332
	body	left	-16	-6	27	7	4.126
		right	18	-10	27	36	9.702
putamen		left	-26	0	13	113	4.446
		right	28	0	-5	58	3.809
anterior cingulate		left	-10	40	21	51	4.301
		right	6	54	1	53	4.927
middle frontal gyrus	orbital part	left	-8	10	-19	7	2.947
lateral frontal gyrus	orbital part	left	-28	8	-19	2	2.804
		right	18	48	-13	2	2.669
anterior insula		left	-32	8	13	2	2.633
		right	28	10	-19	30	4.023
amygdala		left	-28	0	-17	13	3.045
		right	24	2	-19	67	5.816
pallidum		left	-26	-20	1	113	2.441
		right	20	-4	-7	58	3.917

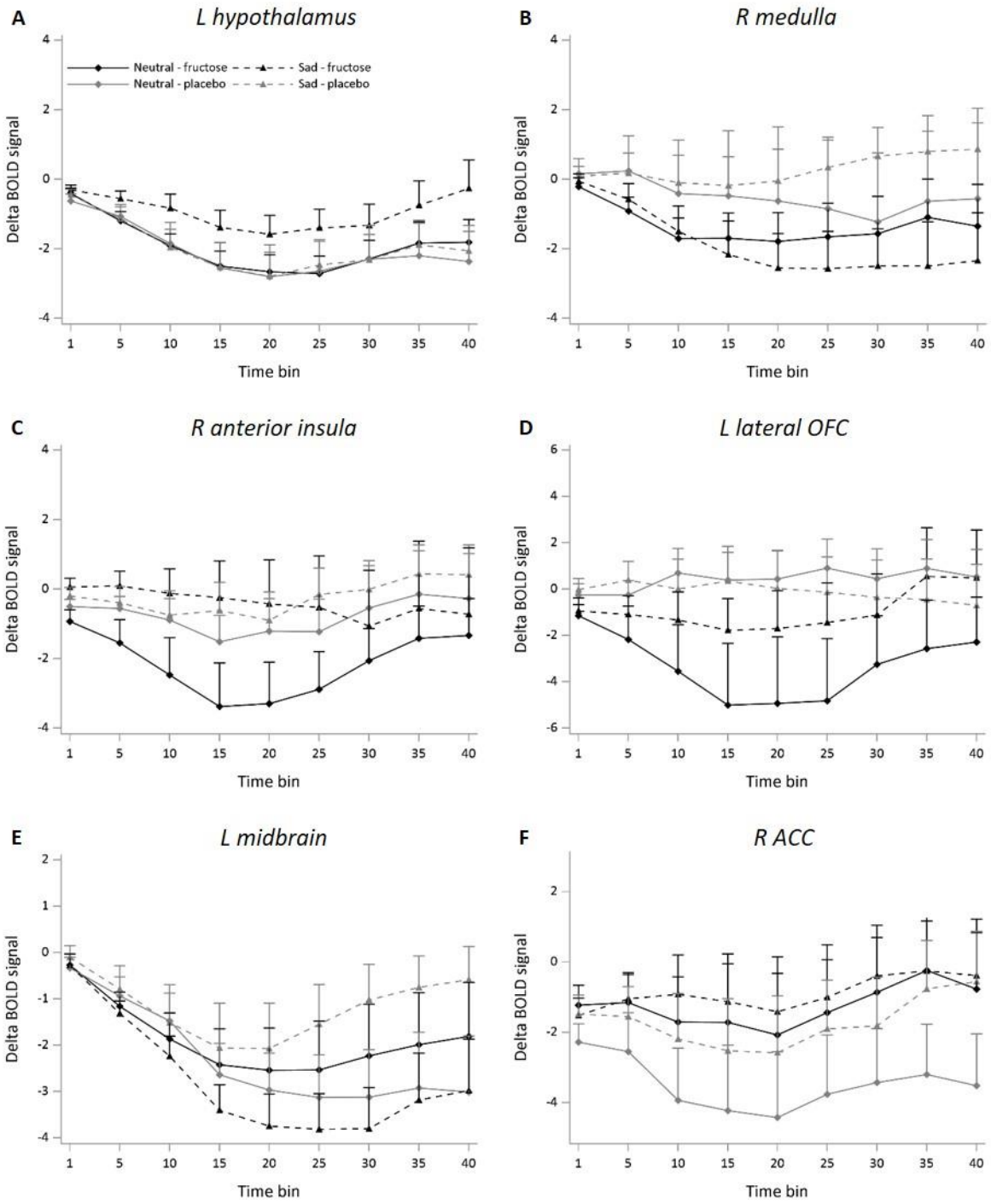

Figure 5.6 Time course of the brain response after intragastric fructose compared to placebo infusion in sad and neutral emotional context

Time bin 1 reflects the first post-infusion 1-minute time bin. Data are represented as mean ± SEM. BOLD: blood oxygenation level-dependent. ACC: anterior cingulate cortex, OFC: orbitofrontal cortex, L: left, R: right

5.5 Discussion

It is generally accepted that nutrients can affect emotional state via their exteroceptive properties (smell, vision, taste), which has been expanded to interoceptive properties by the use of intragastric fatty acids (131). However, research supporting a similar effect for carbohydrates is lacking.

To our knowledge, this is the first study to report an effect of intragastric fructose administration on the neural response to sad emotion induction in sub-cortical regions, including the brainstem (medulla and midbrain) and hypothalamus, and in cortical regions of the reward circuit, including the anterior insula, ACC, basal ganglia and lateral OFC. However, at the self-report level, no significant effects were observed. We further reported effects of fructose administration and emotional context, either alone or interacting, on circulating levels of anorexigenic and orexigenic gut hormones. GLP-1 levels were higher after fructose compared to placebo administration during the neutral, but not sad emotional context, and circulating octanoylated ghrelin levels dropped less during the sad emotional context regardless of whether fructose or placebo was administered.

At the self-report level, the emotion induction procedure did not lead to changes in emotional state, measured as SAM valence and arousal scores. This is not in line with the study of Van Oudenhove et al., where both neural and subjective responses were altered using the same negative emotion induction paradigm. Success of emotion induction is susceptible to high inter-individual variability (140); more specifically, levels of neuroticism can influence its efficacy (141). A possible explanation for the discrepancy between both studies at the self-report level may therefore be found in the susceptibility of the included subjects to the negative emotion induction procedures. Furthermore, different assessment tools were used. Van Oudenhove et al. used a 9-point scale from sad to neutral to happy, where in the current study the self-assessment manikin was used. The latter makes use of pictograms to assess different dimensions of emotion including valence (ranging from sad to happy) and arousal (ranging from feeling very calm to feeling very active) and has been used effectively to measure emotional responses to images and sounds (94). Due to the lack of effect of our emotion induction procedure at the self-report level, we further referred to the induced 'emotional context' rather than 'emotional state'.

GI hormones are key players in the communication along the brain-gut axis and enteroendocrine cells throughout the GI tract express taste receptors similar to their lingual equivalent, thereby stimulating or inhibiting meal-related GI hormone responses (44). In the current study, GLP-1 levels were elevated after fructose administration, which is in line with a previous study by Steinert et al. where the same intragastric load of fructose increased levels of GLP-1. The authors also reported reduced ghrelin levels after fructose administration, which is in contrast with the current study where no effect of fructose

on octanoylated ghrelin levels was found (142). This discrepancy could be explained by the fact that in the previous study total ghrelin levels were analysed, which reflects both octanoylated and desoctanoylated ghrelin levels, with the latter being the biologically inactive form of the hunger hormone, which accounts for 80% of the total ghrelin levels (143). We further confirmed recently published data that showed a stimulating effect of 25 g of fructose on CCK levels (144). Along with these confirmatory findings, we observed an interaction effect of fructose administration and sad emotional context on GLP-1, which has, to the best of our knowledge, never been investigated. Specifically, the fructose-induced rise in GLP-1 levels, compared to placebo administration, was only significantly higher during the neutral emotional context, with the affect being attenuated by sad emotional context. Octanoylated ghrelin levels were higher during the sad emotional context, compared to neutral, regardless of whether fructose or placebo was infused indicating that a sad emotional context, induced increased circulating levels of the orexigenic hormone.

This relative increase in orexigenic signalling, along with a lower level of anorexigenic gut signals, could drive emotional overeating in a sad emotional context. However, this manifests in the absence of the conscious awareness of subjects, as no significant alteration of appetite-related sensation ratings after fructose administration was observed in the current study. This is in agreement with the abovementioned study by Steinert et al. where intragastric administration of 25 g of fructose also did not alter satiety and fullness levels (142). Further, this lack of effect of fructose on conscious appetite-related sensations can be interpreted as confirming the subliminal nature of the stimulus.

Despite the known inhibitory effect of CCK and GLP-1 on food intake and the stimulating effect of ghrelin (38, 145), hedonic food intake did not differ between the 4 conditions. This was not in line with our hypothesis, where we expected higher appetite-related sensation ratings and increased milkshake consumption after the sad emotion induction compared to induced neutral emotions (133, 134). Additionally, we expected that these increased appetite ratings and food intake during the sad state would be counteracted by fructose administration, given a mood-improving role for high-carbohydrate food (135). As mentioned above, our emotion induction paradigm was not successful at the self-report level, hence an attenuating effect of intragastric fructose administration at food intake measurements, was not likely to occur. Furthermore, it is important to note that the current study design was designed to optimally investigate neural responses to intragastric nutrient administration in combination with induced emotions, therefore food intake was only investigated 1 h after infusion of fructose, which might have been too long. This is supported by the observed different effect, of fructose between both emotional states, on circulating GLP-1 levels, which is only found to be significant 20 and 30 min post-infusion.

Despite the insignificant results at the subjective level, a significant nutrient-by-time and a nutrient-by-emotion-by-time 3-way interaction effect was observed in most pre-hypothesised ROIs involved in homeostatic and hedonic control of food intake.

In homeostatic regions, a significant decrease in activity compared to pre-infusion baseline activity was observed after fructose administration. In the hypothalamus and the midbrain, BOLD responses decreased less after fructose infusion compared to placebo administration. In the medulla, brain activity decreased after fructose administration, which was not observed after placebo administration. This is not in line with the first study looking at regional cerebral blood flow (rCBF), as an indirect marker of neuronal activation, after fructose (compared to glucose) administration, as in that study, hypothalamic CBF was only decreased from baseline by glucose, not by fructose (57). However, discrepant results could be explained by different routes of administration; Page et al. used oral ingestion of 75 g of fructose dissolved in 300 ml water. Compared to our intragastric administration, it could indicate that exteroceptive properties were contributing to the results. Besides, different imaging modalities make the direct comparison of both studies challenging, Page et al. assessed rCBF using anterior pulsed arterial spin labelling, where in the current study BOLD responses were measured using phMRI. In most hedonic ROIs, a decreased BOLD response was observed after fructose compared to placebo administration, expect for the right ACC. This is in line with the abovementioned study, as decreased brain activity was only observed in the cingulate cortex after glucose ingestion (57).

In the current study, we expanded the knowledge on the neural effects of fructose, by investigating the interactive effect of subliminal (i.e. non-consciously perceived) administration of fructose with exteroceptively generated emotional contexts. Predominantly, two distinct patterns of brain activity were observed. A similar pattern of the nutrient-by-emotion-by-time 3-way interaction effect was observed in the hypothalamus and brainstem (including the medulla and midbrain) where the post-infusion decrease in brain activity after fructose administration was driven by a bigger decrease after fructose compared to placebo administration during the sad, compared to the neutral, emotional context. This pattern is similar to the observed pattern of the interactive effect of intragastric fatty acid infusion and induced negative emotions in brainstem regions, as reported by Van Oudenhove et al. Although the spatial resolution of 3T fMRI does not permit identification of individual brainstem nuclei, the location of the cluster in the midbrain may indicate involvement of the nucleus of the solitary tract (NTS). The NTS receives vagal sensory input in response to nutrient intake and GLP-1 receptors are present in NTS. The stronger decrease in brain response in the NTS after fructose infusion during the sad emotional context is in line with the attenuated fructose-induced increase in GLP-1 in the sad emotional context compared to the neutral context. The interaction between gut-brain signals and emotion is not limited to subcortical regions. An inverse pattern is observed in cortical regions of the

reward circuit, including the left lateral OFC, right anterior insula and right ACC. In these regions, a stronger decreased brain activity after fructose administration (compared to placebo) was observed during the neutral emotional context. This was not observed during the sad emotional context. This smaller effect of fructose on neural activity in the reward system during the sad emotional context, could serve as the neurobiological explanation for increased food intake during a negative emotional context.

The ROIs were a significant 3-way interaction effect was observed in the current study were highly similar to the ROIs were a 3-way interaction was observed after fatty acid administration in the study of Van Oudenhove et al. In contrast, they did not observe an interaction effect in the insula or amygdala (131). This dissimilarity could be explained by differences in vagal afferents signalling interoceptive information to the among others, the anterior insula, between both nutrients. Vagal afferents are activated by circulating levels of gut peptides, and in the current study, different vagal afferent activation could be reflected by different levels of orexigenic gut hormones between both emotional contexts. However, gut peptide levels were not measured in the study of Van Oudenhove et al., making direct comparisons impossible.

Some limitations of our study require consideration. First, the dosage used in our study (25 g fructose) might have been too low to observe significant effects at the self-report level as daily intake of fructose varies between 11 and 54 g. However, the dosage was chosen to minimise the number of participants who malabsorb fructose, as 25 g is completely absorbed in 50% of the population (146). Second, we did not perform a hydrogen breath test to exclude fructose malabsorption. Therefore, we are not able to completely exclude any malabsorption, however no side effects were reported by any of the participants. Third, our emotion induction paradigm could be considered unsuccessful at the behavioural level, due to the absence of an effect of our emotion induction paradigm on emotional state ratings, despite the fact that this paradigm has been successfully used in previous studies (131, 136). last, for this study, healthy, young, lean and healthy volunteers were included. Additional studies in patients suffering from mood disorders or emotional eating should be performed to further increase the understanding of the interaction of food and mood in this population.

To conclude, this study is the first to indicate that fructose and emotional context interact in homeostatic and hedonic brain regions without a conscious perception of these neural responses, as the intragastric fructose administration did not diminish the effect of negative emotion induction on subjective emotional state ratings. Our findings show that a neurohumoral gut-brain pathway could explain appetite disturbances in mood disorders or emotional overeating in healthy subjects, as a decrease in neural reward responses to fructose administration during the sad emotional context was

observed. Likewise, an interactive effect of fructose infusion and emotional context on circulating GLP-1 levels was observed, together with increased levels of ghrelin during the sad state, regardless of the nutrient administration. Applying a similar study design in patients with functional gastrointestinal disorders, affective disorders, or food intake disorders might be of interest. In those patient populations, a strong comorbidity between gastrointestinal and psychological symptoms exists (69), and investigating the effect of different nutrients on sad emotional state and its neural underpinnings could increase the understanding of these concurrent symptoms.

CHAPTER 6

The effect of acute and sub-acute gluten administration on intestinal and extraintestinal symptoms in NCGS patients and healthy volunteers

Section 6.1 has been published in a slightly different form in the following scientific publications: Biesiekierski JR* and **Iven J.*** Non-celiac gluten sensitivity: piecing the puzzle together. *United European Gastroenterol J* 2015; 3(2):160-5 and Biesiekierski JR* and **Iven J**.* Exploring Non-Coeliac Gluten Sensitivity: A Neuropsychiatric Disorder? *J Dig Dis Hepatol* 2017: JDDH-129
* These authors contributed equally to this work as first authors.

6 The effect of acute and sub-acute gluten administration on intestinal and extraintestinal symptoms in NCGS patients and heathy volunteers

6.1 Summary

Background: Non-coeliac gluten sensitivity (NCGS) is characterised by gastrointestinal (GI) and extra-intestinal (e.g. fatigue) symptoms that subjectively disappear after exclusion of gluten. In a small study, 3-day exposure to gluten increased depression scores in NCGS patients; however acute effects of gluten were not investigated.

Objectives: We aimed to investigate the effect of single-blind acute and sub-acute administration of 16g gluten on psychological and GI symptoms in healthy volunteers (HV) and NCGS patients.

Design: Gluten (16g) or whey protein (placebo) were mixed in 250ml of low fat, unsweetened yoghurt (acute challenge). GI symptoms were assessed using visual analogue scales (VAS; 100mm) every 15' until 180' after administration. Simultaneously, extra-intestinal symptoms were assessed using VAS derived from the Profile of Mood States Questionnaire. The Positive and Negative Affect Schedule was assessed every visit. During the consequent sub-acute challenge, subjects consumed 2 gluten-containing (8g) or gluten-free muffins per day for 5 days. GI symptoms (ordinal values; 0-7) and extra-intestinal symptoms (VAS) were scored daily. After a washout period (2 weeks), subjects crossed over to the alternative dietary arm. Potential mediators (hsCRP, cortisol awakening response, intestinal permeability) of the effect of gluten on (extra-) intestinal symptoms were investigated.

Results: Twenty HV (3 men, 29.7 ± 2.6 years) and 14 NCGS (4 men, 34.0 ± 3.1 years) completed the study. Before the first challenge, NCGS patients showed higher negative affect (p = 0.016) and lower positive affect (p = 0.029) scores compared to HV. After acute gluten challenge, fatigue scores increased in NCGS patients (p = 0.012). During the sub-acute challenge, GI symptoms increased in the NCGS patients compared to healthy controls (abdominal pain: p < 0.001, bloating: p = 0.001), regardless of gluten versus placebo intake. After sub-acute gluten administration, NCGS patients reported lower positive affect scores (p = 0.034). No significant differences between gluten and placebo were observed for intestinal permeability, hsCRP levels or the cortisol awakening response.

