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DEVELOPMENT OF A SPECIFIC AND SENSITIVE ASSAY FOR CHOLECYSTOKININ, AND APPLICATIONS THEREOF

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ABSTRACT

The cholecystokinergic system is an important family of peptides and receptors which plays a critical role in a wide range of physiological and behavioral processes. Cholecystokinin, or "CCK" peptides, originally identified in the gastrointestinal tract, are now considered to be one of the most abundant peptide systems in the mammalian central nervous system. In its role as a neurotransmitter and neuromodulator, cholecystokinin interacts extensively with other neurochemical networks, including the dopaminergic, serotonergic, noradrenergic and GABAergic systems. Prompted by recent findings that implicated the cholecystokinergic system in the pathophysiology of various illnesses, we developed a novel assay system to measure the various forms of cholecystokinin peptides in human plasma and cerebrospinal fluid. Our 3-step system involved: i) extraction of CCK fragments from plasma using reverse phase chromatography; ii) separation of peptides by high performance liquid chromatography; and; iii) detection and quantification of peptides with a double-antibody radioimmunoassay. The system detects CCK-4, sulfated CCK-8 (CCK-8s) and nonsulfated CCK-8 (CCK-8ns) with equal affinity, with the lower detection limit of 2.7 fmol and an ED_{50} of 10.6 ± 2.2 fmol. Using the assay system, we determined that mean CCK-like immunoreactivity (CCK-LI) in the plasma of 12 healthy subjects was 12.9 ± 2.1 pM CCK-4 equivalents. Further, mean concentrations of each individual peptide in plasma were: 1.0 ± 0.2 pM, 3.4 ± 0.8 pM and 1.9 ± 0.4 pM for CCK-4, CCK-8s and CCK-8ns respectively.

After developing the cholecystokinin assay system, we were able to combine our unique methodology with other established techniques to investigate the role of CCK in

illnesses such as premenstrual dysphoric disorder (PMDD), anxiety, bulimia nervosa, and cardiomyopathy.

Briefly, we observed no significant differences in plasma CCK levels between women with PMDD and healthy volunteers. However, we found that, independent of diagnosis, plasma cholecystokinin concentrations were higher in women during their first visit to the clinic to participate in the study, as compared to later visits. This finding suggested that elevated CCK levels reflected an "anticipatory anxiety" response. Moreover, our research with PMDD subjects revealed plasma cholecystokinin levels did not vary with menstrual phase—an important result to CCK researchers who would otherwise be wary of including female subjects in their studies on the basis that menstrual phase may affect cholecystokinin levels and confound results.

In addition, application of our assay system allowed us to determine that oral ingestion of caffeine increased plasma CCK-LI levels 2 - 4 fold in humans. Moreover, we observed substantial variation in post-caffeine cholecystokinin levels among individuals. Although preliminary, these findings suggest that caffeine-induced anxiety may occur via a cholecystokinin-mediated pathway and that certain individuals may be more susceptible to the physiological and behavioural effects of caffeine.

In another study of cholecystokinin and anxiety, we used our CCK assay to determine the effects of ondansetron, a serotonin receptor antagonist, on cholecystokinin levels in plasma. We found that multiple oral doses of ondansetron influence the pharmacokinetic parameters of exogenous CCK. This observation suggests that the cholecystokinergic and serotonergic systems interact to modulate anxious behaviour via a 5-HT_3 receptor-mediated pathway.

We also used the three-step assay system to measure CCK-LI in patients with the eating disorder, bulimia nervosa. Baseline fasted cholecystokinin plasma levels were lower in bulimic woman as compared to control subjects. However, at "satiety", or the post-binge stage, CCK levels in bulimic women were similar to those of control women. These initial data suggest that cholecystokinin, an important satiety hormone, may contribute to overeating in bulimia nervosa.

Finally, our investigation into the role of cholecystokinin in cardiomyopathy revealed that neuronal cholecystokinin receptor density was altered in the cardiomyopathic hamster brain, as compared to age- and sex -matched control animals. Quantitative *in vitro* autoradiography revealed that CCK binding was significantly elevated in the olfactory bulb, frontal cortex and occipital cortex of the cardiomyopathic hamsters, indicating that neural cholecystokinergic dysregulation may be a significant factor in cardiomyopathy.

In summary, we developed a specific and sensitive assay system for cholecystokinin peptides and subsequently used this assay as a powerful methodological tool to investigate the role of the cholecystokinergic system in the pathophysiology of certain psychiatric and cardiovascular illnesses.

RÉSUMÉ

Le système cholécystokinergique, comprenant une famille importante de peptides et de récepteurs, joue un rôle critique dans une variété de processus physiologiques et comportementaux. Les peptides cholécystokinine, ou peptides CCK, d'abord identifiés dans l'appareil gastrointestinal, sont maintenant reconnus comme étant les peptides le plus abondants du système nerveux central du mammifère. Comme neurotransmetteur et neuromodulateur, la cholécystokinine interagit avec une multitude de réseaux neurochimiques, dont les systèmes dopaminergiques, sérotonergiques, noradrénergiques et GABAergiques. Des récentes recherches, impliquant le système cholécystokinergique dans la pathophysiologie de différentes maladies, nous ont incité à développer une nouvelle méthode de mesure des différentes formes des peptides CCK dans le plasma et le fluide cérébrovertébral humains. Notre système comprend trois étapes: i) l'extraction plasmatique des fragments de CCK par chromatographie en phase inverse; ii) la séparation des peptides par chromatographie liquide à haute pression; iii) la détection et la quantification des peptides par essai radioimmunologique avec double anticorps. Le système détecte la CCK-4, la CCK-8 sulfaté (CCK-8s) et la CCK-8 non-sulfaté (CCK-8ns) qui ont une affinité semblable et avec une limite de détection de 2.7 fmol et un ED_{50} de 10.6 ± 2.2 fmol. Avec cet essai, nous avons déterminé que la moyenne d'immunoréactivité apparente de CCK (CCK-LI) dans le plasma de 12 sujets sains était de 12.9 ± 2.1pM d'équivalents CCK-4. De plus, les concentrations plasmatiques

moyennes de chaque peptide etaient de: 1.0 ± 0.2 pM, 3.4 ± 0.8 pM and 1.9 ± 0.4 pM pour la CCK-4, la CCK-8s et la CCK-8ns respectivement.

Après avoir développé cette technique de measure de la cholécystokinine, il nous a été possible de combiner notre méthode unique à d'autres techniques déjà établies pour l'étude du rôle du CCK dans les maladies, telles que le désordre dysphorique prémenstruel (PMDD), l'anxiété, la boulimie nerveuse et la cardiomyopathie.

Brièvement, nous n'avons pas observé de différence significative entre les niveaux plasmatiques de CCK des femmes atteintes de PMDD et ceux des femmes saines. Cependant, nous avons trouvé que, indépendamment du diagnostic, les concentrations plasmatiques de cholécystokinine étaient plus élevées chez les femmes lors de leur première visite à la clinique pour leur participation à l'étude, qu' aux visites subséquentes. Ce résultat suggère que les quantités élevées de CCK réflètent une réponse à une "anxiété anticipée". De plus, notre recherche portant sur les patientes atteintes de PMDD révèlent que les niveaux plasmatiques de cholécystokinine ne changent pas avec le cycle menstruel. Ce résultat est considéré important dans la recherche sur a CCK car les chercheurs pourraient être tentés d'inclure des femmes dans leurs études sous prétexte que la CCK peut influencer le cycle menstruel et ainsi confondre leurs résultats.

De plus, l'application de notre technique nous a permis de déterminer que l'ingestion orale de caféine a pour effet d'augmenter de 2 à 4 fois la CCK-LI plasmatique chez les humains. De plus, nous avons observé une variabilité substantielle des niveaux de cholécystokinine parmi les individus, après consommation de caféine. Ces résultats préliminaires suggèrent que l'anxiété induite par la caféinepourrait résulter

d' un réseau médié par la cholécystokinine, et que certains individus pourraient être plus sensibles aux effets physiologiques et comportementaux de la caféine.

Lors d'une autre étude sur l'anxiété et la cholécystokinine, notre méthode de dosage fut utilisée pour déterminer les effets de l'ondansetron, un antagoniste du récepteur sérotonine, sur les niveaux de CCK dans le plasma. Nous avons découvert qu'après plusieurs doses orales d'ondansetron, les paramêtres pharmacocinétiques du CCK exogène sont modifies. Cette observation suggère que les systèmes cholécystocinergique et sérotonergique intéragissent pour moduler le comportement d'anxiété via le 5-HT_3, un modèle médié par recepteur.

Nous avons aussi utilisé notre technique de mesure de CCK-LI sur les patientes atteintes de la boulimie nerveuse, un désordre alimentaire. Les niveaux plasmatiques basals de cholécystokinine à jeûn étaient inférieurs chez les femmes boulimiques que chez les sujets contrôles. Cependant, à satiété, ou au stage après une grande ingestion de nourriture, les niveaux de CCK chez les femmes boulimiques étaient similaires à ceux des sujets contrôles. Ces premiers résultats suggèrent que la cholécystokinine, une importante hormone impliquée dans la satiété, pourrait contribuer à la suralimentation reliée à la boulimie nerveuse.

Finalement, notre recherche sur le rôle de la cholécystokinine dans la cardiomyopathie révèle que la densité de récepteurs neuronaux de CCK était altérée dans le cerveau du hasmter cardiomyopathique lorsque comparée à celle des animaux contrôles à âge et sexe semblables. L'autoradiographie quantitative *in vitro* a révélé que la liaison du CCK était significativement plus élevée dans le bulbe olfactif, les cortex frontal et occipital des hamsters cardiomyopathiques, indiquant que la dérégulation

neuronale de la cholécystokinine pourrait être un important facteur de la cardiomyopathie.

En résumé, nous avons développé une méthode de dosage spécifique et sensible aux peptides de cholécystokinine et, par la suite, avons utilisé cet essai comme un outil de méthodologie puissant dans la recherche du rôle du système cholécystokinergique dans la pathophysiologie de certaines maladies psychiatriques et cardiovasculaires.

PREFACE

The present dissertation, consisting of eleven chapters, describes the development of a specific and sensitive assay system for cholecystokinin peptides. Various applications of this novel assay system are also discussed. A review of the anatomy, physiology and biochemistry of the cholecystokinergic system is provided as a foundation for discussing the rationale underlying the development of the assay, as well as the need for and utility of such technology in the field of cholecystokinergic research.

Chapters One and Two provide a general overview of the cholecystokinergic system. The evolution of the system, as well the anatomical localization of cholecystokinin peptides and receptors are described.

In Chapter Three, the mechanisms of cholecystokinin release are detailed. The physiological factors and methods involved in releasing cholecystokinin in both the gastrointestinal tract and the central nervous system are described.

Chapter Four focuses on the role of cholecystokinin as a neurotransmitter and neuromodulator. Specifically, the complex interactions among the cholecystokinergic system and the dopaminergic, serotonergic, noradrenergic, GABAergic and opiodergic systems are explored.

Chapter Five reviews the role of second messengers in relation to the cholecystokinergic system. The regulation of cholecystokinin synthesis and release, as well the role of cholecystokinin in signal transduction are briefly discussed.

Chapter Six critiques the current state of technology in cholecystokinin assay systems. The development of our novel cholecystokinin assay system is described, including the rationale underlying the various methods and techniques used in creating and refining the assay. The published manuscript entitled "*Development of a Specific and Sensitive Assay System for Cholecystokinin Tetrapeptide*" is provided at the end of the chapter. This manuscript represents the culmination of the majority of laboratory work undertaken in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*. In addition, this manuscript, along with the other manuscripts included in

this dissertation, represents an original contribution of knowledge to the scientific community in the field of cholecystokinin research.

In Chapters Seven, Eight, Nine and Ten, the various applications of the newly developed cholecystokinin assay system are discussed. Specifically, the utility of the cholecystokinin assay as a unique tool for investigating various illnesses is explored. Chapter Seven discusses the use of the cholecystokinin assay system in investigating Eating Disorders. Chapter Eight focuses on the application of the assay in Premenstrual Dysphoric Disorder and includes a published manuscript entitled, "*Sensitivity to CCK-4 in Women with and without Premenstrual Dysphoric Disorder (PMDD) During Their Follicular and Luteal Phases.*" Chapter Nine describes the utility of the cholecystokinin assay in Anxiety Disorders and includes novel data on the effects of caffeine on the cholecystokinergic system. Chapter Ten describes the use of the assay in investigating Cardiomyopathy. A manuscript-in-submission, entitled "*Altered Cholecystokinin Binding in the Cardiomyopathic Hamster Brain*" is provided at the end of chapter.

Finally, Conclusions are provided in Chapter Eleven. Future Directions in the field of cholecystokinin research using the newly developed cholecystokinin assay system are also discussed.

In compliance with the Guidelines for Thesis Preparation provided by the McGill Faculty of Graduate Studies and Research, the following text is reproduced below:

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> The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
> The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research; a comprehensive

review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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Irrespective of the internal and external examiners reports, if the oral defence committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis. In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis.

ACKNOWLEDGMENTS

I am extremely grateful to Dr. Roberta Palmour for her constant supervision, enthusiasm and optimism throughout my doctoral studies. I am indebted to her for countless scientific discussions, as well as for the long hours spent in editing my manuscripts and thesis. I am thankful for her exceptional generosity and empathy. For giving me the opportunity to be independent while still providing guidance, for being critical without being a critic, for being my teacher and becoming my friend, I am eternally grateful.

I am indebted to Dr. Jolanta Gutkowska for her guidance and supervision during the course of my studies. Dr. Gutkowska's extensive knowledge of biochemistry and her contribution to the field of science are impressive; her expertise, encouragement and realism were always appreciated. I am thankful for her suggestions and revisions of all my scientific literature. I am very grateful to her for providing the unrestricted use of her well-equipped laboratory and resources. Indeed, the majority of my experimental work, including the development of the assay system and all subsequent measurements using this methodology, as well as the ensuing technical knowledge that I acquired can be attributed to the generosity of Dr. Gutkowska.

I also wish to express my appreciation to the members of the Gutkowska laboratory for warmly welcoming me as a member of their professional family. I am especially grateful to Dr. Suhayla Mukkadam-Daher for her invaluable advice in manuscript and protocol preparation; her integrity and tenacity were truly inspirational. I am also grateful to Dr. Marek Jankowski for his valuable insight into receptor

methodology and data analyses. I wish to commend Céline Coderre and Nathalie Charron for their outstanding and unmatched technical assistance. They were truly excellent teachers—even managing to teach me a little French along the way . . . merci.

I would be greatly amiss if I did not extend my gratitude to the following individuals: Dr. Frank Ervin for intellectual enlightenment and exceptional generosity in providing the opportunity to work with his primate colony; Dr. Jacques Bradwejn for his extraordinary vision in the field of cholecystokinin research and his ground-breaking studies, which sparked my own interest in this field; Dr. Jean-Michel Le Mellédo for friendship and insight; Dr. Kurt Sittmann for providing guidance as a member of my supervisory committee; Dr. Tham-Vinh Dam for introducing me to the technique of autoradiography; Dr. Irena Berezowska for aid in establishing my HPLC conditions; Dr. Jose Ma Mejia and Lynda Turner for technical assistance with clinical projects; and to Dr. Franco Vaccarino and the entire Medical Research Council of Canada Anxiety Research Group for providing not only financial support, but also invaluable guidance.