Conclusion: These findings provide new insights into NCGS, which might be characterised by acute gluten-induced increases in extra-intestinal symptoms such as fatigue, rather than GI symptoms. During a sub-acute challenge, GI symptoms were elevated in the patient group regardless of gluten versus placebo intake. Further research on the mechanisms underlying these effects as well as other (dietary) factors involved in symptom generation in NCGS is warranted.

6.2 Introduction

The avoidance of gluten-containing products is a recent worldwide phenomenon. Over the past 10 years, there has been a strong rise in the marketing of gluten-free products, mainly due to the increasing number of people following a gluten-free diet (GFD) (illustrated in Figure 6.1).

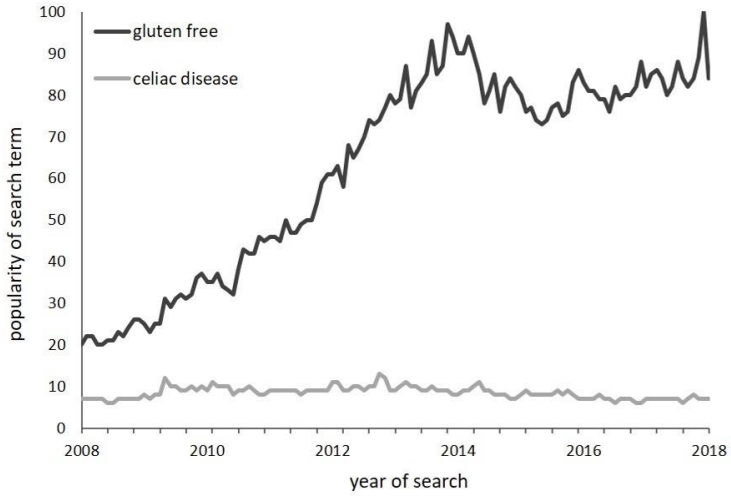

Figure 6.1 Increased popularity of the google search term 'gluten free'.
Worldwide search histories for the terms 'gluten free' and 'celiac disease' (01/01/2008 – 01/01/2018), shown on a Google trends plot (www.google.com/trends). Y-axis values reflect total searches for a term relative to the total number of searches executed on Google over time.

Gluten is the main storage protein of wheat grains. It is a complex mixture of hundreds of related but distinct proteins, mainly gliadin and glutenin, which are referred to as prolamins. Storage proteins similar to gliadin exist in rye, barley and oats. The gliadin fraction, soluble in an alcohol-water solution, is largely resistant to degradation by gastric, pancreatic and intestinal brush-border membrane proteases in the human intestine, and thus remains in the intestinal lumen after ingestion (147, 148). Besides gluten, wheat contains many other components. Therefore, not only gluten-containing products, but also wheat-containing products have long been considered as potential triggers of gut symptoms (149). These non-gluten components include the non-starch polysaccharides, metabolic proteins, starch, minerals, vitamins and a carbohydrate component ("fructans").

While the gluten-related disorders, coeliac disease and wheat allergy are well established, much remains in debate as to whether gluten can trigger gastrointestinal (GI) and/or extraintestinal symptoms in patients without these conditions, or in patients with functional GI disorders (FGID), particularly irritable bowel syndrome (IBS).

Coeliac disease is an immune-mediated disease that occurs when genetically susceptible patients are exposed to dietary gluten; it affects at least 1% of Western populations (148). After digestion, gluten

peptides are deaminated by tissue-transglutaminase and trigger an adaptive T-cell mediated immune response, which leads to the production of interferon-γ and other cytokines, resulting in small intestinal injury and elevated coeliac-specific antibodies (150, 151). Many of the classical clinical symptoms seen in coeliac disease picture malabsorption (diarrhoea, abdominal pain, bloating, wind, distension). More than 95% of patients with coeliac disease carry the human leukocyte antigen (HLA) class II genes, HLA-DQ2 or HLA-DQ8 heterodimers, and the rest express HLA-DQ that contain half of the coeliac associated molecules (150). The only known treatment is a lifelong, strict GFD.

Wheat allergy is an immunoglobulin E-mediated reaction to the insoluble gliadin fraction of wheat with a prevalence estimated around 0.4% of the world's population. The symptoms of wheat allergy develop within minutes to hours after gluten ingestion and include itching and swelling in the mouth, nose, eyes and throat, skin rash, wheezing, and life-threatening anaphylaxis, but do not cause permanent GI damage (152).

Irritable bowel syndrome (IBS) is a symptom-based disorder characterised by recurrent abdominal pain associated with defecation or a change in stool pattern without any identifiable organic cause, as defined by the Rome IV criteria (153). IBS affects around 11% of the population. However, its pathophysiology remains incompletely understood. Proposed mechanisms include visceral hypersensitivity, abnormal GI motility, abnormalities of intestinal flora, low-grade inflammation and altered gut-brain interactions (154). Many IBS patients self-report food intake (especially foods rich in fat and carbohydrates; e.g. dairy products, beans, apples, …) as the trigger of their complaints, however excluding these food items from the diet produces conflicting results (73). Evidence that the carbohydrate part of wheat, fructans, induces symptoms in many IBS patients is convincing (155-157). Short-chain fructans (fructo-oligosaccharides) are poorly absorbed in the small intestine. This malabsorption increases delivery of water and fermentable substrates to the colon, which can result in increased gas production, luminal distension, and, consequently, GI symptoms in patients with IBS (158). Similar behaved carbohydrates have been grouped together and termed FODMAPs: Fermentable Oligo-, Di-, and Mono-saccharides And Polyols. A low FODMAP diet is an evidence-based strategy leading to symptomatic improvement in 74% of IBS patients (159). Wheat- and rye-derived products often contain the highest FODMAP content, predominantly fructans and galacto-oligosaccharides. Cereal products with the lowest FODMAP contents are mostly gluten free, based on rice, oat, quinoa and corn ingredients.

Non-coeliac gluten sensitivity (NCGS) is a condition where intestinal and extraintestinal symptoms are triggered by gluten ingestion in the absence of coeliac disease and wheat allergy, as defined by discussions held at three different international consensus conferences (160-162). The clinical picture

of NCGS is a combination of IBS-like symptoms, including abdominal pain, bloating and bowel habit abnormalities, and systemic manifestations such as tiredness, headache, fibromyalgia-like joint or muscle pain, leg or arm numbness, 'foggy mind,' dermatitis or skin rash, depression, anxiety, and anaemia, which are common in NCGS (162-164). The symptoms occur soon after gluten ingestion, improving or disappearing within hours or a few days after gluten withdrawal and then relapsing following its reintroduction (161). Despite the first reports on NCGS dating from 1978 (165-167), much remains unknown about the condition, and biomarkers are still lacking. Determining the exact prevalence of NCGS is hampered by this lack of biomarkers as well as lack of standardised diagnostic criteria (as are available for IBS). Therefore, prevalence estimates vary. In 2016, a prevalence of 6.2% was reported in The Netherlands, which is lower than the prevalence estimated in the UK (13% in 2014) (168, 169). In addition, it is suggested that NCGS mostly occurs in women (between 1:2.5 and 1:4 female/male ratio) with a median age of onset of 28 years (80).

Currently, there is some evidence for gluten-induced activation of innate immunity (in contrast to the T-cell mediated adaptive immune response in coeliac disease) in the absence of detectable changes in intestinal barrier function in NCGS patients (170). Furthermore, positive coeliac serology (IgG anti-gliadin antibodies) was shown in more than half of the cases, but mostly without specific antibodies associated with coeliac disease (IgG deamidated gliadin peptide antibodies, IgA tissue transglutaminase antibodies or IgA endomysial antibodies) (171). Genetic predisposition to coeliac disease has also been suggested to play a role in NCGS, as patients carrying the HLA-DQ2 allele, but without villous atrophy on duodenal biopsy, have been shown to symptomatically improve on a GFD (172, 173). The best double-blind, randomised placebo-controlled studies have only been published a few years ago (81, 174, 175). Vazquez-Roque et al. reported that in patients with diarrhoea-predominant IBS, consumption of a gluten-containing diet was associated with higher small bowel permeability compared to a GFD (175). This effect cannot be concluded to be gluten-specific, but rather is attributable to the GFD where other food components such as FODMAPs are often co-excluded. In 2011, Biesiekierski et al. showed that ingestion of 16 g of gluten per day induced a rapid onset of GI symptoms and tiredness within the first week of a 6-week intervention (174). A follow-up dietary trial, conducted by the same research group, and designed to be more tightly controlled (using a randomised, double-blind, placebo-controlled, dose-finding, cross-over design) showed significant improvement in GI symptoms and tiredness levels during a low FODMAP-run-in period, but no effect of gluten in inducing change in any symptom in either a 7-day gluten challenge or 3-day re-challenge (81).

In patients with FGIDs, co-morbidity with depressive and anxiety disorders is observed in 30% and 30% - 50% of patients, respectively (69). Short-term exposure (3 days) to gluten specifically increased current levels of depressive symptoms (i.e. negative emotional state), measured on a self-report questionnaire, in patients with self-reported NCGS, with no effect on other indices such as GI complaints or emotional disposition (176). These depressive symptoms may lead to patients being more concerned about their GI symptoms and more sensitive in relation to visceral sensation. Such findings may explain patients reportedly "feeling better" on a GFD despite continuation of GI symptoms. Other possibilities for how gluten may be related to negative affect include: 1) abnormalities of brain serotonin production given that a link between protein ingestion, production of serotonin's precursor tryptophan and serotonin concentration in the brain has been shown (177, 178), 2) gluten exorphins, which are opioid peptides derived from partially digested food, that can cross the blood-brain barrier and modulate neurotransmitter systems in the central nervous system, thereby affecting emotional processes (179), and 3) changes in the gut microbiota, which may in turn affect emotional state through the microbiota-gut-brain axis (180).

While most studies investigating NCGS have used sub-acute (employing challenge periods of 3 days of more) administration of gluten, little is known about its acute effect on GI symptoms and centrally mediated symptoms, and their underlying psychobiological processes. Moreover, it is uncertain if gluten can also cause problems outside the context of NCGS (181), and knowledge on the normal physiological reaction to gluten digestion is lacking, as a healthy control group has not been included in previously performed randomised placebo-controlled trials.

Therefore, in the current study, we aimed to investigate the effect of acute (i.e. single dose) as well as sub-acute (5 days) administration of gluten in a NCGS patient cohort and a healthy control group. The primary aim was to examine the effect of gluten versus placebo administration on extraintestinal symptoms (negative emotional state/depression and fatigue ratings) and GI symptoms (bloating and abdominal pain ratings). Our secondary aim was to investigate the effect of gluten on other extraintestinal symptoms (tension, vigour, anger and positive emotional state ratings) and to explore potential mediators of these effects, including (a change in) intestinal permeability, cortisol levels, inflammation and/or a change in composition of the gut microbiota. We hypothesised that (sub-acute) gluten administration would be associated with an increase in GI and extraintestinal symptom ratings, especially negative emotional state and fatigue ratings, compared to placebo in the NCGS patient group (182). Likewise, we expected to observe a comparable effect of gluten on secondary outcomes

of extraintestinal symptoms (i.e. increased tension but decreased vigour and positive emotional state scores). No effect on anger scores was hypothesised. Additionally, based on the observations of Vazquez-Roque et al., we hypothesised that gluten would increase intestinal permeability in the patient group. Furthermore, we expected that inflammation markers and cortisol levels would not be altered after a short gluten challenge, based on similar observations in the studies of Biesiekierski et al. and Peters et al. (182, 183). As this is the first study reporting effects of one acute, single dose of gluten, we did not have a specific hypothesis about the effect of gluten on acute changes in salivary cortisol. Furthermore, we expected that the abovementioned effects on GI and extraintestinal symptoms as well as on intestinal permeability would be absent in healthy volunteers.

6.3 Materials and methods

Subjects

Thirty-four subjects, 20 healthy volunteers (HV) and 14 NCGS patients, were enrolled in this study between January 2017 and June 2018. Sample size was calculated based on the effect of 3-day exposure to gluten on state depression scores in a NCGS patient group, observed in a study by Peters et al. (Cohen's d = 0.64) (182). This calculation, based on a paired *t*-test, indicated that a sample size of n = 20 NCGS patients was needed to achieve 85% power to detect a similar, medium sized effect of gluten versus placebo administration on mood ratings at a significance level of 0.05. Healthy volunteers were recruited via local advertisements in KU Leuven University buildings and via a database of previous participants of experiments in our research group. NCGS patients were recruited via advertisements in specialised food shops and by referral from gastroenterologists, dietitians or health professionals at the allergy department within UZ Leuven. All candidates were screened beforehand via an online system (Limesurvey) using the following validated self-report questionnaires: short form of the Rome III criteria questionnaire for functional GI disorders, EDI (Eating Disorder Inventory), PHQ-9 (Patient Health Questionnaire), and DEBQ (Dutch Eating Behaviour Questionnaire). For NCGS patients, an extra section was added to quantify GI and extraintestinal symptom levels, previous diagnostic tests (IgA-tTG, HLA and/or duodenal biopsy), adherence to the GFD and any co-existing intolerances for fructose or lactose. Exclusion criteria for HV included: medical history of psychiatric disorders, abdominal or thoracic surgery, hypertension, food- or drug allergies, gastrointestinal, endocrine, neurological, cardiovascular, respiratory or renal diseases, use of medication on regular basis (exception: oral contraception), use of antibiotics, prebiotics or probiotics during the last 3 months, abnormal eating behaviour or eating disorders, smoking, pregnancy, breastfeeding, positive IgA-tTg and excessive alcohol intake. Candidates with first line relatives diagnosed with diabetes, coeliac disease or inflammatory bowel disease were excluded. In addition, NCGS patients must have reported GI symptoms similar to those of IBS and/or extraintestinal symptoms (e.g. fatigue, foggy mind, …)

which improved after the initiation of a GFD, to be eligible for the study. Patients must be adhering to a strict GFD for at least 6 weeks. Prior to inclusion, coeliac disease was excluded in all NCGS patients by serological tests (IgA-tTG or IgG-anti-deamidated gliadin peptides), genotyping to determine the HLA-DQ2/8 status, and by a duodenal biopsy in case of positive serology. All subjects gave written informed consent in compliance with the Declaration of Helsinki and received a compensation for their participation. The study was approved by the Medical Ethics Committee of the Leuven University Hospital, Belgium with reference numbers S58915 (HV) and S60127 (NCGS patients).

Experimental design

The study used a randomised, placebo-controlled, single-blind, cross-over design. HV followed a GFD for 6 weeks and NCGS patients continued their normal GFD. During this period, 5 study visits (1 introduction visit and 4 test visits) were performed at our research unit at the University Hospitals Leuven. All visits were scheduled in the morning, after an overnight fast.

During the introduction visit, subjects were informed about the study design. Gluten-free products were provided to assist GFD adherence, including gluten-free pasta, bread, cookies and cereals (donated by Damhert (Damhert Nutrition nv, Heusden-Zolder, Belgium) and Céréal (Céréal Nutrition & Santé, Vilvoorde, Belgium)). Baseline measurements of emotional state, intestinal permeability and inflammation were collected (see below). An additional blood sample was taken in HV to measure anti-tTg levels and to determine their HLA-genotype. After this visit, a run-in period of 2 weeks followed.

The morning before test visit 1, subjects collected saliva samples to measure the cortisol awakening response (CAR, see below). During the test visit, emotional state was re-assessed. Afterwards, subjects were acutely challenged with 16 g gluten (Tereos, Aalst, Belgium) or 16 g whey-protein (Nestlé Health Science, Resource instant protein, Belgium) mixed into 250 g of low-fat, unsweetened yoghurt (Danone, Rotselaar, Belgium) or lactose free yoghurt (Dilea, Lillois Witterzée, Belgium) in case of lactose intolerant subjects. GI and extraintestinal symptoms were scored before the acute challenge (t = -10) and every 15 min until 3 hours after the start of the acute challenge. Cortisol samples were assessed at time points -10, 60, 120 and 180 min.

The sub-acute administration was performed during the next 5 days. Subjects were provided with 2 individually wrapped, gluten-containing (8 g gluten/muffin) or gluten-free muffins per day and instructed to consume them on two different time points during the day. All muffins were homemade by members of the research team using a gluten-free flour mix (donated by Aveve, Wilsele, Belgium; see Table 6.1 for recipe and nutritional composition of muffins). Preliminary testing in 10 healthy subjects showed that both types of muffins used could not be differentiated, based on taste and

texture (data not shown). All subjects were blinded to both acute and sub-acute challenges for the duration of the study.

Table 6.1 Recipe and nutritional composition of the gluten-free muffins

Ingredients	Calories (kcal)	Carbohydrates (g)	Lipids (g)	Protein (g)	Sodium (g)	Sugar (g)
150 g butter	1122	1	124	1	150	1
120 g sugar	468	120	0	0	0	0
8 g vanilla sugar	31	8	0	0	0	8
8 g baking powder	8	2	0	0	0	0
4 normal sized eggs	352	0	26	30	7	0
175 g gluten-free flour	593	1409	16	9	14	103
1 muffin	**215**	**128**	**14**	**3**	**14**	**9**

One recipe delivers 12 muffins.

After 5 days, test visit 2 occurred. CAR, emotional state, intestinal permeability, and levels of inflammation were re-examined. This test visit was followed by a washout period of 2 weeks. Afterwards, both test visits were repeated (test visit 3 and 4) as described above, with the other dietary intervention.