I wish to thank my friends, particularly Leslie Marvel, Tanja Taivassalo, Sheila Ernest, Caroline Vicanek, Jennifer Urban, and Pascal Guilluame for constant encouragement, support and the many memorable hours spent in both commiseration and celebration. Finally, I offer my deepest gratitude to my family for moral support, inspiration and not making me feel *too* guilty on those countless occasions when it must have seemed that my research took precedence over them.

LIST OF ABBREVIATIONS

ACTH: Adrenocorticotropic hormone

APA: American Psychiatric Association

BH: Bolton Hunter

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

C-Terminus: Carboxy terminus

CCK: Cholecystokinin

CCK-4: Cholecystokinin tetrapeptide

CCK-8ns: Cholecystokinin octapeptide, nonsulfated

CCK-8s: Cholecystokinin octapeptide, sulfated

CCK-A: Cholecystokinin receptor, alimentary type

CCK-B: Cholecystokinin receptor, brain type

CCK-B_1: Cholecystokinin B-receptor, subtype 1

CCK-B_2: Cholecystokinin B-receptor, subtype 2

CCK-LI: Cholecystokinin-like immunoreactivity

CCKz: Elimination rate constant of CCK

CMO: Cardiomyopathic

CNS: Central nervous system

CO_2: Carbon dioxide

CSF: Cerebrospinal fluid

$D_{1,2,3}$: Dopamine receptors, type 1, 2, 3 etc.

DOI: 2,5-HT-dimethyoxy-4-iodoamphetamine HBr

DSM-IV: Diagnostic and Statistical Manual of Mental Disorders IV

DSP-4: N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine

EDTA: Ethylenediamine-tetraacetic acid

G-Protein: Guanosine nucleotide-binding protein

GABA: Gamma-aminobutyric acid

$GABA_A$: Gamma-aminobutyric acid receptor, type A

$GABA_B$: Gamma-aminobutyric acid receptor, type B

GDP: Guanosine 5'-diphosphate

GTP: Guanosine 5'-triphosphate

HPLC: High performance liquid chromatography

i.p.: Intraperitoneal

i.v.: Intravenous

IBMX: 3-isobutyl-1-methylxanthine

LLPDD: Late luteal phase dysphoric disorder

mRNA: Messenger ribonucleic acid

N-Terminus: Amino terminus

PMDD: Premenstrual dysphoric disorder

PMSF: Phenylmethanesulphonyl fluoride

POMC: Proopiomelanocortin

SHR: Spontaneously hypertensive rat

TFMPP: N-(3-trifluoromethylphenyl)piperazine HCl

^{125}I -CCK: Cholecystokinin labelled with radioactive iodine

5-HIAA: 5-hydroxyindoleacetic acid

$5\text{-HT}_{1,2,3}$: Serotonin receptors, type 1, 2, 3 etc.

5-HT: 5-Hydroxytrptamine, Serotonin

8-OH-DPAT: 8-hydroxy-2-(di-n-propyl-amino)tetralin HBr

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

I. In response to a lack of a specific and sensitive assay system for cholecystokinin peptides in the field of cholecystokinin research, we developed a novel assay system to measure the various forms of cholecystokinin peptides in human plasma, cerebrospinal fluid and other tissues. Our novel 3-step system consists of i) extraction of CCK fragments from plasma using reverse phase chromatography; ii) separation of peptides by high performance liquid chromatography; and iii) detection and quantification of peptides with a double-antibody radioimmunoassay. This assay system, unlike its predecessors, detects CCK-4, sulfated CCK-8 (CCK-8s) and nonsulfated CCK-8 (CCK-8ns) with equal affinity, with the lower detection limit of 2.7 fmol and an ED_{50} of 10.6 ± 2.2 fmol.

II. Using our newly-developed cholecystokinin assay system, we determined that mean CCK-like immunoreactivity (CCK-LI) in the plasma of 12 healthy subjects was 12.9 ± 2.1 pM CCK-4 equivalents. Our assay allowed us to establish, for the first time, the mean concentrations of each individual peptide in healthy human plasma: 1.0 ± 0.2 pM, 3.4 ± 0.8 pM and 1.9 ± 0.4 pM for CCK-4, CCK-8s and CCK-8ns respectively.

III. Using our new assay system as a tool to investigate premenstrual dysphoric disorder (PMDD), we discovered that women with PMDD and healthy volunteers had similar CCK-LI levels. We also found that plasma cholecystokinin was increased in all women, regardless of diagnosis, during their initial visit to the study site. These observations

indicate that anticipatory anxiety may significantly increase plasma cholecystokinin levels, and must be accounted for as a potential confounding variable in all studies that involve measurement of CCK concentrations.

IV. Our use of the CCK assay system in the study of premenstrual dysphoric disorder revealed that plasma cholecystokinin levels do not vary with menstrual phase. This is a crucial observation to those researchers who were previously uncertain about the potential confounding effects of menstrual phase in their interpretation of cholecystokinin data. Further, this is an important result to CCK researchers — who would otherwise be wary of including female subjects in their studies for fear that menstrual phase may affect cholecystokinin levels.

V. In a preliminary study of patients with the eating disorder bulimia nervosa, we observed that fasted plasma CCK-LI levels were lower in bulimic woman as compared to healthy control subjects. However, at the post-binge stage, CCK levels in bulimic women were similar to that of control women postprandially. These data suggest that cholecystokinin, an important satiety hormone, may contribute to overeating in bulimia nervosa.

VI. Our assay system allowed us to determine that oral ingestion of caffeine increased plasma CCK-LI levels 2 - 4 fold from baseline fasted levels in healthy human subjects. We also observed substantial variation in post-caffeine cholecystokinin levels among individuals. These observations suggest that caffeine-induced anxiety may occur via a

cholecystokinin-mediated pathway and that certain individuals may be more susceptible to caffeine's physiological and behavioural effects. This differential susceptibility may be due to an individual's history of caffeine consumption and/or may be correlated to an individual's baseline anxiety level.

VII. Our investigation into role of cholecystokinin in cardiomyopathy revealed that neuronal cholecystokinin receptor density is altered in the cardiomyopathic hamster brain, as compared to age- and sex-matched control animals. Quantitative *in vitro* autoradiography revealed that CCK binding is significantly elevated in the olfactory bulb, frontal cortex and occipital cortex of cardiomyopathic hamsters. These findings indicate that neural cholecystokinergic dysregulation may be a significant factor in cardiomyopathy.

CONTRIBUTION OF AUTHORS

The present dissertation includes two multi-authored published manuscripts and one multi-authored manuscript-in-submission. In accordance with the Guidelines for Thesis Preparation provided by the McGill Faculty of Graduate Studies and Research, the following statements outline the contributions of each author:

Title: ***Development of a Specific and Sensitive Assay System for Cholecystokinin Tetrapeptide***
Authors: Salima Merani, Dr. Roberta M. Palmour, Dr. Jacques Bradwejn, Dr. Irena Berezowska, Dr. Franco J. Vaccarino, and Dr. Jolanta Gutkowska. *Peptides* 18;869-875, 1997.
Contributions: This paper represented the crux of my doctoral research. My contributions to this paper included: designing the cholecystokinin assay system, formulating the experimental protocol, conducting the experiments in the laboratory, collecting blood samples from subjects, measuring cholecystokinin levels in plasma, collecting data, analyzing data, writing the manuscript and preparing the manuscript for submission. Drs. Palmour and Gutkowska were my direct supervisors during the laboratory experiments, and in addition, were involved in the editing of the manuscript. The assay was developed in the laboratory of Dr. Gutkowska, using the technical resources generously provided by her. Drs. Bradwejn and Vaccarino provided cholecystokinin tetrapeptide for initial testing, aided in the recruitment of human subjects and edited the final version of the manuscript. Dr. Berezowska synthesized the Bolton Hunter cholecystokinin peptide used in the radioimmunoassay.