During the entire study period, participants rated their daily emotional state and GI symptoms using a diary (see below). To review the adherence to the GFD and study food (muffins), participants logged their daily food intake using the online application MyFitnessPal (Under Armour, Baltimore, USA). To investigate the composition of the gut microbiota, faecal samples were collected after the introduction visit (baseline) and twice for a period of 8 consecutive days, starting 2 days before test visits 1 and 3. Faecal samples were stored at -80°C until microbial analysis using 16S rRNA gene sequencing. Gut microbiota analysis is beyond the scope of this doctoral thesis and will not be discussed further. The study protocol is summarised in Figure 6.2.

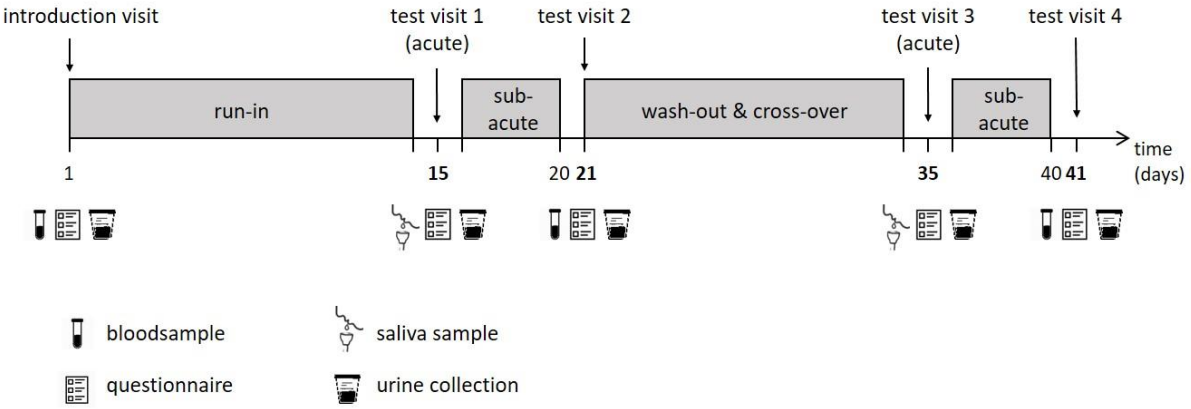

Figure 6.2 Overview of the study protocol
Blood samples: inflammation marker (hsCRP). Questionnaires: Positive and Negative Affect Schedule, visual analogue scales (VAS) derived from the profile of mood state to assess extraintestinal symptoms and VAS for gastrointestinal symptoms. Urine collection: intestinal permeability (lactulose/mannitol ratio). Saliva samples: cortisol. Acute test visit: acute challenge with 16 g of gluten or whey protein mixed in 250 ml low fat, unsweetened yoghurt. Sub-acute challenge: 2 gluten-free or gluten-containing (8 g gluten) muffins, consumed on 2 different time points during the day. Test visits 1 and 3 and 2 and 4 were identical, except for the dietary intervention.

Assessment of extraintestinal and intestinal symptoms

Gastrointestinal symptoms

On test visits 1 and 3, subjects scored their GI symptoms (bloating, belching of air, cramps and abdominal pain) using 100 mm visual analogue scales (VAS) ranging from “not at all” (0 mm) to “extreme” (100 mm) immediately before (t = -10) and every 15 min for 3 hours after the acute challenge. Daily ratings for GI symptoms (abdominal pain, bloating and influence of intestinal symptoms on daily life) were assessed using a questionnaire, developed for IBS symptoms, using ordinal values, graphically displayed by variations of smiley emoticons, with 7 categories (where 0 = no symptom or no influence to 6 = unbearable or complete influence) (see Appendix A).

Extraintestinal symptoms

Positive and negative affective state (PA, NA) during the five days before the test visit were evaluated at the beginning of every test visit using the positive and negative affect schedule (PANAS). After the acute administration (test visits 1 and 3), 100 mm visual analogue scales (VAS) ranging from “not at all” (0 mm) to “extreme” (100 mm), derived from the profile of moods state (POMS) were completed before (t = -10) and every 15 min after the acute administration for 3 hours, to assess vigour, tension, anger, fatigue and depression (184). Daily ratings using the same VAS were collected.

Biological samples

Intestinal permeability

Intestinal permeability was measured using a standard differential urinary sugar excretion test (185, 186). The test solution consisted of 5 g of lactulose (Eurogenerics, Brussels, Belgium) and 2 g of mannitol (ABC chemicals, Wauthier-Braine, Belgium) dissolved in 150 ml of bottled water (Coca-Cola enterprises, Chaudfontaine, Belgium). Participants did not drink anything 30 min prior to the test. The solution was ingested all at once, 5 min after participants emptied their bladder. One glass of water was given 30 min after participants drank the sugar solution. Urine was collected for a period of 2 hours in a container treated with 750 mg neomycin (SA Aca Pharma NV, Nazareth, Belgium) to avoid bacterial infections. After collection, the total urine volume was noted and urine was filtered using a syringe-filter (Millex-HP, 0.45 µm, Merck Millipore, Overijse, Belgium) in 1.5 ml aliquots. For analysis purposes, the internal standard cellobiose (800 mg/l) was added to the urine samples (400 µl). All samples were diluted to 500 µl in demineralised water. Twenty µl of each diluted sample was analysed using a high performance liquid chromatography (HPLC) set-up (Alliance 2695, Waters, Zellik, Belgium), which was equipped with a polymer-based column (Shodex Asahipak NH2P-50 4E, 250 mm × 4.6 mm, 5 µm particle size; BGB Analytik Benelux B.V., Harderwijk, The Netherlands), as previously described (187). The chromatographic separation was carried out isocratically with 75% acetonitrile/25% MilliQ water for 16 min. The effluent was analysed in an evaporative light scattering detector (ELSD 3300, Grace, Deerfield, Massachusetts, USA) with a N_2 flow of 1.5 l/min at 40°C. Data were processed using Empower V.2.0 (Waters). The limits of quantification, the lowest concentration that could be quantified with a coefficient of variation below 20%, were 12.5 mg/l and 15mg/l for mannitol and lactulose, respectively. For each test visit, the lactulose-mannitol ratio (LMR) was calculated as the lactulose concentration divided by the mannitol concentration. The LMR in the urine collection 2 h after ingestion of the sugars was used to quantify small intestinal permeability (185).

Inflammation

During the introduction visit, and after each sub-acute administration period (test visit 2 and 4), blood samples were collected to measure circulating levels of the acute-phase protein high sensitive C-reactive protein (hsCRP), as a marker of low-grade inflammation (188). Blood samples were collected in 10 ml lithium heparin-tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), centrifuged for 10 min at 4°C at 1900 xg and plasma was stored at -80°C. Plasma samples were analysed using particle enhanced immunoturbidimetric assay (Cobas integra system, Roche Diagnostics, Vilvoorde, Belgium) in the laboratory of the University Hospitals Leuven, following the standard clinical practice.

Salivary cortisol

The CAR was used to indicate the hypothalamus-pituitary-adrenal (HPA)-axis activity, which is dysregulated in depression (189). The CAR was assessed through saliva sampling, reflecting the active unbound form of cortisol, in the morning on the day before the 1st and 3th test visits (acute challenge) using salivettes (Sarstedt AG & Co, Nümbrecht, Germany) (190). Samples were collected immediately after awakening (t = 0) and 30, 45 and 60 min afterwards, to determine the cortisol awakening curve. This was repeated on the morning of the 2nd and 4th test visits. Subjects did not drink, consume breakfast or brush their teeth before the samples were collected. Furthermore, saliva samples were collected immediately before the acute challenge (t = -10) and 60, 120 and 180 min later. Saliva samples were centrifuged at 4° C, 3000 xg for 10 min. Cortisol levels were determined using a commercially available ELISA assay (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions.

Coeliac disease serology and genotyping

During the introduction visit, blood samples for HLA were collected and genotyping was performed at the laboratory of the Red Cross Flanders, Belgium. In case of positive genotyping for HLA-DQ2/8 for NCGS patients, duodenal biopsies were collected via gastroscopy at the endoscopy unit of the University hospitals Leuven.

At the same time, blood samples to measure IgA anti-tissue-transglutaminase and IgG anti-deamidated gliadin were collected in a 10 ml SST5-tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were analysed using a chemiluminescent immunoassay (Quanta Flash, Inova Diagnostics, San Diega, CA, USA) at the laboratory of UZ Leuven, following the standard clinical practice.

Statistical analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). Significance level was set at a *p*-value ≤ 0.05. The time courses of extraintestinal and GI symptom responses were analysed as change from baseline. To analyse symptom scores during test visits 1 and 3 (acute administration), time point -10 was used as baseline for all later time points (time points 15 until 180 min). For test visits 2 and 4 (after sub-acute administration), the mean of the last two days of the run-in or washout period was used as baseline for all consecutive days (day 1 till 6, where day 1 is the day of the acute administration). To analyse salivary cortisol levels after the acute challenge, time point -10 served as baseline for later time points (t = 60, 120, 180). Additionally, for PA, NA, LMR and CAR, differences between test visit 1 and 3 were evaluated to investigate if, after the washout period, values returned to levels observed before the 1st acute intervention, prior to performing the comparison between the measurements at the end of the sub-acute gluten and placebo administration (i.e. test visits 2 and 4).

Similarly, hsCRP levels at the end of the sub-acute gluten and placebo administration were compared. In case of very strongly zero inflated data or little to no variability for a given variable, the data for that variable were excluded from analysis and hence results are not reported in the results section. When significant life events were reported in the diaries of subjects that could influence their symptom scores, data of that specific day were removed.

Linear mixed models were used to analyse outcome variables that followed a Gaussian distribution. In case the outcome variable, its logarithmic transformation or BoxCox transformation was not normally distributed, data were converted into tertiles, quartiles or quintiles, grouping it into three, four or five categories. This data were then analysed using generalised linear mixed models with a cumulative logit link function for ordinal response variables. The variance-covariance structure providing the best fit was chosen based on the minimum value of Akaike's information criterion (AIC). Nutrient (within-subject – gluten, placebo), time (within-subject - amount of time points depending on the variable) and group (between-subject - HV, NCGS patients) were added as categorical independent variables to the model. Visit (categorical, within-subject), nutrient-by-visit interaction and gender (categorical, between-subject) were added to control for putative visit, carry-over or gender effects. In case of significant differences between baseline scores for GI or extraintestinal symptoms scores between groups and/or nutrients for a certain outcome variable, baseline scores of that variable were added to the model as continuous covariates to control for these baseline differences. Effects of interest included main effects of group and nutrient (testing differences in responses between groups over both nutrients or nutrients over both groups, over the entire time course), as well as the two-way nutrient-by-group interaction effect (testing whether the difference in response between gluten and placebo is significantly different between groups) and the two-way nutrient-by-time and group-by-time interaction effects (testing whether between-group or between-nutrient differences vary over time). Following up on the nutrient-by-group interaction effect, reflecting our main effect of interest, *post hoc* tests were performed to compare both nutrients within each group and to compare both groups within each nutrient administration.

6.4 Results

Subjects

Seventy HV were screened to participate in this study, of whom 49 did not meet inclusion criteria. One healthy volunteer was excluded from this study and replaced, due to lack of compliance with protocol guidelines. Thirty-three NCGS patients were screened, 7 did not meet inclusion criteria. Eleven eligible patients pulled out because of practical reasons, dropped out after serology and genotyping screening, or declined a gastroscopy, after positive genotyping. Recruitment of NCGS patients is currently still ongoing. Characteristics of all included subjects are shown in Table 6.2 and subject flow is shown in

Figure 6.3. None of the subjects had increased levels (defined as > 20 CU) of immunoglobulin A tissue transglutaminase. All patients adhered to the gluten-free diet during the study. The blinding technique was successful, as indicated by the fact that subjects did not perform above chance level when asked to identify the order of the dietary interventions: 17 out of 34 participants (50%) were able to identify the order correctly. Six out of 14 NCGS patients (43%) were able to correctly identify the order due to a self-experienced relapse of symptoms. One patient ceased the sub-acute administration period of the second dietary arm (gluten) because of intolerable symptoms (including high levels of fatigue, diarrhoea and incapability to work).

Table 6.2 Subjects characteristics

	Healthy volunteers (N = 20)	**NCGS (N = 14)**	***p*-value**
Characteristics			
Age in years	29.65 ± 2.56	34 ± 3.07	0.25
Men – n (%)	3 (15%)	4 (28%)	0.41
BMI in kg/m³	22.08 ± 0.48	22.25 ± 0.65	0.83
HLA status			
Positive	10 (50%)	3 (21%)	0.15
HLA-DQ2 positive	6	1	
HLA-DQ8 positive	4	2	
Elevated serum coeliac antibodies (> 20 CU)			
Tissue transglutaminase (IgA)	0	0	

There were no significant differences between both groups for age or BMI (one-sample t-test). The proportion of men versus female and HLA-positive versus HLA-negative subjects did not significantly differ between groups (Fisher exact test). All data are represented as mean ± SEM. HLA: Human leukocyte antigen, NCGS: Non-coeliac gluten sensitivity.

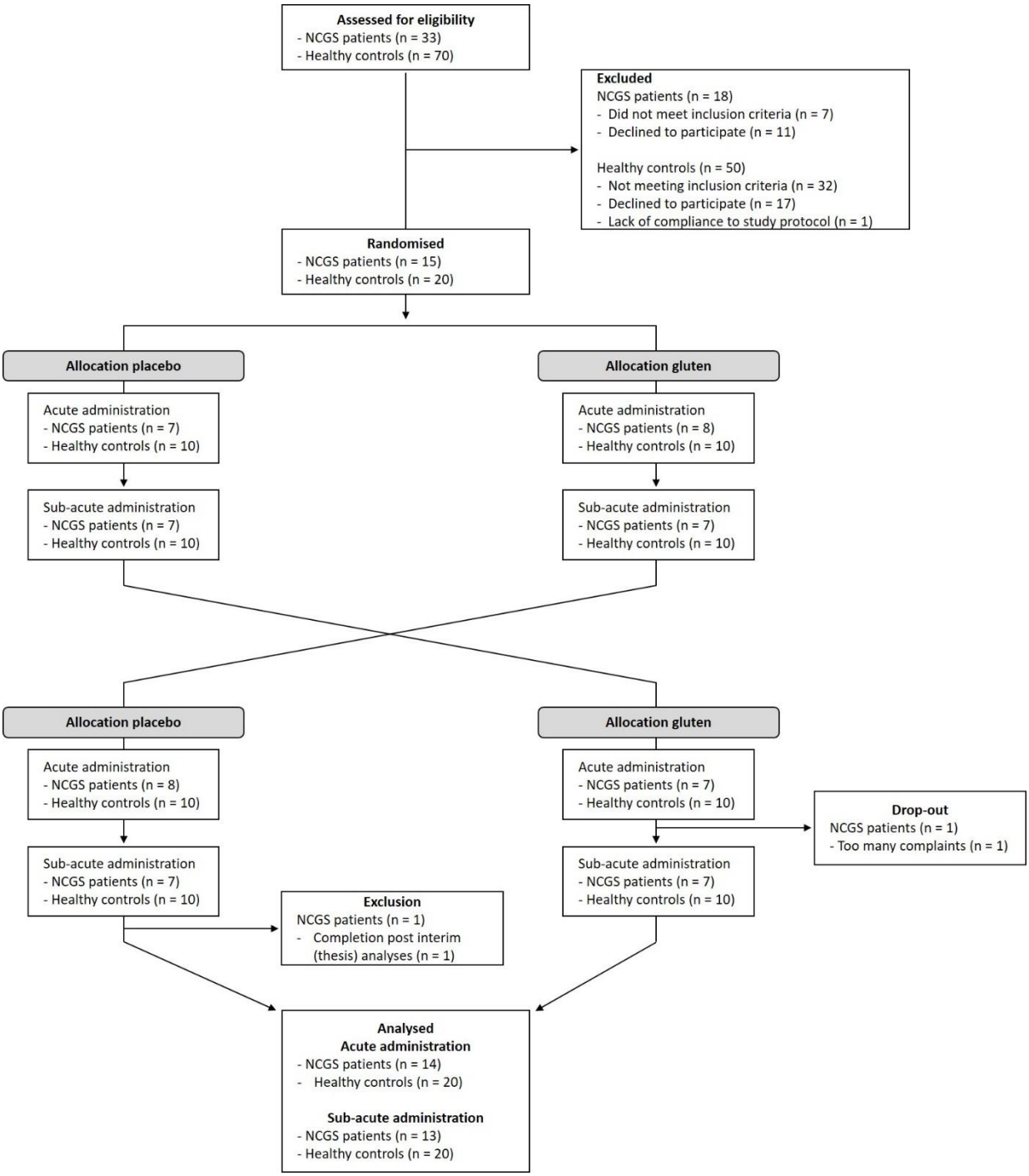

Figure 6.3 Subjects flow and reasons for exclusion and withdrawals.

Extraintestinal symptoms

Fatigue

Acute administration

After the acute challenge, NCGS patients showed a stronger increase in fatigue scores (relative to baseline) compared to HV (main effect of group; F (1, 31) = 7.94, p = 0.008, Figure 6.4A), but no difference in fatigue scores was observed after acute gluten compared to placebo administration (main

effect of nutrient; F (1, 32) = 1.93), p = 0.16). No significant group-by-nutrient interaction effect was observed (F (1, 32) = 1.27, p = 0.27), however, explorative *post hoc* analysis revealed that gluten, but not placebo, induced a stronger increase in fatigue scores in the NCGS patient group compared to HV (F (1, 32) = 7.20, p = 0.012 for gluten and F (1, 32) = 1.59, p = 0.22 for placebo) (Figure 6.4B).

Sub-acute administration

During the sub-acute administration, NCGS patients did not show a different response in fatigue scores in comparison with HV (main effect of group; F (1, 31) = 0.19, p = 0.67). Further, no main effect of nutrient was observed, indicating that the effect of 5 day gluten administration did not differ from the effect of placebo on fatigue scores (F (1, 31) = 0.08, p = 0.78). The group-by-nutrient interaction effect was also not significant (F (1, 31) = 0.19, p = 0.67).

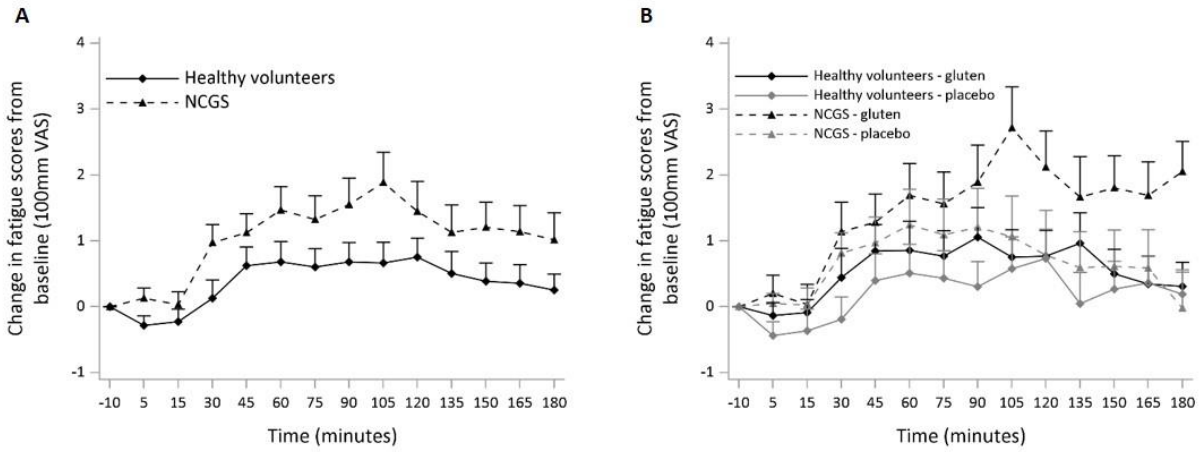

Figure 6.4 Change in fatigue scores after acute gluten versus placebo challenge
NCGS patients showed a significant stronger increase in fatigue scores after the acute challenge (**A,** main effect of group, p = 0.008), due to elevated fatigue scores after gluten, but not after placebo administration in the NCGS patient group compared to healthy volunteers (**B,** *post hoc* analysis of main effect of nutrient, p = 0.012 for gluten, p = 0.22 for placebo). Fatigue scores were analysed as change from baseline on a 100 mm VAS. Data are represented as mean ± SEM. NCGS: Non-coeliac gluten sensitivity, VAS: visual analogue scale.

Vigour

Acute administration

Vigour scores after the acute challenge were not different between NCGS patients and HV (main effect of group; F (1, 31) = 1.20, p = 0.28), nor did vigour scores differ between acute gluten and placebo administration (main effect of nutrient; F (1, 32) = 1.54, p = 0.22). A non-significant trend towards an interaction between group and nutrient was observed (F (1, 32) = 3.10, p = 0.088). *Post-hoc* analysis revealed that the acute gluten challenge provoked a trend towards a stronger decrease in vigour scores in the NCGS patient group compared to HV, which was not observed after the placebo challenge

(F (1, 32) = 4.00, p = 0.054 for gluten and F (1, 32) = 0.16, p = 0.69 for placebo, respectively) (Figure 6.5).

Sub-acute administration

During sub-acute administration, vigour scores did not differ between NCGS patients and HV (main effect of group; F (1, 31) = 0.03, p = 0.86), nor between gluten and placebo administration (main effect of nutrient, F (1, 31) = 0.00, p = 0.98). No significant interaction effect between group and nutrient was observed (F (1, 31) = 0.11, p = 0.75).

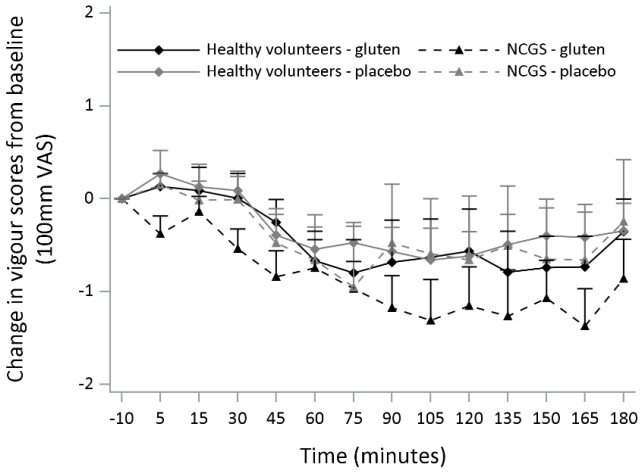

Figure 6.5 Change in vigour scores after acute gluten versus placebo challenge
Post hoc analyses revealed a trend for stronger decrease in vigour scores after gluten challenge in the NCGS patients compared to healthy volunteers (p = 0.054), which was not observed for the acute placebo administration (p = 0.69). Vigour scores were analysed as change from baseline on a 100 mm visual analogue scale (VAS). Data are represented as mean ± SEM. NCGS: Non-coeliac gluten sensitivity.

Tension

Acute administration

For analysis purposes, acute tension scores (change from baseline on a 100 mm VAS) were divided into four categories represented by the following ordinal values: −2: score ≤ -0.4, -1: -0.4 < score < 0, 0: score = 0, 1: score > 0. After the acute administration, gluten induced a less strong decrease in tension scores (relative to baseline) compared to placebo administration (main effect of nutrient; F (1, 777) = 5.82, p = 0.016) (Figure 6.6A). The change in tension scores was not different between NCGS patients and HV (main effect of group, F (1, 777) = 0.89, p = 0.34). No significant interaction between group and nutrient was observed (F (1, 777) = 1.02, p = 0.31). However, explorative *post-hoc* analysis showed that acute gluten compared to placebo administration induced a less strong decrease in tension scores in the NCGS patient group, but not in the HV (t (777) = 2.22, p = 0.027 for NCGS patients versus t (777) = 1.12, p = 0.26 for HV) (Figure 6.6B).

Sub-acute administration

A non-significant trend for a less strong decrease in tension scores, relative to before the intervention, after acute gluten compared to acute placebo administration was observed (main effect of nutrient; F (1, 30) = 3.42, p = 0.07). No main effect of group was observed, indicating that there was no difference in tension scores between NCGS patients and HV during the sub-acute administration (main effect of group; F (1, 31) = 0.51, p = 0.48). No significant interaction between group and nutrient was observed (F (1, 30) = 0.04, p = 0.84).

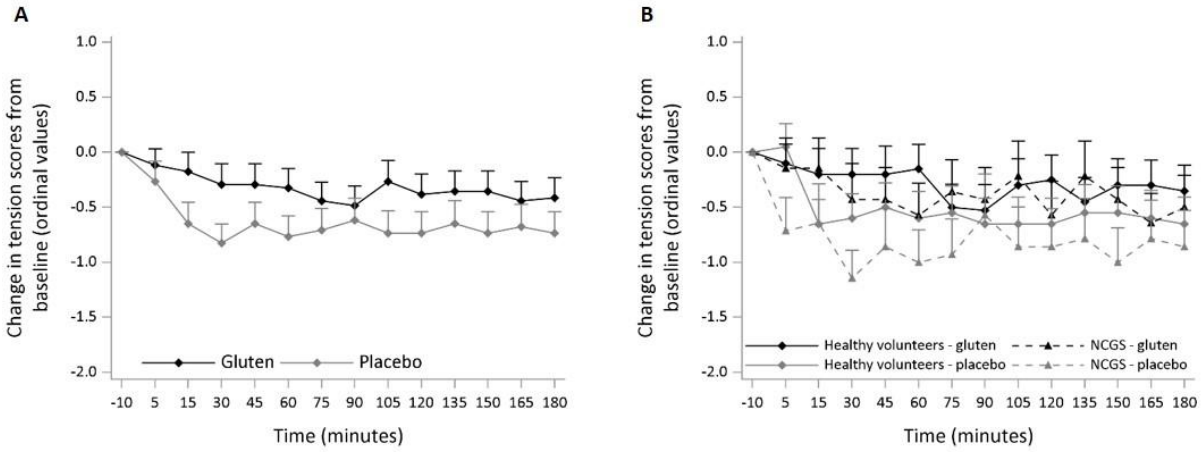

Figure 6.6 Change in tension scores after acute gluten versus placebo challenge
Acute gluten challenge resulted in a less strong decrease in tension scores compared to placebo (**A**, main effect of group, p = 0.016). Tension scores significantly rose after gluten administration in the NCGS patients (p = 0.027) but not in healthy volunteers (p = 0.26) (**B**). Acute tension scores (change from baseline on a 100 mm VAS) were divided into four categories represented by the following ordinal values: −2: score ≤ -0.4, -1: score > -0.4 and < 0, 0: score = 0, 1: score > 0. Time point 5 represents the end of the acute challenge. Data are represented as mean ± SEM. NCGS: Non-coeliac gluten sensitivity, VAS: visual analogue scale

Positive and negative emotional state

Before the first acute administration (i.e. during the introduction visit and at the start of test visit 1), NCGS patients reported higher NA and lower PA scores compared to HV (main effect of group, F (1, 32) = 6.48, p = 0.016 and F (1, 32) = 5.23, p = 0.029 for NA and PA respectively) (Figure 6.7A and B).

NA scores did not differ between groups after both sub-acute administration periods (main effect of group, F (1, 32) = 1.37, p = 0.25) or between nutrients (main effect of nutrient, F (1, 32) = 1.92, p = 0.18). Neither was a significant group-by-nutrient interaction effect found (F (1, 32) = 0.53, p = 0.47) (Figure 6.7C).

PA scores did not differ between NCGS patients and HV after both sub-acute administration periods (test visit 2 and 4) (main effect of group, F (1, 32) = 2.23, p = 0.15) or between nutrients (main effect

of nutrient, F (1, 32) = 2.04 p = 0.16). However, a significant group-by-nutrient interaction effect was observed for PA scores (F (1, 32) = 4.42, p = 0.044). *Post-hoc* analyses showed that NCGS patients, but not HV, reported lower PA scores after gluten compared to placebo administration (F (1, 32) = 5.30, p = 0.028 for NCGS patients and F (1, 32) = 0.27, p = 0.60 for HV) (Figure 6.7D).

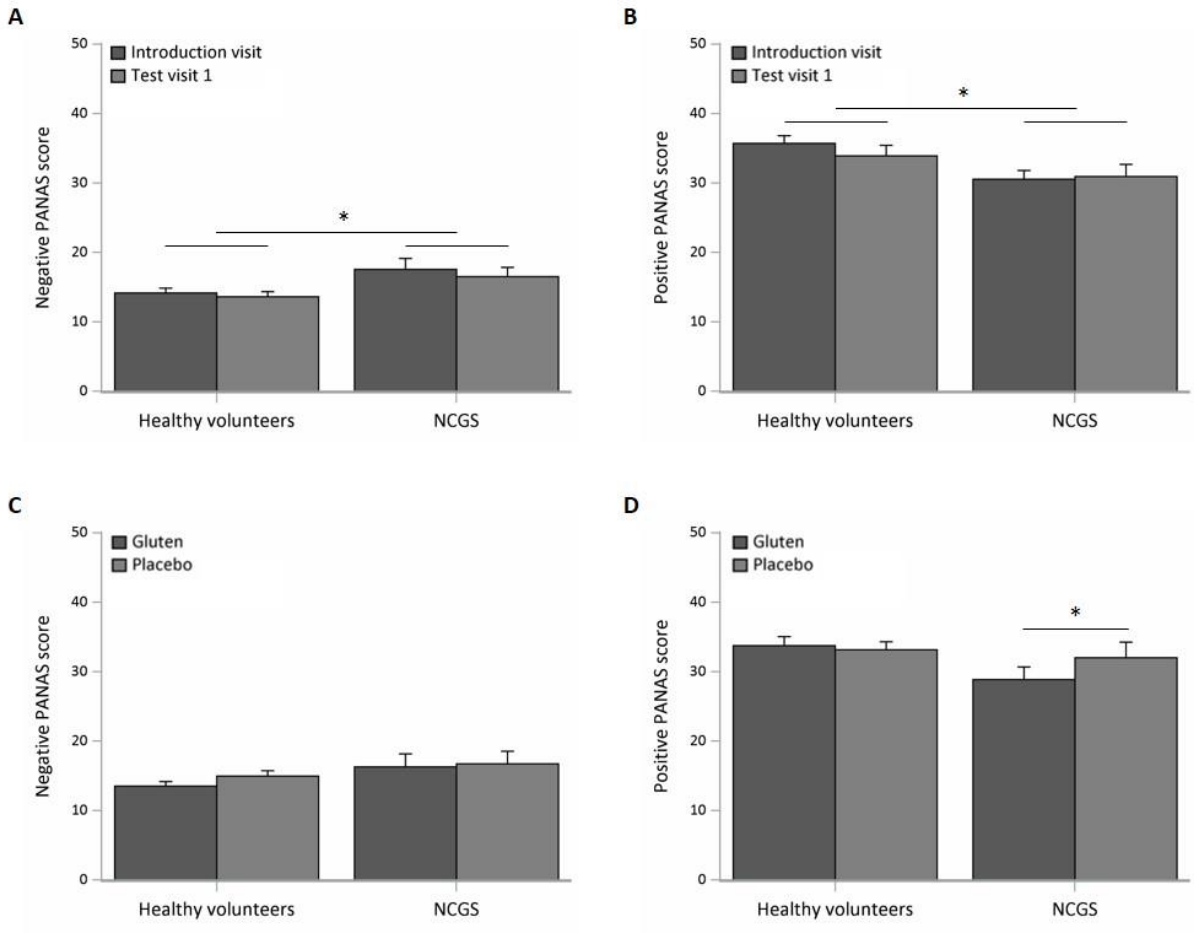

Figure 6.7 Change in positive and negative affect scores
During the introduction visit and the first test visit, NCGS patients showed higher negative affect (**A**) and lower positive affect (**B**) scores compared to healthy volunteers (p = 0.016 and p = 0.029 respectively). Negative affect scores did not differ between groups after both sub-acute administration periods (**C**). Positive affect scores were lower in the NCGS patient group compared to healthy volunteers after gluten, but not after placebo, administration (**D**, p = 0.028 for NCGS patients and p = 0.60 for healthy volunteers). Visit 0 represents the introduction visit Data are represented as mean ± SEM. *: p < 0.05. PANAS: positive and negative affect schedule, NCGS: Non-coeliac gluten sensitivity.

Intestinal symptoms

Bloating

Acute administration

For analyses purposes, acute bloating scores (change from baseline on a 100 mm VAS) were divided into five categories represented by the following ordinal values: −2: score ≤ -0.75, -1: -0.75 < score < 0, 0: score = 0, 1: 0 < score ≤ 0.5, 2: score > 0.5. After the acute challenge, a trend for a stronger increase in bloating scores (relative to baseline) in NCGS patients compared to HV was found (main effect of group; F (1, 777) = 3.11, p = 0.078). No main effect of nutrient was observed (F (1, 777) = 1.25, p = 0.26), which indicates that there was no difference in bloating scores between both dietary arms. There was no significant group-by-nutrient interaction (F (1, 777) = 0.25, p = 0.62).

Sub-acute administration

During the sub-acute administration, NCGS patients reported a stronger increase in bloating scores than HV (main effect of group; F (1, 31) = 13.28, p = 0.001) (Figure 6.8A). No main effect of nutrient was observed, indicating that there was no different effect of gluten compared to placebo on bloating scores during the sub-acute administration (main effect of nutrient; F (1, 31) = 0.01, p = 0.93) and no significant interaction between group and nutrient was observed (F (1, 31) = 0.11, p = 0.74) (Figure 6.8B).

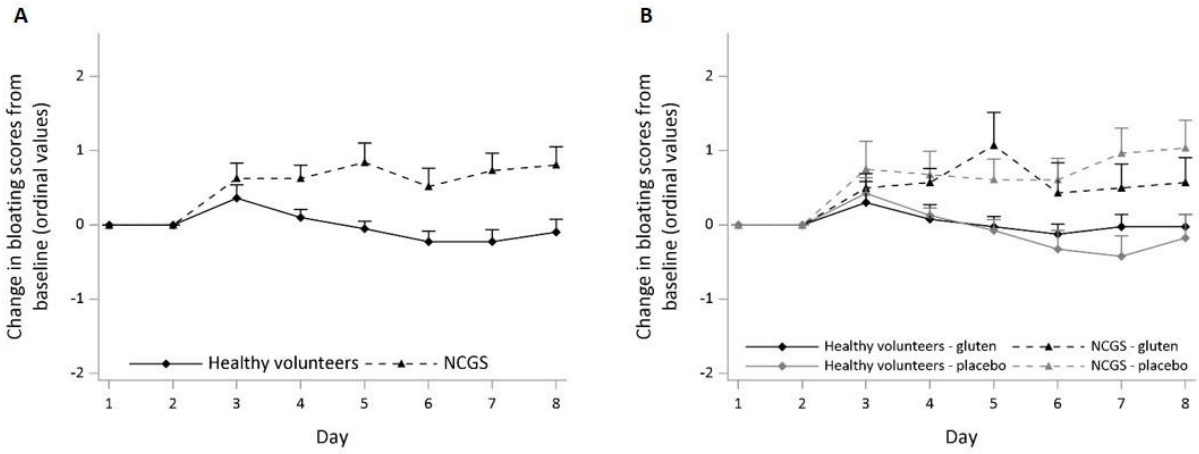

Figure 6.8 Change in bloating scores after sub-acute administration in NCGS patients versus healthy volunteers

NCGS patients reported a stronger increase in bloating scores compared to healthy volunteers (**A**, main effect of group, p = 0.001), caused by a stronger increase in bloating scores after both sub-acute administrations (**B**). Sub-acute bloating scores were analysed as change from baseline as ordinal values with 7 categories (0-6). Day 1 and 2 represent baseline values, day 3 the acute administration and day 4 till 8 the sub-acute administration period. Data are represented as mean ± SEM. NCGS: Non-coeliac gluten sensitivity.