Title: ***Sensitivity to CCK-4 in Women with and without Premenstrual Dysphoric Disorder (PMDD) During Their Follicular and Luteal Phases***
Authors: Dr. Jean-Michel Le Mellédo, Salima Merani, Dr. Diana Koszycki, Dr. Francois Bellavance, Dr. Roberta M. Palmour, Dr. Jolanta Gutkowska, Dr. Susanne Steinberg, Dr. Daniel Georges Bichet and Dr. Jacques Bradwejn. *Neuropsychopharmacol* 20;81-91, 1999.
Contributions: This paper represented the application of the newly developed cholecystokinin assay system. My contributions to this paper included: developing the methodology used in collecting blood samples from human subjects to prevent cholecystokinin degradation, formulating the experimental collection procedure, measuring cholecystokinin levels in plasma, collecting data, analyzing cholecystokinin levels, writing the "*Methods For Total CCK-Like Immunoreactivity Measurements*" section of the manuscript and editing the manuscript. Drs. Palmour and Gutkowska were

my direct supervisors during the laboratory experiments, and were also involved in the editing of the manuscript. Measurements of cholecystokinin levels were performed in the laboratory of Dr. Gutkowska, using the technical resources generously provided by her. Dr. Jean-Michel Le Mellédo formulated the experimental design, interviewed the subjects, collected the blood samples, analyzed data, wrote the remainder of manuscript and prepared the manuscript for submission. The remaining authors aided in recruiting human subjects, performing psychiatric evaluations of the subjects, administering cholecystokinin injections, measuring estradiol, progesterone, luteinizing hormone and follicular hormone concentrations and editing the manuscript.

Title: ***Altered Cholecystokinin Binding in the Cardiomyopathic Hamster Brain***
Authors: Salima Merani, Dr. Marek Jankowski and Dr. Jolanta Gutkowska
Contributions: This manuscript-in-submission represented another application of the newly developed cholecystokinin assay system. My contributions to this manuscript included: formulating the hypothesis, formulating the experimental design, conducting the autoradiography experiments in the laboratory, collecting data, analyzing data, writing the manuscript and preparing the manuscript for submission. Dr. Jankowski aided in the analysis of data, preparation of graphs and production of autoradiographic images. Dr. Gutkowska was my direct supervisor during the laboratory experiments, and was also involved in the editing of the manuscript.

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CHOLECYSTOKININ PEPTIDES: AN OVERVIEW

CHAPTER ONE

CHOLECYSTOKININ PEPTIDES: AN OVERVIEW

1.1 Historical Perspective

In 1928, Ivy and Oldberg discovered a hormone in the mammalian upper intestine which had the ability to cause gallbladder contraction. They named this substance cholecystokinin. Fifteen years later, Harper and Raper (1943) demonstrated that a factor in porcine intestinal tissue was able to stimulate pancreatic enzyme secretion. They named this "new" hormone pancreozymin. In 1966, Jorpes and Mutt isolated a substance from the porcine intestine that possessed the ability to cause both gallbladder contraction and pancreatic enzyme release. It was thus discovered that cholecystokinin and pancreozymin were, in fact, the same hormone. Because the term "cholecystokinin" preceded "pancreozymin", the novel cholecystokinin-pancreozymin hormone was called cholecystokinin.

Initial attempts to purify cholecystokinin, frequently abbreviated to "CCK", from porcine intestinal tissue resulted in the isolation of a triacontatriapeptide, a thirty-three amino acid form of CCK referred to as CCK-33 (Mutt and Jorpes, 1971). Further studies demonstrated that cholecystokinin was, in fact, a heterogeneous molecule which existed in a number of different forms in intestinal extracts, including the carboxy-terminal octapeptide (CCK-8) and various larger fragments (Fig. 1). These variants contained a 5 amino-acid C-terminal sequence which was homologous to that found in gastrin, an

important hormone in the gastrointestinal tract originally isolated in 1905 (Edkins 1905; 1906).

In 1975, Vanderhaeghen and co-workers demonstrated the presence of material in the vertebrate central nervous system which reacted with anti-gastrin antibodies. This gastrin-like immunoreactivity was further investigated by employing a series of antibodies with differential cross-reactivities towards gastrin and CCK. These experiments revealed that the gastrin-like immunoreactivity found in canine, porcine and rodent cerebral extracts was actually generated by the synthesis of cholecystokinin peptides in the brain (Dockray, 1976). Scientists later demonstrated that both CCK-33 and CCK-8 were present as intact peptides in the pig (Muller *et al.*, 1977), dog, monkey (Straus and Yalow, 1978) and human brain (Rehfeld, 1978). In fact, specific quantification of neural CCK-like immunoreactivity soon revealed that the various forms of cholecystokinin constituted the most abundant system of peptides in the mammalian brain (Beinfeld *et al.*, 1981). Even today, the function of such copious quantities of cholecystokinin in the brain remains unclear.

1.2 Evolution and Species Heterogeneity

From its first isolation in porcine intestine, cholecystokinin has been found in many invertebrate and vertebrate phyla (Johnsen and Rehfeld, 1993). The cholecystokinin/gastrin family of peptides, which is characterized by a common, biologically active C-terminal pentapeptide, has been identified in the protochordate *Ciona intestinalis*, thereby dating these peptides as greater than 500 million years old

(Johnsen and Rehfeld, 1990). It is postulated that the divergence of cholecystokinin and gastrin occurred prior to the evolution of amphibia, as two different members of this family were identified in the frog *Rana catesbeina*. Gene duplication of a common sequence has been proposed as the mechanism for this divergence, as the amino acid sequences of frog CCK and gastrin are remarkably similar (Johnsen, 1994).

The various forms of intestinal and cerebral CCK isolated in amphibia and reptiles, including heptapeptide (CCK-7), octapeptide (CCK-8) and larger forms such as CCK-69 and CCK-70, were found to share a 70% homology between the frog and turtle, which in turn were quite similar to human cholecystokinin (50% identity). The C-terminal octapeptide was identical to that in humans (Johnsen, 1994). Studies of avian CCK in the brain revealed similar results (Fan *et al.*, 1987). In mammals, CCK has been identified in a number of species including rat, hamster, guinea pig (Zhou *et al.*, 1985), chinchilla (Fan *et al.*, 1987), cat (Eberlein *et al.*, 1988), hog, dog, monkey (Straus and Yalow, 1978) and man (Rehfeld, 1978). The C-terminal octapeptide is once again highly conserved among species: the sequence is identical among mammals, with the exception of the guinea pig (Zhou *et al.*, 1985) and chinchilla (Fan *et al.*, 1987), in which valine replaces methionine at the sixth position from the C-terminus. The strict preservation of the last five amino acids from the C-terminus along both temporal and species lines indicates the importance of this pentapeptide in terms of biological activity.

There is, nonetheless, species variation with respect to the various forms of CCK which have been isolated from mammalian tissue. For instance, in addition to CCK-8, CCK-22 has been found in rat (Liddle *et al.*, 1984), pig (Cantor and Rehfeld, 1989) and

rabbit (Rehfeld, 1994). CCK-33 has been found in chinchilla (Fan *et al.*, 1987), pig (Mutt and Jorpes, 1971) and dog (Eysselein *et al.*, 1984). CCK-39 has been reported in guinea pig (Dockray *et al.*, 1981) and dog (Eysselein *et al.*, 1984). The presence of CCK-58 has been demonstrated in cat and dog (Eberlein *et al.*, 1988).

In humans, reports of cholecystokinin isolation vary considerably. The specific isoforms of CCK vary by tissue of origin and method of isolation, but all forms clearly originate from a common precursor. Cantor and Rehfeld (1987), for example, identified CCK-8, CCK-22, CCK-33 and CCK-58 in human plasma, while Jansen *et al.* (1987) found CCK-22, CCK-33/39, CCK-58, but no CCK-8. Isolation of CCK-8, CCK-22 and CCK-33/39, with no detection of CCK-58 has also been reported (Liddle, 1985). Another study reported CCK-8, CCK-33/39, CCK-58, but no CCK-22 (Eberlein *et al.*, 1987). This latter group suggested that CCK-58 accounts for almost 50% of all CCK-like immunoreactivity, but goes undetected due to *in vitro* degradation during processing of blood and plasma. We have recently demonstrated that cholecystokinin tetrapeptide (CCK-4), sulfated and nonsulfated forms of the octapeptide (CCK-8s and CCK-8ns respectively), as well as larger molecular forms can be isolated from human plasma (Merani *et al.*, 1997). It is believed that CCK-8 is the major form of cholecystokinin in the human central nervous system (Lindefors *et al.*, 1991; Miller *et al.*, 1984), but at least one group of researchers has reported that CCK-12 predominates in cerebrospinal fluid (Geracioti *et al.*, 1993). CCK-8, CCK-39, CCK-58 (Eysselein *et al.*, 1990) and CCK-83 (Eberlein *et al.*, 1992) have been found in human intestinal extracts.

1.3 Biogenesis, Processing and Degradation

The cholecystokinin gene has been localized to human chromosome 3q (Lund *et al.*, 1986). It is approximately 7 kb in size and consists of 3 exons. The first exon contains the 5' untranslated region of the CCK mRNA. The second exon encodes the signal peptide and prohormone, while the third exon contains the biologically active C-terminal region (Deschenes *et al.*, 1984; Takahashi *et al.*, 1986). In humans, the precursor to the various CCK isoforms is a preprohormone of 115 amino acids with a molecular weight of approximately 13, 000 daltons. This preprocholecystokinin undergoes a series of post-translational modifications, including cleavage of the N-terminal signal peptide, N-terminal flanking sequence and C-terminal flanking peptide, tyrosine sulfation, amidation of the C-terminal phenylalanine and a series of peptidase cleavages. The purification and chemical characterization of CCK-83 from human intestinal mucosa revealed a molecule which was both sulfated and amidated — suggesting that these modifications likely occur prior to amino-terminal processing of the prohormone (Eberlein *et al.*, 1992).

A number of different enzymes have been implicated in the post-translational processing of the CCK precursor. Procholecystokinin contains nine regions with a single basic amino acid residues and three with paired basic amino acids, but it has been demonstrated that some of these sites may not be used as cleavage sites. Enzymes which have been shown to cleave peptides C-terminal to a single basic amino acid residue include endopeptidases which generate CCK-58, CCK-39, CCK-33, CCK-22 and CCK-8 (Eberlein *et al.*, 1992). CCK-8, either sulfated or nonsulfated, can further be processed to

CCK-6, CCK-5 and CCK-4 by serine peptidases (Camus *et al.*, 1989). A nontrypsin protease may be responsible for the formation of CCK-12 from CCK-33 (Turkelson *et al.*, 1990). In addition, specific metallopeptidases (E.C.3.4.24.15), aminopeptidases (E.C.3.4.11.2), neutral endopeptidases (E.C.3.4.24.11) are suspected in the metabolism of cerebral CCK-8 into CCK-4 (Konings *et al.*, 1993). More recently, a cytosolic isoform of tripeptidyl peptidase II (E.C. 3.4.14.10) was identified and shown to hydrolyze neuronal CCK-8 to generate CCK-5 and CCK-3. This cleavage occurs at the two peptide bonds where the methionine residues donate the carboxyl group (Rose *et al.*, 1996). In light of these findings, it is likely that several enzymes are involved in the processing of the cholecystokinin precursor.

1.4 Anatomical Localization of Cholecystokinin Peptides

In the majority of studies which assess either qualitative or quantitative levels of cholecystokinin, the term "cholecystokinin-like immunoreactivity", or CCK-LI, is frequently reported. This is due to the fact that many of the methods used to measure CCK do not have the sensitivity or specificity required to differentiate among the individual cholecystokinin fragments. In order to be consistent with the literature, the term CCK or CCK-LI will be used interchangeably in this dissertation to designate CCK-like immunoreactivity that cannot be accurately identified as CCK-4, CCK-8, CCK-33 etc.

Peripheral Distribution of Cholecystokinin Peptides

In the mammalian periphery, cholecystokinin-like immunoreactivity is found in high concentrations in the intestinal tract. In the gastrointestinal system, CCK is abundant in the duodenum and jejunum, with lower concentrations found in the ileum (Eng *et al.*, 1982; Calam *et al.*, 1982; Eysselein *et al.*, 1990). CCK has been localized, via immunohistochemical staining, to endocrine cells of the proximal intestine, and to nerves in the ileum and colon (Larson and Rehfeld, 1979). The synthesis of CCK has been demonstrated in mucosal endocrine I-cells (Buchan *et al.*, 1978), duodenal cells and pancreatic A-cells (Tsumuraya *et al.*, 1986). CCK-LI has been reported in the adrenal gland (Colombo-Benkmann *et al.*, 1996), lung (Wang and Cutz, 1993) and respiratory tract (Balaguer *et al.*, 1992). Cholecystokinin innervation has been demonstrated in the urinary bladder, ovary, uterus (Doss *et al.*, 1991), testis and sperm (Persson *et al.*, 1988). CCK can also be found in plasma (Cantor, 1989; Jansen and Lamers, 1983; Merani *et al.*, 1997) and cells of the immune system (Brambilla *et al.*, 1993; Panerai and Sacerdote, 1993). The presence of cholecystokinin precursor mRNA has been identified in the heart, lung, kidney and the small intestine of the rat (Funakoshi *et al.*, 1994).

Distribution of Cholecystokinin Peptides in the Central Nervous System

Cholecystokinin is one of the most abundant peptides in the mammalian central nervous system (Beinfeld *et al.*, 1981). The distribution of central cholecystokinin peptides is heterogeneous. High levels of CCK-LI are found in the cerebral cortex.

Approximately 1% of neurons in this region are estimated to contain CCK (Parnavelus, 1986). Cholecystokinin is also concentrated in the amygdala, hippocampus, olfactory bulb, hypothalamus, nucleus accumbens, septum, periaqeductal grey, parabrachial nucleus, superior and inferior colliculi. Moderate to low levels have been noted in the anterior pituitary and cerebellum (Beinfeld *et al.*, 1981; Lindefors *et al.*, 1993; Qureshi *et al.*, 1993). The retina has also been shown to contain CCK (Marshak *et al.*, 1990). Cholecystokinin peptides have been found in the brainstem and spinal cord (Cortes *et al.*, 1991; Zouaoui *et al.*, 1990). Cerebrospinal fluid sampling has been used in a number of studies as a source of accessible CCK to examine cholecystokinin concentrations in various studies of normal and pathological states (Lydiard *et al.*, 1992, 1993; Geracioti *et al.*, 1993). Although a variety of molecular forms have been isolated from the central nervous system, smaller forms of CCK, particularly the sulfated octapeptide, seem to predominate in the brain and CSF (Beinfeld *et al.*, 1981), while larger fragments (CCK-33, CCK-58) constitute the majority of CCK-LI in the intestinal tract (Brownstein and Rehfeld, 1984; Eysselein *et al.*, 1990). A few studies, however, have reported the predominance of larger forms of cholecystokinin in the brain (Lindefors *et al.*, 1991; Rehfeld *et al.*, 1988).

Widespread distribution of preprocholecystokinin mRNA has been reported in the rodent central nervous system, including various brain regions (Ingram *et al.*, 1989), brain stem and spinal cord motoneurons (Abelson and Micevych, 1991). In general, the localization of neurons positive for CCK peptide immunoreactivity parallels that of CCK mRNA distribution (Ingram *et al.*, 1989), suggesting that the central nervous system

serves as a site of cholecystokinin synthesis. Some studies have reported the absence of cholecystokinin mRNA in human thalamus and caudate putamen; however, the possibility that minute undetectable levels of mRNA exist in these regions has not been excluded (Lindefors *et al.*, 1991; Savasta *et al.*, 1990).