Abdominal pain

Sub-acute administration

Due to too strong zero inflation and little variability for the change in abdominal pain scores during the acute administration, only sub-acute data are reported.

During the sub-acute period, NCGS patients reported a stronger increase (relative to baseline) in abdominal pain scores compared to HV (main effect of group; F (1, 31) = 17.57, $p < 0.001$) (Figure 6.9A). Abdominal pain scores were not different during gluten compared to placebo intake (main effect of nutrient; F (1, 30) = 1.47, $p = 0.23$). No significant interaction between group and nutrient was observed (F (1, 30) = 0.16, $p = 0.69$) (Figure 6.9B).

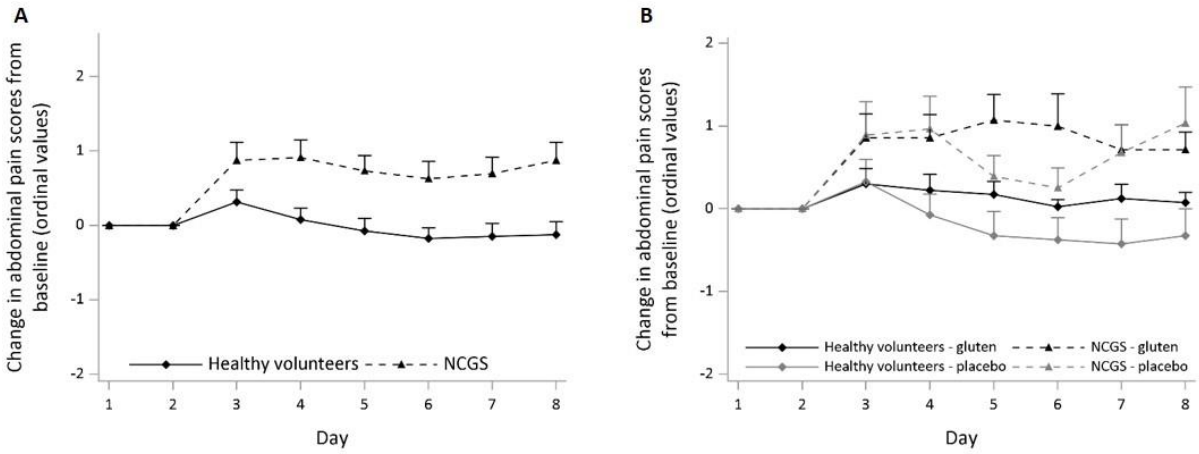

Figure 6.9 Change in abdominal pain scores after sub-acute administration in NCGS patients versus healthy volunteers

After the sub-acute challenge, NCGS patients reported a stronger increase in abdominal pain scores compared to healthy volunteers (**A**, main effect of group, $p < 0.001$). Abdominal pain scores did not differ between sub-acute gluten and placebo administration (**B**). Sub-acute abdominal pain scores were analysed as change from baseline as ordinal values with 7 categories (0-6). Day 1 and 2 represent baseline values, day 3 the acute administration and day 4 till 8 the sub-acute administration period. Data are represented as mean ± SEM. NCGS: Non-coeliac gluten sensitivity.

Biological samples

Intestinal permeability

Before the first acute administration (i.e. during the introduction visit and at the start of test visit 1), no significant differences in LMR were observed between NCGS patients and HV (main effect of group, F (1, 30) = 1.46, $p = 0.24$).

LMR after sub-acute administration did not differ between NCGS patients and HV (main effect of group, F (1, 32) = 2.41, $p = 0.13$) or between nutrients (main effect of nutrient, F (1, 32) = 1.23 $p = 0.28$). No significant group-by-nutrient interaction effect was observed (F (1, 32) = 0.22, $p = 0.64$).

Salivary cortisol

Before the start of each dietary arm, the CAR was not different between groups, as measured on the morning before test visit 1 and 3 (main effect of group, F (1, 32) = 0.00, p = 0.99).

The CAR did not differ between groups after both sub-acute administration periods (main effect of group, F (1, 32) = 0.14, p = 0.71) or between nutrients (main effect of nutrient, F (1, 29) = 0.19, p = 0.69). Moreover, no significant nutrient-by-group interaction effect was found (F (1, 29) = 0.30, p = 0.59).

Cortisol responses to acute gluten versus placebo challenge did not differ (main effect of nutrient, F (1, 29) = 1.39, p = 0.25), nor did cortisol responses differ between NCGS patients and HV (main effect of group, F (1, 30) = 0.38, p = 0.54). In the 3 hour time period after the acute challenge, salivary cortisol levels decreased compared to baseline (main effect of time, F (2, 55) = 11.36, p < 0.001).

hsCRP

No significant difference in hsCRP levels was observed between NCGS patients and HV at the start of the study (main effect of group, F (1, 31) = 0.65, p = 0.43).

For analyses purposes, hsCRP levels were divided into four categories represented by the following ordinal values: 0: score < 0.18, 1: 0.179 < score < 0.73, 2: 0.729 < score < 1.86, 3: score > 1.859.

HsCRP levels were not significantly different after sub-acute administration of gluten or placebo (main effect of nutrient, F (1, 28) = 0.15, p = 0.70) or between groups (main effect of group, F (1, 28) = 2.74, p = 0.11). Neither was a significant group-by-nutrient interaction effect found (F (1, 28) = 2.67, p = 0.11).

6.5 Discussion

Gluten has been identified as the exogenous trigger in coeliac disease, where the predisposing genetic factor (HLA-DQ2/8), the auto-antigen (tissue transglutaminase) and the mechanism (T-cell mediated immune response against the gliadin fraction of gluten) are well established. In contrast, for NCGS, a relatively new entity in which patients experience IBS-like GI symptoms and extraintestinal symptoms after dietary gluten intake and where symptoms disappear on a GFD, the evidence about the role of gluten and the mechanisms involved remain controversial and incompletely understood (82).

This single-blind, randomised, placebo-controlled, cross-over trial is, to the best our knowledge, the first study that investigated the effect of both acute and sub-acute gluten administration on extraintestinal and GI symptoms in NCGS patients as well as in a healthy control group. Our results indicated that NCGS patients, compared to HV, reported a combination of higher NA and lower PA scores before the start of the study as well as during the run-in period. After a single challenge with 16 g gluten, compared to placebo administration, NCGS patients also reported alterations in other

extraintestinal symptom scores (including increased fatigue and tension, and decreased vigour scores). However, GI symptoms (bloating scores) were not significantly different after acute gluten compared to placebo administration in the patient group. An effect of gluten administration on GI and extraintestinal symptoms was lacking in the healthy control group. After a subsequent sub-acute administration period of 5 days, the patient group reported decreased levels of PA after gluten, but not placebo administration whereas no differences were observed for NA. There were no alterations in other extraintestinal symptoms reported by NCGS patients nor HV after both nutrients. However, NCGS patients reported increased levels of bloating and abdominal pain after sub-acute gluten as well as sub-acute placebo administration, compared to HV. Hypothesised potential mediators of these effects, (intestinal permeability, hsCRP levels and the CAR) were not altered in NCGS patients nor in HV after sub-acute gluten and placebo administration.

After acute administration of gluten, NCGS patients reported a stronger increase in fatigue scores compared to placebo administration, which was not observed in HV. This is in line with a prospective, multi-centre study by Volta et al., in which 64% of 486 NCGS patients reported fatigue as one of the most common experienced extraintestinal symptoms (191), and with Biesiekierski's pioneer study in which self-reported NCGS patients showed increased tiredness within the first week of a 6 week gluten challenge (183). However, the finding of increased fatigue after a single acute gluten administration is, to the best of our knowledge, novel. In addition, NCGS patients reported a stronger decrease in vigour scores (i.e. feeling less mentally strong and energetic) after acute gluten administration, compared to HV. This observation is in line with the observed higher levels of fatigue, as previous studies described a negative correlation between vitality ratings, which is closely related to the mood dimension 'vigour', and fatigue scores rated on the POMS (192). Fatigue can be considered as a systemic process where low-grade inflammation is known to play a role in its development. HsCRP serves as a marker for systemic low-grade inflammation and has been shown to be associated with new-onset fatigue (193). However, no effect on circulating hsCRP levels was observed after sub-acute gluten or placebo administration, which is in line with Biesiekierski's abovementioned study (183). This is also in line with results from a study using human duodenal biopsies showing that after gliadin exposure, no increased inflammation was observed in the mucosa of NCGS patients (194). Furthermore, leptin, a peptide hormone produced by adipocytes has been identified as a potential trigger of elevated fatigue. A previous study in an IBS cohort reported a positive relation between leptin levels and fatigue scores, regardless of age, gender or BMI (195). This is supported by recent findings in chronic fatigue syndrome patients, where a positive association between circulating leptin levels and disease severity has been shown (196). This warrants the examination of leptin levels in future randomised controlled trials in NCGS patient cohorts.

Based on a previous study by Peters et al., we hypothesised to observe an increase in depression/negative emotional state scores after (sub-acute) gluten compared to placebo administration (182). Negative affect scores measured using the PANAS, were higher in NCGS patients before the start of the study and after the run-in period, but did not significantly differ after sub-acute gluten and placebo interventions, contrary to what we hypothesised. On the other hand, positive affect scores were decreased after gluten compared to placebo administration in the NCGS patient group, indicating that gluten had a negative effect on a positive emotional state. Unfortunately, variability in the observed depression scores, measured repeatedly using the POMS, was too low to permit adequate statistical analysis. Due to the unavailability of a validated Dutch version of the Spielberger State Trait Personality Inventory (STPI; as used in the study by Peters et al.), we used a different assessment tool (POMS) to assess daily depression scores. However, NCGS patients, but not HV, reported a significantly less strong decrease in tension scores after the acute gluten challenge compared to placebo. According to the POMS, tension is related to the mood dimension anxiety, which is often reported as an extraintestinal symptom in NCGS (184). This is not in line with the Peters study, which only reported a change in depression scores, but not in other indices, including anxiety. The "leaky gut" (i.e. increased intestinal permeability as an indicator of small intestinal injury) has been suggested as a potential mediator of the effect of gluten on extraintestinal symptoms, such as depression, in NCGS patients (197). Due to an increased transcellular, mucosal passage, gluten peptides could cross the blood-brain barrier and either cause neuro-inflammation or affect the opiate system and potentially other neurotransmission systems, thereby affecting emotional processes (198). Besides, locally produced cytokines, as result of (low-grade) inflammation, can rapidly activate primary vagal afferent nerves, which constitutes an important part of the brain-gut axis, thereby inducing a distortion of the communication between the gut and the brain (199). However, no significant difference in intestinal permeability (indicated by the LMR), after both sub-acute interventions, was observed between NCGS patients and HV. This is in line with the findings in the first study of Biesiekierski et al. (183). (Low-grade) systemic inflammation (measured by hsCRP) was shown to be associated with depression (200, 201). However, as hsCRP levels did not differ between both dietary interventions or between both groups, systemic inflammation is unlikely to be the mediating mechanism of the decreases in positive affect scores in response to gluten in NCGS patients, nor for the increased negative affect or decreased positive affect scores in NCGS at baseline. As *a priori* proposed mediators such as intestinal permeability or hsCRP levels were not significantly altered by our dietary challenges, the "leaky gut" hypothesis and consequent low-grade inflammation, cannot explain the occurrence of extraintestinal symptoms in our NCGS patient cohort. Further investigation of the role of gut microbiota is warranted, but falls beyond the scope of this doctoral thesis. Previous research detected intestinal dysbiosis in patients with coeliac disease, which was normalised under a

GFD (202). Moreover, probiotics are able to modulate activity of brain regions involved in processing of emotions. Therefore, it is suggested that an alteration of gut microbiota composition might play a role in the pathophysiology of extraintestinal symptoms in NCGS (203).

Besides the attenuating effect on positive affect, an effect of sub-acute administration of gluten on other extraintestinal symptoms in NCGS patients compared to HV was, against our hypothesis, lacking. This is in contrast with our findings after the acute challenge. A potential explanation is the difference in vehicles used: low fat, unsweetened natural yoghurt versus (gluten-free) muffins. Besides, during the acute administration test visit, symptoms were recorded in a controlled, standardised, neutral and scientific environment. In contrast, sub-acute symptoms were assessed retrospectively at the end of each day in uncontrolled, non-standardised conditions. Additionally, a different administration schedule was used during the sub-acute administration (2 x 8 g at different times during the day versus 1 x 16 g in the morning). A single sub-acute dose of 8 g could have been too low to trigger an increase in extraintestinal symptoms as has been observed after the acute dose of 16 g. It should be noted that participants were explicitly asked to consume the muffins on 2 different time points during the day, ideally as 10 a.m. and 4 p.m. snacks to meet the requirements of a sub-acute administration.

For most GI symptom scores after acute gluten or placebo administration, variability between the observations was too low and/or too strongly zero inflation was observed, preventing adequate statistical analysis. This can be potentially explained by the currently imbalanced number of NCGS patients and HV in our interim analysis, with the latter typically experiencing no or very low levels of symptoms. Following sub-acute administration, NCGS patients reported GI symptoms (bloating and abdominal pain), whereas HV did not. However, this effect cannot be described as gluten-specific, as they reported similar levels of GI complaints after both dietary interventions. This is in line with the results of a recent study where NCGS patients reported symptoms after consumption of 2 gluten-containing-muffins (5.5 g/muffin) a day for 4 days, but also after consumption of gluten-free muffins in a cross-over study with four periods of double-blind provocations (204). Our results are also in line with a tightly controlled, cross-over Australian trial where increased GI symptoms were found regardless of whether participants received gluten-containing or gluten-free foods, and only 8% of the sample size showed a gluten-specific response (81). However, the follow-up study from the same investigators was more tightly controlled and showed that people who avoid gluten-containing food are more likely to react to poorly absorbable FODMAPs, which are abundant in gluten-containing compared to gluten-free food, instead of to gluten. The benefits of the GFD observed in patients diagnosed with NCGS may therefore be a consequence of the reduction in FODMAP intake instead of the elimination of gluten. This hypothesis is further supported by a more recent study where fructans rather than gluten were found to be the trigger of GI symptoms in self-reported NCGS patients after

separate reintroductions (205). In the current study, both our vehicles were very low in FODMAP content, however we did not control for any other confounding dietary factors. Biesiekierski et al. postulated a potential relationship between gluten and FODMAPs in NCGS patients. The authors hypothesised that gluten might induce visceral hypersensitivity by triggering the enteric nervous system (especially mechanoreceptors). Next, FODMAPs could be involved in the manifestation of GI symptoms, so gluten might induce symptoms only in the presence of moderate levels of FODMAPs (81). Finally, stress is considered as a mediator of GI symptoms as well, via alteration of visceral hypersensitivity, among others. Salivary cortisol levels are frequently used as a biomarker of psychological stress, and alterations in the function of stress-responsive systems such as the HPA axis have been observed in IBS patients, although this dysregulation did not appear to have a primary role in GI symptom generation (206-208). In the current study, variation between the observed stress scores, measured using the POMS, was too low to permit adequate statistical analysis. However, potential stress-related biological mediators, the cortisol response to the challenge and the CAR, were unaffected after both (sub-) acute challenges, indicating that a blunted cortisol response is not mediating GI nor extraintestinal symptom development in NCGS, in line with earlier findings in IBS mentioned above (207).

Against our expectations of higher levels of GI symptoms during the sub-acute gluten, but not placebo arm, NCGS patients reported symptoms during both arms, pointing toward a nocebo effect likely occurring in this group. This effect refers to negative effects after placebo intervention due to negative expectations (209). In a meta-analysis by Molina-Infante et al., data from 10 randomised, double-blind, placebo-controlled clinical studies in NCGS patients were analysed. In 40% of the studies, NCGS patients showed a nocebo response (210). In our NCGS sample size, 43% reported complaints after placebo administration, which they (incorrectly) identified as the gluten arm. This highlights the power of patients' expectations and a strong anticipatory symptomatic response. Our study cohort included self-reported NCGS patients of whom not everyone had been subjected to a full gastroenterological work-up before the study. The nocebo response is hard to eliminate in double-blind placebo controlled trials, which are considered the gold standard for dietary studies (211) and especially for studies within the NCGS patient population, as suggested by the Salerno experts' criteria (198), until potential diagnostic biomarkers are identified. A longer study duration or a three-arm placebo controlled trial, which includes a no treatment group, or uses 2 to 3 placebo challenges, could be possible approaches to minimise the nocebo effect in future studies.

The main limitation of this study as reported in this doctoral thesis is the unequal distribution between HV and NCGS patients. However, data reported in this chapter is an interim analysis, and recruitment of NCGS patients is ongoing. Next, attempts to exclude all exteroceptive properties by administering

an acute dose of gluten intragastrically, bypassing all sensory modalities, were not successful. First, intragastric administration by means of a nasogastric tube was impossible due to gluten's insolubility in water. Second, to administer 16 g gluten a day, as used in the current study, subjects would have had to swallow 21 medium-sized gluten-filled gelatine capsules. To administer the same amount of whey protein, 40 capsules would be required. This amount of capsules is likely to lead to difficulties in adherence. Therefore, we opted for a mixture of (uncooked) gluten protein with natural yogurt. However, this does not reflect the consumption of gluten in a real life setting. Besides, two different vehicles were used for acute and sub-acute administration. As mentioned above, both vehicles were very low in FODMAP content based on ingredients, however, as we did not directly control for any other confounding dietary factors (e.g., high fat, high chemical foods (212, 213)) or background FODMAP intake, their potential effect cannot be excluded. Doing so is a well-known challenge in nutrition trials and is best achieved by providing all meals for the study duration, which is an expensive and demanding challenge. For the current study, we instructed all subjects to maintain their normal dietary lifestyle and only replace gluten-containing products with a gluten-free alternative. Participants were instructed to keep their diets otherwise consistent throughout the study duration and their dietary intake was monitored by research personnel evaluating the online diet diaries.

Some recommendations for future studies merit attention. First, as the NCGS patient population is a very heterogeneous population, future clinical trials should not refrain from strict patient selection. Rather than a diagnosis solely based on exclusion of other gluten-related disorders (coeliac disease, wheat allergy), a double-blind placebo-controlled randomised challenge trial is currently recommended as the gold standard to confirm the NCGS diagnosis (198). However, given the high nocebo levels reported in the current study and previously performed studies (as indicated by the abovementioned recent meta-analysis (210), we would advise to include an additional study arm, as described above. Second, the vehicle should be chosen carefully. Data from previously performed studies are hard to compare, as a range of different challenges have been used, including gluten-free food supplemented with wheat flour (which contains FODMAPs), gluten protein, via muffins or capsules (182, 204, 214). As mentioned above, the amount of capsules required to administer 16 g of gluten or whey protein could lead to difficulties in adherence. Besides, the use of capsules would be disheartened as blinding could easily be broken. Study foods should be low in other dietary components with a potential to trigger GI symptoms, not only including FODMAPS but also dairy products like cheese and milk, lectins (e.g. in potatoes), capsaicin and fried and fatty foods in general. The best suited-vehicle to deliver both an acute gluten and gluten-free challenges is still to be discovered and should be palatable, gluten-free and easy to contain equally distributed cooked gluten. Furthermore, the gluten-challenge used should be analysed to exclude presence of other non-gluten

protein fractions present in wheat, including α-amylase trypsin inhibitors (ATIs), which represents 2-4% of the total wheat protein and has been suggested to evoke GI symptoms. It has been reported that ATIs may induce immune responses and induce intestinal inflammation via activation of a toll-like receptor 4 (TLR4) pathway resulting in the release of pro-inflammatory cytokines from monocytes, macrophages and dendritic cells derived from coeliac and non-coeliac patients (5). However, the role of ATIs in humans in vivo is not yet established and is in need of further clinical studies.

Despite increasing awareness and research, key questions regarding NCGS remain unanswered. In line with previous studies, the current results did not confirm the occurrence of gluten-induced GI symptoms outside of coeliac disease. One single dose of 16 g gluten or 8 g gluten twice a day did not induce more GI complaints than placebo, in self-reported NCGS patients nor in healthy volunteers. These results therefore do not provide support for the existence of the controversial disease entity NCGS in its current form, and emphasise the need to re-evaluate both the definition and diagnosis of NCGS. The potentially unsuitable definition and name of NCGS has received attention in the past, and it has been proposed to be replaced with non-coeliac wheat sensitivity (181, 215). However, the latter would not take the well established induction of similar symptoms by rye and barley (fructans) into consideration. To improve the diagnostic accuracy, other dietary factors (especially fructans) should be highlighted more, as gluten alone did not increase levels of GI symptoms. A proposed next step is to add an extra phase between exclusion of coeliac disease and wheat allergy and dietary gluten exclusion, where a low FODMAP diet is introduced. Thereby, gluten intake will be simultaneously reduced but not completely excluded. If patients do not report feeling better, gluten exclusion and re-introduction, should be performed to consider gluten as trigger. However, looking beyond exclusion of one or two dietary components (gluten and/or FODMAPs) should be taken into consideration to elucidate if patients reporting gluten-induced symptoms should follow a strict gluten-free diet. The low FODMAP diet has been shown to be successful in the relief of IBS symptoms, nevertheless equal benefit was reached in a Western European setting with traditional dietary advice for IBS, and a combination of both has been recommended (216). Personalised diets have been suggested as a possibility to manage GI complaints in IBS, which could be expanded to complaints provoked by gluten in which case dietary advice for IBS could be complemented with exclusion of (high amounts of) gluten (217). It remains a matter of debate whether NCGS could be considered a sub-group of IBS rather than a distinct entity, as previous studies reported that some IBS patients benefit from a GFD as well (218). As psychological interventions have been shown to successfully relieve symptoms in IBS patients, cognitive behavioural therapy (CBT) could also be considered in NCGS patients (65). Changing thinking styles (contributing to the nocebo response, amongst others reported in the current study) has the potential to change the way patients behave and feel (32). Additionally, including systematic exposure

in CBT has been proven to increase the beneficial effect of CBT in IBS patients, resulting in improvement of self-reported GI symptoms, pain catastrophizing, avoidance behaviour and quality of life (219, 220). Treating NCGS symptoms by exposure to feared and highly avoided stimuli (e.g. gluten-containing food in different quantities) might be a suitable alternative for exclusion diets. As NCGS patients are known to be enormously adherent to a strict GFD and often initiate this diet without education or follow-up by a dietician and/or gastroenterologist, it is important to unravel if strict GFD adherence is necessary as a GFD can result in nutritional inadequacies (221, 222). Besides relief of GI symptoms, exposure based CBT has been proven to relieve depression symptoms and general functioning in IBS patients, as well as fibromyalgia symptoms and fatigue, depression and disability in fibromyalgia patients (223, 224). These results further highlight the potential benefit of exposure based CBT in the NCGS patient population for relief of GI as well as extraintestinal symptoms, since fatigue, depression and fibromyalgia-like joint pains are often reported as extraintestinal symptoms in this patient population (225).

In the current study, the acute gluten challenge significantly increased fatigue and tension scores and simultaneously decreased vigour scores in NCGS patients, but not in a healthy control group. Besides, positive affect scores were decreased after 5 days of gluten administration, compared to placebo, in the NCGS patient cohort. These findings may implicate that extraintestinal symptoms are triggered by isolated gluten ingestion, however this could not be explained by previously proposed hypotheses such as increased permeability, induced inflammation or altered cortisol levels. After sub-acute administration of both gluten and placebo, NCGS patients, but not HV, reported significantly higher GI symptom scores, yielding toward a contribution of the nocebo effect in this observation. To conclude, this study does not confirm a role of gluten as the culprit for GI symptoms, thereby questioning the diagnosis of NCGS as a unique disease entity. Our findings highlight the need for further studies disentangling different components that trigger symptoms in self-reported NCGS patients, which could lead to elimination of an (unnecessary) restrictive gluten-free diet in this patient population.

6.6 Appendices

Appendix A: Questionnaires to measure gastrointestinal symptoms as used in the daily daries

Hoe ernstig was bij U de buikpijn vandaag?

Niet aanwezig *Ondraaglijk*

Hoe ernstig was bij U het opgeblazen gevoel in de buik vandaag?

Niet aanwezig *Ondraaglijk*

Hoeveel invloed hadden uw darmklachten op uw leven vandaag?

Geen invloed *Volledig*

Hoeveel stress had U vandaag?

Geen stress *Hoogst denkbare*

Hebt U vandaag ongewone of prikkelende voeding gegeten?

☐ Neen ☐ Ja: welke: ....

Enkel voor vrouwen: had U vandaag Uw maandstonden?

☐ Neen ☐ Ja:

CHAPTER 7

General discussion and future prospects

7 General discussion and future prospects

"Der Mensch ist, was er ißt" - "You are what you eat".

This famous statement by L. Feuerbach, a German philosopher, indicates that a connection between the food we consume and our psychological state or well-being has been recognised since 1863 (226). Centuries later, our understanding of the interplay between the gastrointestinal (GI) tract and the brain has dramatically increased, however the specific impact of different nutrients and/or tastants on the brain is inadequately understood. Disturbed gut-brain signalling is implicated in, among others, functional gastrointestinal disorders (FGID) and disorders of appetite regulation and food intake, including obesity (4); both pose a high economic burden on health care resources (227, 228). Despite being heavily studied during the last decade, the mechanisms underlying these disorders are still poorly understood and (pharmacological) treatment options remain inadequate. An increased understanding of the integration of interoceptive with exteroceptive signals (e.g. taste and sight of food items), together with input from the reward system and affective and cognitive neurocircuits, is vital to understand the high comorbidity between psychological and gastrointestinal (GI) symptoms in FGIDs and disorders of food intake (69, 229).

Therefore, the primary aim of this doctoral thesis was to gain further insight into the gut-brain signals induced by different nutrients and tastants and their effect on appetite, emotions and GI symptoms. For this purpose, a variety of behavioural, neuropsychological and biochemical methods were combined, thereby allowing exploration into the integration between central and peripheral mechanisms along the brain-gut axis. Tastants and nutrients have a clear common denominator in brain-gut axis signalling mechanisms, however, for both, we were interested in quite different aspects (i.e. the effect of intragastric tastant administration on neural responses and appetite and the effect of intragastric nutrient administration on emotional state and GI symptoms) and therefore we will discuss them separately in the following paragraphs.

7.1 Tastant-induced gut brain signals

Previously, it has been discovered that intragastric bitter administration decreases appetite and circulating levels of orexigenic gut peptides and alters gastric motility via activation of taste receptors located on enteroendocrine cells (EEC) (84, 90). However, the (neural) factors mediating this taste-signalling pathway were not known. This knowledge-gap was addressed in chapter 3 of the current doctoral thesis.

Our findings indicated that intragastric administration of the bitter tastant, quinine-hydrochloride (QHCl), decreases prospective as well as actual hedonic food intake in healthy women by interfering

with homeostatic and hedonic brain circuits. Moreover, we showed that this effect may be mediated by a quinine-induced reduction in orexigenic hormone levels. Subsequently, we investigated neural anticipatory food reward responses after intragastric administration of the same dose of the same bitter tastant (chapter 4). We demonstrated that, without subjects being consciously aware, QHCl alters neural anticipatory food reward responses during a food incentive delay (FID) task, especially during the receipt phase of this task. However, this was not observed in key reward circuitry, but increased brain activity was found in brain regions involved in inhibitory control and attentional processing.

These novel results suggest that resting state brain activity in the core reward circuit was increased by intragastric QHCl, compared to placebo administration. Nevertheless, when food reward responses were provoked and the reward system was stimulated using food cues, no differences in brain responses in reward related regions were observed after QHCl administration compared to placebo. Previous studies indicated that anticipatory food reward is encoded in the orbitofrontal cortex (OFC), amygdala, cingulate gyrus, dorsal striatum, nucleus accumbens (ventral striatum), midbrain (including the ventral tegmental area (VTA)), fusiform gyrus and parahippocampal gyrus (97, 230, 231). Virtually all of these regions were activated when anticipatory food reward was elicited during the passive viewing task as well as the food incentive delay task. These results confirm earlier findings on the encoding of anticipatory reward. Contrary to what we hypothesised, no significantly stronger anticipatory reward responses were observed in key hedonic brain regions after QHCl administration compared to placebo. However, an increased brain activity was found during the receipt of rewarding food, in the superior parietal cortex and dorsolateral prefrontal cortex (dlPFC), which could implicate a stronger cognitive inhibition. A regions of interest (ROI) mask containing the regions where a significant difference between QHCl and placebo was observed during anticipation of food reward, was used to reanalyse the resting state brain activity acquired using phMRI. This was done in order to investigate if QHCl altered neural responses in these regions outside the core reward system in our first study. We found that increased brain activity was also observed in the superior parietal cortex and dlPFC, after QHCl administration, outside the context of anticipatory food reward. This consistent effect of QHCl on those regions, whether in rest or during stimulation, suggests that an increased cognitive inhibition process during the receipt of rewarding food may underlie the anorexigenic effect of bitter tastants. To further explore the inter-relationship and integrated performance of the core reward circuitry and cognitive inhibition network after QHCl administration, functional connectivity analyses will be performed.

Future studies investigating the effect of QHCl on consummatory food reward (i.e. food reward elicited in response to food intake (97)), using delivery of small sips of a pleasant or tasteless solution (230,

232) are recommended. A meta-analysis by Diekhof et al. investigated the commonalities and differences in regional brain activity during passive anticipation and actual consumption of rewards in the ventral striatum and medial orbitofrontal cortex/ventromedial prefrontal cortex (mOFC/vmPFC). These two functionally related regions form the core network for encoding reward magnitude (i.e. an objective property of reward value that refers to the invariant ratio of different reward sizes, 'less versus more'). The authors showed that the ventral striatum was consistently activated during passive anticipation and receipt of reward and that different reward magnitudes are represented in this region. On the other hand, the mOFC/vmPFC was only involved in the processing of reward magnitude during actual reward consumption (233). Based on these findings, we anticipate an activation of neural consummatory food reward responses in the mOFC/vmPFC after QHCl compared to placebo administration. Additionally, based on our findings in chapter 4, we expect a stimulating effect of QHCl on brain activity in the cognitive inhibition network during consumption of rewarding food, resulting in anorexigenic properties. Besides, one study discovered that two distinct motivational neural systems exists: one orchestrating approach and another avoidance behaviour. The former is activated when one is exposed to a stimulus in a rewarding context, i.e. when subjects were highly motivated to consume the food and the food was rated as pleasant. The latter is coordinating avoidance behaviour when exposed to (the same) stimulus in a non-rewarding context, i.e. when the food was eaten inconsistently with subjects' motivation (e.g. when satiation was already reached) and the food was rated as unpleasant (234). This was particularly observed in the OFC, where distinction was observed between the medial and lateral part. The medial OFC was activated in response to the rewarding outcome, which is in line with the results from the abovementioned meta-analysis. The lateral part of the OFC was activated when the stimulus shifted from rewarding to aversive (234). QHCl modulated resting activity in both the medial and lateral part of the OFC outside of the context of anticipatory reward (chapter 3), which emphasises the ability of QHCl to impact on, besides cognitive inhibitory regions, regions involved in affective evaluation.

Our findings, together with the preceding studies in our research group, confirm the functionality of bitter tastants in the GI tract, which has played a key role in survival given bitter foods were considered as potential toxins (45). With the identification of the anorexigenic properties of bitter tastants and the underlying brain mechanisms, we also brought attention to new opportunities to create potential pharmaceutical applications in obesity. However, several aspects first require attention. First, dose effect studies are required to indicate which dose elicits appropriate responses for both sexes, given the difference in bitter sensitivity between male and females (as raised in chapter 3). In male subjects, an effect of intragastric bitter tastants on hunger has been described previously, however this was only when increased doses were used compared to females, indicating a dose-effect response (235).

Second, our research described in chapters 3 and 4 of this doctoral thesis only investigated a single dose of QHCl in the morning after an overnight fast. Therefore, studies investigating the effect of repeated administration of bitter tastants on appetite and food intake are warranted, once the optimal dose is discovered. This should also be investigated in a clinical trial where QHCl capsules are provided and where subjects do not change their dietary habits, reflecting the effect of QHCl on food intake in a real life setting instead of in an acute experimental design. Third, a combination of two bitter tastant agonists, both shown to influence hunger ratings and associated gut peptides, should be considered. QHCl binds to 9 receptors of the taste 2 receptor (TAS2R) family, of which 5 are in common with denatonium benzoate (DB), which binds to 8 different TAS2R (236). A combination of both molecules could activate a broader spectrum of TAS2R within the gut, increasing its activity. Besides, combination with another natural component further exploring the threshold or a build-up effect could be taken into consideration. For example, capsaicin, the pungent component in chili peppers, has been shown to decrease desire to eat scores, *ad libitum* food intake and overconsumption (237).

Given the anorexigenic properties of intragastrically administered bitter tastants in healthy volunteers, performing similar paradigms as described in chapter 3 and 4 of this doctoral thesis in an obese patient population would be of interest. In obesity, bitter taste sensitivity and taste signalling in the gastric mucosa have been shown to be altered (238, 239). Moreover, brain responses to anticipation of food reward are increased in key reward regions in obese subjects (23). In addition, impaired prefrontal metabolism (involved in inhibitory control) and increased activity in gustatory regions has been described (21, 23, 110). This could underlie an increased hedonic responsiveness driving (chronic) overeating, especially when exposed to food cues. Exposure to food cues has increased dramatically as a result from the change in environment from one where food sources were limited and unreliable to an environment where highly palatable, inexpensive, energy-dense foods are abundant (240). As our internal satiety signals have remained stable over the last 50 years, they may not overcome this increased sensory and nutritional stimulation produced by food, which increases the incidence of hedonic, non-homeostatic feeding (241, 242). As we have shown in chapter 4, QHCl increases brain activity/responses in the dorsolateral prefrontal cortex (dlPFC), therefore comparing the effect of QHCl in this specific region between normal-weight, healthy subjects and obese patients is of interest, especially as obese subjects have consistently less postprandial activation in the left dlPFC, regardless of their meal size (123, 243). These findings shows that intragastric bitter administration may have the potential capacity to prevent overeating of highly palatable foods in obesity by increasing cognitive inhibition.