In summary, the cholecystokinergic system of peptides consists of a heterogeneous molecule that exists in a number of different forms. While larger molecules, such as CCK-33 and CCK-58, predominate in the gastrointestinal system, smaller form such as CCK-4 and CCK-8 appear to constitute the majority of cholecystokinin in the mammalian central nervous system. Although different forms of cholecystokinin can be found in different regions and in different species, the last four to five amino acids from the C-terminus appear to be strictly conserved This observation suggests that this sequence may be essential to the biological activity of the peptide, and that any assay that endeavours to measure cholecystokinin in biological tissue should be capable of detecting and quantifying the cholecystokinin tetrapeptide.

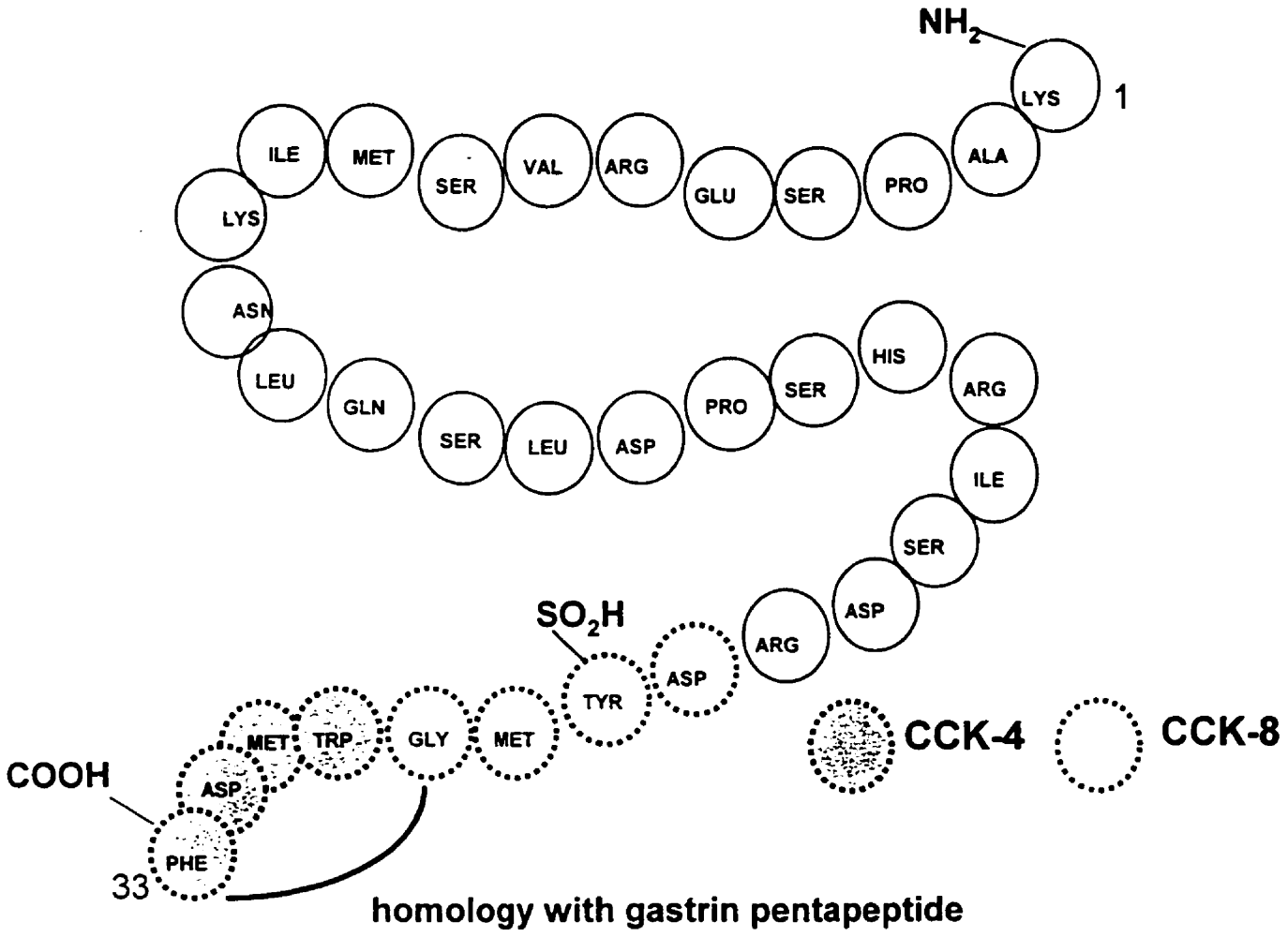

Figure 1. Schematic representation of CCK-33, with CCK-4 and CCK-8 highlighted.
NH_2—N terminus; COOH—C terminus. Adapted from Noble *et al.*, 1999.

CHAPTER TWO

CHOLECYSTOKININ RECEPTORS: AN OVERVIEW

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2.1 Historical Perspective

The presence of two distinct cholecystokinin receptors was first demonstrated in 1980 by Innis and Snyder. By comparing agonist binding in the brain and pancreas, they observed that the cerebral CCK receptor exhibited a differential peptide specificity from the pancreatic receptor. The existence of both receptor subtypes in the brain was subsequently documented by Moran and coworkers (1986), who found that a small number of binding sites in the brain had properties similar to that of peripheral CCK receptors. The pancreatic-type CCK receptors were termed CCK-A, or alimentary, receptors as opposed to the CCK-B, or brain, receptors that predominated in the brain (Figs. 2 and 3). The CCK-B receptor is sometimes referred to as the CCK-B/gastrin receptor due to the avidity of gastrin at this binding site. Indeed, cloning studies have confirmed that the CCK-B and the gastrin receptor are, in fact, the same receptor (Lee *et al.*, 1993).

The discovery that CCK receptors displayed pharmacological heterogeneity instigated much research and development into synthetic agonist and antagonists of the specific receptor subtype. Consequently, a number of well-characterized compounds are currently available and as such, provide indispensable tools in the elucidation of CCK-A and CCK-B receptor distribution and function. It is now well-established that CCK-A

receptors are found mainly in the periphery, but are also present in a limited number of brain regions. Likewise, CCK-B receptors are widely distributed throughout the central nervous system, but can also be found in some peripheral tissues.

2.2 Molecular Characterization of Cholecystokinin Receptors

The rat pancreatic CCK-A receptor (Fig. 2) was originally cloned by Wank and co-workers (1992a). The open reading frame of the CCK-A receptor cDNA sequence encodes a 444 amino acid protein. The cDNA sequence of the rat CCK-A receptor is identical whether it is derived from the brain or gastrointestinal system. The human CCK-A receptor encodes 428 amino acids (de Weerth *et al.*, 1993a). The CCK-A receptor gene resides on a syntenic region of human chromosome 4 and mouse chromosome 5 (Huppi *et al.*, 1995). Studies with CCK-A receptor knock-out mice reveal that mice lacking functional CCK-A receptors, generated by targeted gene disruption, appear morphologically normal, suggesting that absence of CCK-A receptors is not lethal (Kopin *et al.*, 1999).

Cloning studies of the rodent pancreatic CCK-A revealed that the primary receptor structure includes seven transmembrane hydrophobic regions, confirming that this receptor is a member of the G-protein coupled receptor family. Four putative phosphorylation sites for protein kinase C have been identified in the CCK-A receptor (Wank *et al.*, 1992a).