The proposed research investigating the effect of QHCl on consummatory food reward in obesity can be extended to patients with binge eating disorder (BED), which often leads to obesity. BED is the most

prevalent eating disorder, characterised by compulsive episodes of excessive consumption of highly palatable foods together with a strong sense of loss of control, but without the presence of compensatory purging behaviours (244). In BED, aberrant neural responses to both food cues and anticipated food receipt occur. These altered responses, coupled with diminished recruitment of prefrontal cognitive control circuitry, are believed to contribute to binge eating of palatable foods (245). It is of interest to investigate if intragastric QHCl administration could impact on cognitive inhibition regions in this patient population as observed in healthy volunteers. The FID paradigm as used in chapter 4 of the current doctoral thesis has been applied in bulimic-type eating disorders (including bulimia nervosa and BED), where increased neural activation was observed in the medial OFC and posterior cingulate cortex (246). However, the authors did only include a very limited amount of ROIs, which did not include the dlPFC and superior parietal cortex. Moreover, bulimia nervosa patients were enrolled in this study as well. Bulimia nervosa and BED are similar, but not identical food intake disorders, and therefore it is of interest to conduct this experiment within a sole BED patient population. Functional magnetic resonance imaging (fMRI) data analysis should be performed within a broader mask of ROIs, including the regions where an effect of QHCl compared to placebo was observed during the FID task (bilateral precuneus, superior parietal cortex and dlPFC), as reported in chapter 4.

7.2 Nutrient-induced gut-brain signals

To evaluate the rewarding properties of food and to generate affective responses, exteroceptive visual, gustatory, olfactory and somatosensory information is integrated with homeostatic satiety signals (including gut peptides) (247, 248). The effect of consciously ingested fat has been extensively studied in the past and it is well known that fatty foods stimulate our reward system (229, 249). This has been expanded to interoceptive properties by means of intragastric fatty acid administration, where negative induced emotions were attenuated at the subjective and neural level (131). This could explain why fatty acids are craved, especially when one is feeling down, and are consequently labelled as 'comfort foods'. However, sweet foods are also often ingested during a negative emotional state, and previous research has indicated that normal weight women increased consumption of sweet foods when exposed to negative emotions (250, 251). This implied that an increased understanding of the effect of different nutrients on neural and subjective responses to negative mood could unravel appetite disturbances, for example in eating and mood disorders. However, the vast majority of previous research has been conducted using consciously perceived food-related stimuli or actual consumption of food, which makes it impossible to disentangle the effect of different aspects of feeding (interoceptive-homeostatic, exteroceptive-hedonic, cognitive) on emotional state. This led to the study paradigm outlined in chapter 5, where a non-consciously perceived, purely interoceptive

administration of fructose was used to investigate subjective and neural responses to an induced negative emotion. Where chapters 3 and 4 were to investigate the effect of intragastrically administered bitter tastants on appetite and neural response related to this, in chapter 5 the effect of carbohydrates on brain responses in homeostatic and hedonic regions using phMRI was investigated and in that sense, these chapters are related to each other. But more specifically, the main focus in chapter 5 was to investigate the effect of intragastrically administered carbohydrates on emotional state and therefore, we discuss them separately in this chapter.

We did not detect differences in self-reported emotional state ratings, neither in appetite-related sensations, after induction of negative or neutral emotions. Besides, circulating levels of orexigenic gut peptides (i.e. octanoylated ghrelin) were higher during the sad emotional context, regardless of which condition was administrated (fructose versus placebo). Moreover, circulating levels of anorexigenic hormones were only elevated after fructose during the neutral, but not negative emotional context.

However, an interactive effect of interoceptive fructose-induced signals and an exteroceptively induced sad emotional context on neural responses was observed in subcortical regions (including medulla and midbrain), the hypothalamus, and key regions of the hedonic brain circuit (including the anterior insula, ACC and lateral OFC). Our observed post-infusion decrease in brain activity in the hypothalamus and brainstem is in line with earlier studies investigating carbohydrate-induced gut-brain signals, where a blood oxygenation level-dependent (BOLD) signal decrease was observed after intragastric glucose administration (50). These brain regions are also responding to intragastric fatty acid administration (49), albeit with a response in the opposite direction. The mechanism behind the opposite effects of carbohydrates and fatty acids on brainstem regions and the homeostatic hypothalamus, remains to be elucidated. This post-infusion fructose-induced decreased BOLD response in the hypothalamus and brainstem was driven a bigger difference between fructose and placebo administration during the sad, compared to the neutral, emotional context. This observed pattern is in line with the pattern observed after fatty acid infusion (131). In the reward circuitry, an inverse pattern was observed, with attenuated response after fructose administration during the sad emotional context compared to neutral. This decreased activation of the reward system could underline overeating when one is feeling sad.

As self-report levels of emotional state were not altered after our current applied emotion induction paradigm, we were not able to investigate the potential attenuating effect of an intragastric dose of 25 g of fructose on a negative emotional state at the self-report level. Some implications for future studies can be formulated based on our present findings. First, other paradigms could be considered. The current applied design was optimal to investigate the time response of the effect of fructose on

brain activity, as measured using pharmacological magnetic resonance imaging (phMRI). A classical fMRI block design, as used in chapter 4, could also be applied to investigate neural responses to emotional stimuli. Blocks combining classical music pieces with e.g. Velten mood induction statements (252) and emotional pictures from the International Affective Picture System (IAPS), which are emotional, normative and internationally accessible visual stimuli which are considered the most reliable and valid system in the experimental study of emotions, could be used (253). This combination could be less monotone and prevent disinterest compared to the long phMRI design used in chapter 5. Another tool proven to be effective for studying negative emotions are film clips (254). Second, it has been shown that emotional responses differ by age. Older adult age, compared to young adult age, was associated with a more intense experience of negative emotions provoked by film clips and older adults reported higher arousal in the case of sadness clips compared to younger people (254). The mean age of our study population in chapter 5 was 22.6 years old and it might be advised to include older people as well in future studies. Third, it should be noted that the fructose dosage used, chosen to largely exclude malabsorption, might not have been high enough. Twenty-five grams of fructose did not alter interoceptive feelings of hunger and other appetite-related sensations. Emotions and appetite-related sensations firmly interact with each other, and furthermore, according to Craig's theory, emotions are a reflection of homeostatic interoceptive signals (i.e. 'homeostatic emotions') (10). Therefore, the lack of differences in emotional outcomes can also partially be a consequence of the lack of a difference in appetite-related sensations. Repeating this paradigm with different dosages could help explore these hypotheses. A recent study showed that habitual fructose intake is 45 g / day in The Netherlands, slightly less than in the United States (255). As daily intake, regardless of emotional state, is almost twice as high as our study's dosage, higher concentrations might be needed to provoke acute effects on appetite-related sensations and/or affective state by fructose consumption.

At the neural level, fructose administration did induce different responses in homeostatic and hedonic brain regions depending on the emotional context. It is of interest to know if this response is altered in disturbed nutritional states, e.g. patients with eating disorders or obesity. These patient populations, as well as FGID patients, where psychological complaints go hand-in-hand with GI symptoms, could be more susceptible to negative emotion inductions compared to the healthy volunteers included in our study design. Furthermore, as FGID patients often show co-existing anxiety (69), investigating the effect of fructose on other specific emotional states such as anxiety would be of interest to understand the effect of different nutrients on comorbid psychological symptoms in FGIDs.

Besides, as suggested in a commentary on the study of Van Oudenhove et al., individuals who are chronically sleep deprived tend to ingest comfort foods late in the evening (229). Investigation of the effect of fructose administration in relation to the circadian rhythm might be of interest, given that in

the current study design all experiments were performed in the morning. Gut peptide levels also fluctuate with the circadian rhythm (256) and in our current study, gut peptide levels were influenced by the emotional context. Exploration of the differences in circulating levels of gut peptides involved in the regulation of food intake, at different time points during the day in different emotional states, might give insight into a pattern of consuming food to cope with negative emotional states.

Our study on the effect of fructose on neural and behavioural responses to negative emotions contributed to the previous knowledge on the effect of subliminal administered nutrients on emotional state that was previously limited to fatty acids. However, the effect of the third macronutrient, proteins, on emotional state remained inadequately known as well. In chapter 6 of this manuscript, we aimed to investigate the effect of the gluten protein on intestinal but also extra-intestinal (including emotional state) symptoms. As the literature on the effect of proteins on emotional state was more limited compared to the knowledge on the effect of fructose on brain responses (58), we addressed this research question in an off-line setting. The aim of this final chapter was not to investigate appetite, but the effect of the gluten protein on (extra-)intestinal symptoms and is therefore related to chapter 5, but to a lesser extent to chapters 3 and 4. For this reason, we discuss this last chapter separately from the effect of tastant-induced gut-brain signals.

As previously mentioned, comorbidity between GI symptoms and psychological symptoms is often observed in FGID. This is a key factor in the definition of non-coeliac gluten sensitivity (NCGS), where IBS-like symptoms and extraintestinal symptoms such as fatigue, a foggy mind, and also depression, occur after ingestion of gluten and disappear when the protein is eliminated from the diet (82). In chapter 6 of this doctoral thesis, we investigated the effect of sub-acute gluten administration on GI and extraintestinal symptoms in NCGS patients and a healthy control group; the latter had never been included in earlier NCGS studies. We also examined, to the best of our knowledge for the first time, the effect of acute administration of a single dose of gluten on the same outcomes. Since no gluten-specific effects were observed on GI symptoms in our NCGS patient cohort nor in healthy volunteers, we do not provide support for the existence of the controversial disease entity NCGS as a gastrointestinal disorder in its current form.

Future studies are needed to explore the mechanism behind the generation of extraintestinal symptoms in (self-reported) NCGS, which were observed in our study after a single dose of gluten compared to placebo, but not after a 5-day challenge. Our hypothesised potential mediators, including cortisol, intestinal permeability and inflammation markers were not altered. Applying a phMRI design as used in chapter 3 could unravel if different gluten-induced gut-brain signals are underlying the extraintestinal effects.

In terms of management of NCGS, new strategies beyond a sole strict gluten-free diet should be considered. It has been shown that a diet low in fermentable oligo-, di- and mono-saccharides and polyols (FODMAP), which successfully manages IBS complaints (217), is also beneficial for the improvement of symptoms in NCGS patients (81). In addition, some evidence suggests an additional benefit to a simultaneous adherence to a low FODMAP and gluten-free diet (257). Future studies should focus on tearing apart the specificity and sensitivity to the response to the different components of wheat, including the carbohydrate fructans (one of the components excluded in the low FODMAP approach), the gluten proteins and the non-gluten proteins (ATIs).

Outside of coeliac disease and NCGS patients, a gluten-free diet has gained popularity in the healthy population as well and has been referred to as 'the most popular diet in modern history' (258). A quarter of the American population report to avoid gluten, partly owing to the belief that gluten can have harmful health effects (259), although less than half of these individuals are affected by gluten related disorders (including coeliac disease, wheat allergy and NCGS) (258). Our results, as described in chapter 6, indicate no effect of gluten ingestion in a healthy control group. Gluten-free diets are thought to be nutritionally inadequate and poor in fibre and vitamin B12 and D, but rich in saturated fatty acids (260). However, the avoidance of gluten may result in reduced consumption of beneficial whole grains, which may affect cardiovascular risk (259). Therefore, anyone following a GFD should be monitored to ensure nutritional adequacy.

In conclusion, we provided evidence that Feuerbach was right; as the current doctoral thesis provided proof that the food one consumes, specifically sugars, (subconsciously) affects our emotional processing. Likewise, we increased the previous knowledge on the anorexigenic properties of bitter tastants and paved the way for new study designs in patient populations with disturbed eating behaviour, including obesity. Furthermore, we highlighted the unlikely existence of (self-reported) NCGS, as no GI symptoms were observed in NCGS patients after gluten exposure. Additionally, as no different responses after gluten or placebo administration were found in healthy volunteers, we suggested that the current self-prescription of the gluten-free diet may be unnecessary.

CHAPTER 8

References

8 References

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CHAPTER 9

Summary

9 Summary

The brain-gut axis is a complex, bi-directional, neuro-humoral communication system between the gastrointestinal (GI) tract and the brain, which represents an important part of an integrated interoceptive system through which our brain continuously receives information about the physiological state of the body. This information is integrated with exteroceptive sensory signals as well as input from reward, affective and cognitive neurocircuits, which constitutes the biological basis of a close link between interoception and emotions. Anorexigenic and orexigenic gut peptides constitute an important part of this cross-talk and signal to homeostatic and reward-related (hedonic) brain areas. Besides the regulation of homeostasis and GI function, gut-brain signalling can also interfere with emotional states, as affective neurocircuitry is strongly intertwined with interoceptive homeostatic-afferent circuits. This interplay may explain the overlap of digestive symptoms with affective symptoms in several GI disorders. One of the key roles of the brain-gut axis is the regulation of appetite and food intake. However, the mechanisms underlying the influence of specific nutrient-induced gut-brain signals on various psychobiological processes are poorly understood.

The present doctoral thesis aimed to elucidate the influence of different (subliminal) nutrient- and tastant-induced gut-brain signals on GI symptoms, appetite-related sensations, and food intake, but also more broadly on affective state. More specifically, we took an integrative approach to explore the integration between central and peripheral mechanisms along the gut-brain axis, combining neurophysiological, psychological, behavioural, and biochemical methods.

It has been shown that, beside nutrients, intragastric tastant administration also generates neurohumoral gut-brain signals, among others, via alteration of circulating gut peptide levels, which in turn activate receptors present on the afferents of the vagus nerve, or act directly on receptors in the brain. Quinine-hydrochloride (QHCl), a bitter tastant, has recently been shown to decrease hunger ratings and circulating plasma levels of orexigenic gut peptides, but the mechanisms were poorly understood. We investigated this knowledge gap by examining the effect of intragastric administration of QHCl on brain activity in homeostatic and hedonic brain regions, and on hunger and food intake in healthy females (chapter 3). Our study was the first to demonstrate that QHCl alters brain activity in homeostatic and hedonic regions, even when administered intragastrically to bypass effects of taste and smell. In addition, we showed that these brain responses covary with QHCl-induced decreases in circulating levels of orexigenic gut hormones. Furthermore, these differential brain responses covaried with decreased prospective food consumption scores after QHCl, and predicted the magnitude of the decrease in hedonic food intake observed after QHCl versus placebo. Given the observed brain responses in reward-related regions, we assessed the effect of intragastric administration of QHCl on

anticipatory food reward responses (chapter 4). We showed that intragastric administration of QHCl, alters neural anticipatory food reward responses during a food incentive delay task (FID). Compared to placebo, QHCl increased brain activity in the precuneus (during the anticipation phase of reward) and in the superior parietal cortex and the middle frontal gyrus, including the dorsolateral prefrontal cortex (during the receipt phase of reward), but not in the core reward circuit. Neural responses during the passive viewing task were not significantly different for high-calorie compared to non-food items after QHCl administration compared to placebo. These findings highlight a potential role for cognitive inhibitory regions as mediator of the orexigenic effect of bitter tastants. Future studies investigating the effect of intragastrically administered QHCl on consummatory food reward responses are warranted.

In the second part of this thesis, we wanted to investigate the effect of intragastric administration of fructose on subjective and neural responses to an experimentally induced negative emotional state in healthy volunteers (chapter 5). It is generally accepted that exteroceptive properties of food can influence emotional state, and this has been expanded to exteroceptive properties as our group previously demonstrated that subliminal intragastric infusion of fatty acids interferes with affective processes, since responses to sad emotion induction were attenuated at the subjective and neural level. However, studies investigating similar effects of other nutrients such as carbohydrates are sparse. We reported an effect of intragastric fructose administration on the neural response to sad emotion induction in homeostatic regions as the hypothalamus and the hedonic brain circuit, including the anterior insula, anterior cingulate cortex and lateral orbitofrontal cortex. However, at the self-report level, no significant effects were observed. We further reported effects of fructose administration, either alone or interacting with emotional state, on circulating levels of anorexigenic and orexigenic gut hormones. GLP-1 levels were higher after fructose compared to placebo administration during neutral, but not sad emotional context, and circulating octanoylated ghrelin levels dropped less during the sad emotional context regardless of whether fructose or placebo was administered.

The effect of other nutrients, such as proteins, on emotional state remains incompletely understood as well. The gluten protein may affect emotional state in patients with Non-Coeliac Gluten Sensitivity (NCGS), as has been shown in one small study where 3-day exposure to gluten increased depression scores compared to placebo in patients with NCGS. Potential mechanisms have not previously been explored; therefore, we aimed to investigate the effect of acute and sub-acute administration of gluten on emotional state and GI symptom levels in healthy volunteers and a NCGS patient group in the last part of this doctoral thesis (chapter 6). We showed that acute gluten administration altered extraintestinal symptoms (including increased fatigue and decreased vigour scores) in the NCGS

patient cohort. An effect on GI symptoms was lacking after one single dose of gluten. After a subsequent sub-acute administration period of 5 days, NCGS patients reported decreased levels of positive emotional state scores, but other extraintestinal symptoms were not altered. In contrast to after the acute challenge, NCGS patients reported increased levels of bloating and abdominal pain after sub-acute gluten as well as sub-acute placebo administration, compared to HV. Hypothesised potential mediators of these effects (intestinal permeability, hsCRP levels and the cortisol awakening response) were not altered in NCGS patients, nor in HV, after sub-acute gluten and placebo administration. Our study did not confirm a role of gluten as the culprit for GI symptoms, thereby questioning the definition and diagnosis of NCGS as a distinct disease. Our findings highlight the need for further studies disentangling different components that trigger symptoms in self-reported NCGS patients, which could lead to elimination of an (unnecessary) restrictive gluten-free diet in this patient population.