A recent study of the human CCK-A receptor has identified the tryptophan (TRP) and glutamine (GLN) residues in the 39th and 40th positions, respectively, as the primary

determinants for CCK octapeptide binding. These residues are thought to be involved in establishing a bond network with the N-terminus, as the CCK heptapeptide binding is unaffected when site-directed mutagenesis was employed to alter these sites (Kennedy *et al.*, 1997).

In 1992, Kopin and colleagues cloned the CCK-B receptor from canine parietal cells. The canine CCK-B receptor protein contains 453 amino acids. Further research by Wank and co-workers (1992b) established that the cDNA sequence of the rodent brain CCK-B receptor encodes a 452 amino acid protein. The cDNA sequence of the CCK-B receptor is identical whether it is derived from the brain or gastrointestinal system. The human CCK-B receptor contains a 1344 base pair open reading frame which encodes a 447 amino acid protein and shares 89.5% identity with the rat CCK-B receptor (Pisegna *et al.*, 1992; Denyer *et al.*, 1994). The CCK-B receptor gene has been localized to a syntenic region of human chromosome 11 and distal mouse chromosome 7 (Huppi *et al.*, 1995). Molecular analysis of the human CCK-B receptor has identified a single gene for the CCK-B receptor with no closely related homologues (Denyer *et al.*, 1994). Studies with CCK-B receptor knock-out mice demonstrate that mice lacking functional CCK-B receptors appear "viable, fertile and grossly normal into adulthood", suggesting that, like CCK-A receptors, CCK-B receptors are not essential to viability (Langhans *et al.*, 1997). It remains unclear whether the viability of either the CCK-A or CCK-B receptor knock-out mice stems from the ability of the remaining receptor (CCK-B or CCK-A respectively) to compensate for the nonfunctional receptor.

Cloning studies revealed that the CCK-B receptor, similar to the CCK-A receptor, contains seven transmembrane hydrophobic regions and is a member of the G-protein coupled receptor superfamily (Fig. 3). The nucleotide sequences of the CCK-A and CCK-B receptor cDNA show 54% identity, while the amino-acid sequences show 48% homology. The two amino acid sequences diverge substantially in the third intracellular loop, a region noted for G-protein coupling specificity (Wank *et al.*, 1992b).

The canine and human CCK-B receptor share 90% amino-acid identity. The two receptors have comparable agonist binding affinities, but differ in antagonist binding. A single amino acid in the sixth transmembrane domain of the CCK-B receptor has been reported as the critical determinant for non-peptide antagonist affinity (Beinborn *et al.*, 1993). This crucial amino acid corresponds to valine in the 319th position in the human CCK-B receptor, and leucine in the 355th position in the canine receptor. Substitutions of these amino acids, while significantly altering antagonist binding, did not affect agonist binding. Interestingly, the rat CCK-B receptor has a valine in position 319, and has affinities for non-peptide antagonists comparable to that of humans. These findings suggest that the residues or conformations which confer antagonist specificity are distinct from those which underlie agonist binding (Beinborn *et al.*, 1993).

Specific regions of the CCK-B receptor which are important in agonist binding have also been identified. The analysis of splice variants in the N-terminal region of the CCK-B receptor revealed that deletions of the N-terminal extracellular region and the upper part of the first transmembrane domain altered agonist binding (Miyake, 1995). In addition, a 5 amino acid sequence in the second intracellular loop was shown to be

essential for the high affinity of the CCK-B receptor for gastrin (Silvente-Poirot and Wank, 1996).

2.3 Pharmacological Characterization of Cholecystokinin Receptors

The differential pharmacology of the two cholecystokinin receptors led to the identification of the CCK-A and CCK-B receptor subtypes. Innis and Snyder (1980) demonstrated that in the brain, gastrin and pentagastrin displayed nanomolar affinity for the CCK receptor, while in the pancreas these peptides were almost inactive. In fact, they showed that gastrin, pentagastrin and CCK-4 were 500-2000 times more potent in the brain than in the pancreas. A number of studies have since confirmed the findings of Innis and Snyder, and have expanded upon their findings. For instance, it has been reported that the CCK-A receptor has a 500-1000 fold higher affinity for sulfated CCK analogues as compared to nonsulfated counterparts, whereas the CCK-B receptor has only a 3-10 fold higher affinity for sulfated peptides. The CCK-A receptor may also be recognized by its high affinity for the synthetic antagonist L-364,718 (devazepide), while the CCK-B receptor has a high affinity for the antagonist L-365,260 (Saito *et al.*, 1980; Moran *et al.*, 1986; Chang and Lotti, 1986; Lotti and Chang, 1989).

The majority of research on CCK-A receptors has been conducted on the rat pancreas. It has been reported that amidation of the cholecystokinin C-terminal phenylalanine is essential to the interaction of the peptide with the CCK-A receptor. CCK peptides with a sulfated tyrosine at the seventh position from the C-terminus show the greatest affinity to the receptor. In an *in vivo* study of rat pancreatic secretion, similar

affinities and potencies were noted for CCK-8s, CCK-33 and CCK-39 (Solomon *et al.*, 1984). The authors suggested that the length of the amino-acid chain beyond the octapeptide does not affect the binding of the cholecystokinin molecule to the pancreatic CCK-A receptor (Solomon *et al.*, 1984). A more recent study, however, has demonstrated that CCK-58 binds more avidly to CCK-A receptors in canine pancreatic acini as compared to CCK-8s, indicating that a stable conformational structure might contribute to enhanced binding of the larger peptide (Keire *et al.*, 1999).

It is important to note that cholecystokinin peptides which are not sulfated at the seventh tyrosine, including CCK-8ns and shorter fragments, also bind at the CCK-A receptor. However, the lack of sulfation results in a marked reduction of affinity; some studies have reported a 1000-fold lower affinity with these fragments (Bonetto *et al.*, 1999; Steigerwalt and Williams, 1981). In a guinea-pig gallbladder contraction bioassay, Bonetto and co-workers (1999) recently demonstrated that sulfated CCK-8 was 150 times more potent than nonsulfated CCK-8; sulfated CCK-33 was 100 times more potent than nonsulfated CCK-33; and sulfated CCK-58 was 35 times more potent than nonsulfated CCK-58.

Gastrin peptides are also capable of binding at the CCK-A receptor. This is not surprising considering the structural similarity between CCK and gastrin, including the presence of an identical pentapeptide C-terminal amino acid sequence in both molecules. However, gastrin molecules bind to the CCK-A receptor with a 500-fold lower affinity than sulfated cholecystokinins. This is consistent with the finding that a sulfated tyrosine at the seventh position from the C-terminus confers CCK peptides with high affinity.

Unlike CCK peptides, the affinity of sulfated gastrins is only slightly greater than their nonsulfated counterparts (Steigerwalt and Williams, 1981).

In contrast to the CCK-A receptor, the CCK-B receptor displays high affinity for gastrin. In fact, the CCK-B receptor has a 300-fold higher affinity for gastrin than does the CCK-A receptor. Site-directed mutagenesis revealed that a 5 amino acid sequence in the second extracellular loop of the CCK-B receptor conferred the majority of selectivity for gastrin. Interestingly, this particular alteration of the sequence did not affect L-365,260 antagonist binding, suggesting that different regions are responsible for agonist and antagonist specificity (Silvente-Poirot and Wank, 1996).

The CCK-A and CCK-B receptors are undoubtedly pharmacologically heterogeneous; however, each receptor also displays differential affinities for agonist binding among different species. For instance, in the mouse and rat, CCK-4 has been reported as being 5-20 fold weaker than CCK-8s at the CCK-B receptor; in the guinea pig and hamster, CCK-4 is 200-500 weaker than CCK-8s (Williams *et al.*, 1986; Steigerwalt and Williams, 1981).