CHAPTER 10

Nederlandstalige samenvatting

10 Nederlandstalige samenvatting

De term ‘brain-gut axis’ verwijst naar het complex, bidirectioneel, neuro-humoraal communicatiesysteem tussen het maagdarmstelsel en de hersenen, dat een belangrijk deel vertegenwoordigt van een geïntegreerd interoceptief systeem. Via dit systeem ontvangen onze hersenen continu informatie over de fysiologische toestand van het lichaam. Deze informatie wordt gecombineerd met exteroceptieve sensorische signalen en met input van neurologische circuits die betrokken zijn in beloning, cognitie en emotie. Deze integratie vormt de biologische basis voor een sterke connectie tussen interoceptie en emoties. Anorexigene (eetlust beperkende) en orexigene (eetlustopwekkende) gastro-intestinale hormonen vormen een belangrijk onderdeel van deze communicatie tussen het maagdarmstelsel en de hersenen en brengen informatie over naar homeostatische en belonings-gerelateerde (hedonische) hersengebieden. Naast het regelen van homeostase en gastro-intestinale functies kunnen hersen-darm signalen ook interfereren met emotionele toestanden, aangezien affectieve neurocircuits sterk verweven zijn met interoceptieve homeostatisch-afferente circuits. Dit samenspel kan de overlap tussen gastro-intestinale symptomen en affectieve symptomen bij verschillende aandoeningen van het maagdarmstelsel verklaren. Een van de belangrijkste taken van de ‘brain-gut axis’ is de regulatie van eetlust en voedselinname. De mechanismen die ten grondslag liggen aan de invloed van door voedingsstoffen geïnduceerde hersen-darm signalen op verschillende psychobiologische processen zijn echter onvoldoende begrepen.

Het doel van dit proefschrift was om de invloed van hersen-darm signalen, uitgelokt door verschillende voedingsstoffen en smaakstoffen, op gastro-intestinale symptomen, eetlust-gerelateerde sensaties en voedselinname, maar ook meer in het algemeen op de affectieve toestand, te onderzoeken. Hiervoor hebben we neurofysiologische, psychologische, gedrags- en biochemische methoden gecombineerd om de integratie tussen centrale en perifere mechanismen van de ‘brain-gut axis’ te onderzoeken.

Het is aangetoond dat niet alleen voedingsstoffen, maar ook intragastrisch toegediende smaakstoffen, neuro-humorale darm-hersensignalen genereren, onder andere via een wijziging in de concentratie van gastro-intestinale hormonen die door ons maagdarmstelsel geproduceerd worden. Deze hormonen activeren op hun beurt receptoren die aanwezig zijn op de afferenten van de nervus vagus, of werken direct in op receptoren in de hersenen. Onlangs werd aangetoond dat quinine-hydrochloride (QHCl), een bittere smaakstof, het hongergevoel onderdrukt en de concentratie van orexigene gastro-intestinale hormonen reduceert, maar de mechanismen hierachter waren onvoldoende gekend. We onderzochten deze mechanismen door het effect van intragastrisch toegediende QHCl op hersenactiviteit in homeostatische en hedonische hersenregio's en op honger en voedselinname na te gaan bij gezonde vrouwen (hoofdstuk 3). Onze studie was de eerste die

aantoonde dat QHCl de hersenactiviteit in homeostatische en hedonische regio's verandert, zelfs wanneer het intragastrisch wordt toegediend om effecten van smaak en geur uit te sluiten. Bovendien toonden we aan dat deze veranderde hersenactiviteit samenging met gedaalde concentraties van orexigene gastro-intestinale hormonen. Bovendien gingen deze gewijzigde neurale responsen ook gepaard met verminderde scores voor voedselconsumptie na toediening van QHCl en voorspelden ze de grootte van de daling in hedonische voedselinname die werd waargenomen na toediening van QHCl in vergelijking met placebo. Gezien de waargenomen hersenreacties in het beloningscircuit, hebben we het effect onderzocht van een intragastrische toediening van QHCl op beloningsreacties uitgelokt door de anticipatie van voedsel (hoofdstuk 4). We toonden aan dat intragastrische toediening van QHCl een invloed heeft op neurale beloningsreacties na de anticipatie van voedsel tijdens een 'food incentive delay task' (FID). In vergelijking met placebo verhoogde QHCl de hersenactiviteit in de precuneus (tijdens de anticipatiefase van de beloning) en in de superieure pariëtale cortex en de mid-frontale gyrus, inclusief de dorsolaterale prefrontale cortex (tijdens de ontvangstfase van de beloning), maar niet in het klassieke beloningscircuit. Neurale reacties tijdens een passieve visuele taak waren niet significant verschillend voor calorierijke vergeleken met caloriearme voedselproducten na toediening van QHCl in vergelijking met placebo. Deze bevindingen benadrukken een mogelijke rol voor inhiberende cognitieve hersenregio's in het anorexigene effect van bittere smaakstoffen. Toekomstige studies die het effect onderzoeken van intragastrisch toegediende QHCl op beloningsreacties uitgelokt door de consumptie van voedsel zijn aangewezen.

In het tweede deel van dit proefschrift wilden we het effect onderzoeken van intragastrisch toegediende fructose op subjectieve en neurale reacties op een experimenteel geïnduceerde negatieve emotionele toestand bij gezonde vrijwilligers (hoofdstuk 5). Het is algemeen aanvaard dat exteroceptieve eigenschappen van voedsel, zoals smaak en geur, invloed kunnen hebben op onze emotionele toestand. Dit is uitgebreid tot interoceptieve eigenschappen doordat onze onderzoeksgroep eerder heeft aangetoond dat een intragastrische infusie van vetzuren affectieve processen beïnvloedt, meer bepaald werden subjectieve en neurale responsen op een experimenteel geïnduceerde negatieve emotionele toestand verminderd. Studies die vergelijkbare effecten van andere voedingsstoffen zoals koolhydraten onderzoeken zijn echter schaars. We rapporteerden een effect van intragastrisch toegediende fructose op de neurale respons op een experimenteel geïnduceerde negatieve emotionele toestand in homeostatische gebieden als de hypothalamus en het beloningscircuit, waaronder de anterieure insula, de anterieure cingulate cortex en de laterale orbitofrontale cortex. Op het subjectieve niveau (i.e. op basis van vragenlijsten) werden echter geen significante effecten waargenomen. We rapporteerden ook effecten van de toegediende fructose, alleen of in wisselwerking met de emotionele toestand, op de concentratie van orexigene en

anorexigene gastro-intestinale hormonen. GLP-1-concentraties waren hoger na fructose in vergelijking met na placebo toediening tijdens de neutrale, maar niet tijdens de negatieve emotionele context. De ghreline concentraties daalden minder tijdens de negatieve emotionele context ongeacht of fructose of placebo werd toegediend.

Het effect van andere macronutriënten, zoals eiwitten, op de emotionele toestand blijft ook onvolledig begrepen. Het gluten eiwit kan invloed uitoefenen op de emotionele toestand bij patiënten met niet-coeliakie gluten-sensitiviteit (NCGS), zoals werd aangetoond in een kleine studie waar patiënten met NCGS verhoogde depressie-scores rapporteerden na blootstelling aan gluten gedurende drie dagen. Potentiële mechanismen zijn niet eerder onderzocht; daarom wilden we het effect onderzoeken van acute en subacute toediening van gluten op de emotionele toestand en gastro-intestinale symptomen bij gezonde vrijwilligers en een groep NCGS patiënten in het laatste deel van dit proefschrift (hoofdstuk 6). We toonden aan dat acute gluten-toediening extra-intestinale symptomen veranderde (waaronder verhoogde vermoeidheid en verminderde kracht) bij NCGS patiënten. Een effect op gastro-intestinale symptomen werd niet waargenomen na acute toediening van gluten. Na een daaropvolgende subacute toedieningsperiode van 5 dagen rapporteerden NCGS patiënten een verlaagde score voor positieve emotie, maar andere extra-intestinale symptomen waren niet veranderd. In tegenstelling tot na de acute toediening, rapporteerden NCGS patiënten een verergering van hun opgeblazen gevoel en buikpijn na zowel subacute gluten als na subacute placebo toediening, in vergelijking met gezonde vrijwilligers. Potentiële mediatoren van deze effecten (intestinale permeabiliteit, hsCRP-waarden en de cortisol-ontwakingsreactie) waren niet veranderd bij NCGS patiënten, noch bij gezonde vrijwilligers, na subacute gluten en placebo toediening. Ons onderzoek bevestigde dus niet dat gluten de boosdoener is voor gastro-intestinale symptomen, waardoor de definitie en diagnose van NCGS als een afzonderlijke ziekte in twijfel kan worden getrokken. Onze bevindingen benadrukken de behoefte aan verdere studies om de verschillende componenten, die symptomen kunnen veroorzaken bij zelf-gerapporteerde NCGS-patiënten, te ontrafelen. Dit zou kunnen leiden tot de eliminatie van een (onnodig) restrictief glutenvrij dieet bij deze patiëntenpopulatie.

CHAPTER 11

Acknowledgements, personal contributions and conflict of interest statement

11 Acknowledgements, personal contributions and conflict of interest statement

Without the support, guidance and collaborations with many different experts and colleagues, I would never have been able to complete this PhD project successfully. My sincere gratitude and appreciation go out to all of them, who have directly or indirectly supported me along the path of this project. I will discuss author contributions on a per-chapter basis.

Chapter 1: General introduction

Part of this chapter has been published in a slightly different form as: Weltens N*, Iven J*, Van Oudenhove L°, Kano M°. The gut-brain axis in health neuroscience: implications for functional gastrointestinal disorders and appetite regulation. *Annals of the New York Academy of Sciences*. * These authors contributed equally to this work as first authors. ° These authors contributed equally to this work as senior authors.

This rest of the content of this chapter was written by J. Iven and thoroughly revised by J.R. Biesiekierski, L. Van Oudenhove and J. Tack.

Chapter 2: Research objectives

The research objectives of this doctoral project were conceived by J.R. Biesiekierski, L. Van Oudenhove, J. Tack and J.Iven.

The content of this chapter was written by J. Iven and thoroughly revised by J.R. Biesiekierski, L. Van Oudenhove and J. Tack.

Chapter 3: Intragastric quinine administration decreases hedonic eating in healthy women through peptide-mediated gut-brain signalling mechanisms

This chapter has been published as the following scientific publication: Iven J*, Biesiekierski JR*, Zhao D, Deloose E, O'Daly OG, Depoortere I, Tack J, Van Oudenhove L. Intragastric quinine administration decreases hedonic eating in healthy women through peptide-mediated gut-brain signalling mechanisms. *Nutritional Neuroscience.* 2018:1-13. doi: 10.1080/1028415x.2018.1457841. * These authors contributed equally to this work as first authors.

J. Iven, J.R. Biesiekierski, O. O'Daly, I. Depoortere, J. Tack and L. Van Oudenhove conceived and designed the study protocol. L. Van Oudenhove, I. Depoortere and J. Tack acquired funding and provided technical services and materials. J. Iven recruited participants. J. Iven and J.R. Biesiekierski performed experiments. J. Iven, D. Zhao, E. Deloose and L. Van Oudenhove analysed and interpreted data. J. Iven took the lead in writing the manuscript and prepared tables and figures with support from

J.R. Biesiekierski and L. Van Oudenhove. All authors supported in interpretation of the results and critically revised the manuscript.

In addition, the authors would like to thank the staff at the Radiology Department, including R. Peeters, S. Ghysels, G. Putzeys and K. Byloos for their collaboration and technical support as well as J. Tóth for his assistance in the analysis of the metabolic hormone data. We wish to thank H.G. Ly and N. Weltens for their assistance with scanning during the first study visits.

Chapter 4: Intragastric quinine alters brain responses to the anticipation and receipt of reward in attentional and executive regions, but not in core reward circuitry

Authors: J. Iven, J.R. Biesiekierski, P. Dupont, I. Depoortere, J. Tack and L. Van Oudenhove

J. Iven, J. Biesiekierski and L. Van Oudenhove conceived and designed the study protocol. L. Van Oudenhove, I. Depoortere and J. Tack acquired funding and provided technical services and materials. J. Iven recruited participants and performed experiments. P. Dupont programmed scripts for functional brain imaging analysis. J. Iven and L. Van Oudenhove analysed and interpreted data. J. Iven took the lead in writing this chapter and prepared tables and figures with support from J.R. Biesiekierski and L. Van Oudenhove.

In addition, the authors would like to thank the staff at the Radiology Department, including R. Peeters, S. Ghysels, G. Putzeys and K. Byloos for their collaboration and technical support as well as J. Tóth for his assistance in the analysis of the metabolic hormone data. We also wish to thank J. Simon for providing us with their version of the food incentive delay task, used for this study protocol. In line with that, we would like to thank A.G. Liuzzi from the Laboratory for Cognitive Neurology, for her assistance with the Presentation software used for this task and J. Zaman from the Health Psychology group for his assistance with the Affect 4.0 program used for the passive viewing task.

Chapter 5: Intragastric fructose administration interacts with emotional state in homeostatic and hedonic brain regions

Authors: J. Iven, J.R. Biesiekierski, D. Zhao, J. Tack and L. Van Oudenhove

J. Iven, J. Biesiekierski and L. Van Oudenhove conceived and designed the study protocol. J. Tack and L. Van Oudenhove acquired funding and provided technical services and materials. J. Iven recruited participants. J. Iven and J.R. Biesiekierski performed experiments. J. Iven, D. Zhao and L. Van Oudenhove analysed and interpreted data. J. Iven took the lead in writing this chapter and prepared tables and figures with support from J.R. Biesiekierski and L. Van Oudenhove.

In addition, the authors would like to thank S. Sirjacobs for her help with recruitment of participants and performance of experiments during her master thesis project. Furthermore, we would like to

thank the staff at the Radiology Department, including R. Peeters, S. Ghysels, G. Putzeys and K. Byloos for their collaboration and technical support as well as J. Tóth and E. Deloose for their assistance in the analysis and interpretation of the metabolic hormone data.

Chapter 6: The effect of acute and sub-acute gluten administration on intestinal and extraintestinal symptoms in NCGS patients and healthy volunteers

Authors: J. Iven, A. Geeraerts, T. Vanuytsel, J. Tack, L. Van Oudenhove, J.R. Biesiekierski

Part of this chapter has been published in a slightly different form in the following scientific publications: Biesiekierski JR* and Iven J.* Non-coeliac gluten sensitivity: piecing the puzzle together. *United European Gastroenterology Journal.* 2015; 3(2):160-5 and Biesiekierski JR* and Iven J.* Exploring Non-Coeliac Gluten Sensitivity: A Neuropsychiatric Disorder? *Journal of Digestive Diseases and Hepatology*. 2017: JDDH-129. * These authors contributed equally to this work as first authors.

J. Iven, J. Biesiekierski, J. Tack, T. Vanuytsel and L. Van Oudenhove conceived and designed the study protocol. J. Biesiekierski and J. Tack acquired funding and provided technical services and materials. J. Iven and A. Geeraerts recruited healthy subjects. J. Iven, A. Geeraerts, J. Tack and T. Vanuytsel recruited NCGS patients. J. Iven and A. Geeraerts performed experiments. J. Iven, A. Geeraerts and L. Van Oudenhove analysed and interpreted data. J. Iven took the lead in writing this chapter and prepared tables and figures with support from J.R. Biesiekierski and L. Van Oudenhove.

The authors would like to thank N. Pauwels and C. Breynaert for their assistance in the recruitment of NCGS patients. We thank S. Van Wouwe for her assistance with recruitment of healthy volunteers and performance of experiments during her master thesis project. We would also like to thank J. Tóth, A. Luypaert and K. Verbeke for their help with analysis of biological samples, including cortisol levels and intestinal permeability. We wish to thank K. Claes and the team of the Laboratory of the University Hospitals Leuven as well as M.P. Edmonds and the team of Red Cross Flanders for the analysis and genotyping of blood samples. Furthermore, we would like to thank Prof. J. Raes and his team for their help with collection of faecal samples, especially L. De Commer who took care of all practical issues regarding sampling material. Besides, we would like to thank the team of Prof. J. Raes in advance for their assistance in future analyses of the gut microbiota data, which was beyond the scope of this doctoral thesis.

Chapter 7: General discussion and future prospects

The content of this chapter was written by J. Iven and thoroughly revised by J.R. Biesiekierski, L. Van Oudenhove and J. Tack.

Chapter 9: Summary

The content of this chapter was written by J. Iven and thoroughly revised by J.R. Biesiekierski and L. Van Oudenhove.

Chapter 10: Nederlandstalige samenvatting

The content of this chapter was written by J. Iven and revised by L. Van Oudenhove.

Conflict of interest

The authors have no conflicts of interest to declare.

Curriculum vitae

Julie Iven was born on February 25, 1990 in Sint-Truiden, Belgium. After finishing secondary school (Latin-Mathematics-Science) at Katholieke Centrum Scholen, Sint-Truiden, she started her training in Biomedical Sciences at the University of Leuven, Belgium. She obtained her bachelor's degree in 2012 and her master's degree cum laude in 2014 (Master thesis: Visceral sensations and discrimination in a context of fear conditioning). Subsequently, she started her PhD training at the Translational Research Center for Gastrointestinal Disorders (TARGID), KU Leuven, under supervision of Prof. Dr. Jan Tack, Prof. Dr. Lukas Van Oudenhove and dr. Jessica R. Biesiekierski in October 2014. Her scientific work has contributed to several meeting abstracts and peer-reviewed publications.

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* These authors contributed equally to this work as first authors. ° These authors contributed equally to this work as senior authors.

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