Both CCK-A and CCK-B receptors exist in multiple affinity states with respect to CCK-8s binding. High, low and very low affinity states were demonstrated in COS cells transfected with either CCK-A or CCK-B receptors, as well as in rat and guinea pig pancreatic acini (Huang *et al.*, 1994; Pandya *et al.*, 1994; Yu *et al.*, 1990). Because multiple affinity states are a common feature of G-protein coupled receptors, it is likely that the binding of GTP versus GDP to guanine-nucleotide binding regulatory proteins (G-proteins) regulates the conversion between the high-affinity and low-affinity state of

cholecystokinin receptors. The discovery of these different affinity states has led to the speculation that multiple subtypes of the CCK-A and CCK-B receptor exist. However, genomic and cDNA library hybridization, as well as Northern and Southern hybridization studies with rat, guinea pig and human species did not uncover evidence of any other putative CCK receptors (Wank *et al.*, 1994), and thus far only two genes —the CCK-A and CCK-B receptor genes— have been cloned (Noble *et al.*, 1999).

2.4 Anatomical Localization of Cholecystokinin Receptors

Peripheral Distribution of Cholecystokinin Receptors

CCK-A receptors are widely distributed throughout the mammalian gastrointestinal tract. As one of the major physiological functions of cholecystokinin is the induction of gallbladder contraction (Jorpes and Mutt, 1966), it is not surprising that cholecystokinin receptors have been localized to this organ. CCK-A receptors have been identified in the gallbladder of the guinea-pig (von Schrenck *et al.*, 1988), dog (Sonobe *et al.*, 1995), cow (Steigerwalt *et al.*, 1984), and man (Reubi *et al.*, 1997).

The pancreas, another major target organ of cholecystokinin, also contains numerous CCK-A receptors. CCK-A receptors are mainly found on pancreatic ß cells and are involved in the stimulation of pancreatic enzyme release (Sakamoto *et al.*, 1985). The pancreas of the rat (Hadjiivanova *et al.*, 1992), guinea-pig (von Schrenck *et al.*, 1988), pig (Philippe *et al.*, 1997) and man (Monstein *et al.*, 1996) have all been shown to contain CCK-A receptors. A comparative study of CCK-A receptors from the guinea pig

gallbladder muscle and pancreatic acinar cells revealed that the two tissues possessed pharmacologically identical CCK-A receptors (von Schrenck *et al.*, 1988).

Cholecystokinin has an important regulatory role in the stomach, including the modulation of gastric acid secretion and gastric emptying (Valenzuela and Defilippi, 1981; Sandvick and Wandum, 1991). CCK-A receptors have been localized throughout the guinea pig (Motomura *et al.*, 1997), canine (Mantyh *et al.*, 1994) and human (Reubi *et al.*, 1997) gastrointestinal tract.

The CCK-A receptor subtype predominates in the gastrointestinal tract; however, recent localization studies have reported the presence of CCK-B receptors in this region. Although the rodent pancreas is devoid of CCK-B receptors (Hadjiivanova *et al.*, 1992), the presence of CCK-B receptors has been demonstrated in the pancreas of the dog (Fourmy *et al.*, 1984), pig (Philippe *et al.*, 1997), cow (Le Meuth *et al.*, 1993), and man (Tang *et al.*, 1996). In the stomach, CCK-B receptors have been identified in rodent gastric mucosa, particularly on chief cells and parietal cells, where they modulate gastric acid secretion (Sandvick and Wandum, 1991). CCK-B receptors have also been localized to the gastric mucosa of the guinea-pig (Helander *et al.*, 1997), canine (Mantyh *et al.*, 1994) and human gastrointestinal tract (Reubi *et al.*, 1997).

In addition to the gastrointestinal tract, CCK-B receptors have been found in cells of the immune system. The human T Jurkat cell line, a model of T lymphocytes (Dornand *et al.*, 1995), and human monocytes (Sacerdote *et al.*, 1991) both express CCK-B receptors.

A number of cancer cell lines also express cholecystokinin receptors. CCK-B receptors have been found in medullary thyroid carcinomas, small cell lung cancers, astrocytomas and occasionally in stromal ovarian cancers, gastroenteropancreatic tumors, and breast, endometrial, and ovarian adenocarcinomas. CCK-A receptors have also been localized to cancerous tumors, though with lesser frequency than CCK-B receptors. The presence of CCK-A receptors has been detected in gastroenteropancreatic tumors, meningiomas and neuroblastomas (Reubi *et al.*, 1996; Reubi *et al.*, 1997a).

Distribution of Cholecystokinin Receptors in the Central Nervous System

Cholecystokinin binding sites, as with cholecystokinin peptides, are widely distributed throughout the mammalian central nervous system. Though the majority of this binding is represented by CCK-B receptors, some central regions also possess CCK-A receptors.

Though CCK-A receptors are primary distributed in the periphery, CCK-A receptors have been found in the central nervous system. In the rodent brain, CCK-A receptors have been localized to a small number of regions in the area postrema and nucleus tractus solitarus (Hill *et al.*, 1987). CCK-A receptor binding has been detected in the bovine brain, particularly in the nucleus accumbens, striatum, cortex, cerebellum, hippocampus and brainstem; however, the majority of receptors are of the CCK-B subtype (Barret *et al.*, 1989; Morency *et al.*, 1994). In the monkey brain, CCK-A receptors seem to be more widely distributed. CCK-A binding sites have been found in

the substantia nigra, ventral tegmental area, hypothalamic nuclei, caudate nucleus, ventral putamen and spinal cord (Hill *et al.*, 1990).

A recent investigation into the distribution of cholecystokinin receptors in the rodent central nervous system has uncovered a more widespread distribution of CCK-A receptors than previously suspected. Mercer and Beart (1997) discovered that CCK-A receptors were widely distributed in the rat forebrain, including the nucleus accumbens, septum, stria terminalis, habenula, substantia nigra, ventral tegmental area and lateral geniculate nucleus. CCK-A receptors were particularly concentrated in the rat medulla and spinal cord. In this particular study, CCK-A receptors were visualized by immunohistochemical studies (Mercer and Beart, 1997). It is important to note, however, that immunohistochemistry may be more sensitive than standard receptor autoradiography, a common technique employed by previous studies which examined receptor distribution. This may explain the detection of a higher number of CCK-A receptors in this study, as compared to its predecessors. It is important to note, however, that immunohistochemistry detects receptor protein, functional or not, while binding experiments only detect receptors in conformations capable of binding ligand.

CCK-B receptors are widely distributed throughout the mammalian central nervous system. A number of studies have reported the presence of CCK-B receptors in rodent (Woodruff *et al.*, 1991), guinea-pig (Innis and Snyder, 1980), hamster (Miceli and Steiner, 1989), bovine (Morency *et al.*, 1994), monkey (Mercer *et al.*, 1996) and human brain (Kinze *et al.*, 1996). In a recent investigation of post-mortem human brain tissue, Kinze and co-workers reported a high and homogeneous CCK-B receptor density in the

cerebral cortex. The highest levels of CCK-B binding were detected in the caudate nucleus, cortex parietalis and putamen. Low levels of CCK-B binding sites were found in the hippocampus. CCK-B receptor binding was undetectable in the thalamus and corpus callosum (Kinze *et al.*, 1996).

In a study designed specifically to compare cholecystokinin binding differences among different species, it was demonstrated that the rat and mouse brain possessed high concentrations of CCK-B receptors in the cerebral cortex, olfactory bulb, caudate, hypothalamus and hippocampus. The guinea pig also showed the highest density of CCK-B receptors in the cerebral cortex. In contrast to other species, the guinea pig possessed a high density of cholecystokinin receptors in the cerebellum (Williams *et al.*, 1986). In another comparative study, Morency and colleagues compared their findings on CCK receptor distribution in the bovine brain with other species. While the overall distribution of CCK-B receptors was similar to that of other mammalian brains, differences were observed in the cellular distribution of CCK binding within particular structures, such as in laminae of the cortex and specific regions of the hippocampus (Morency *et al.*, 1994).

Species differences have also been noted in the spinal cord. In the rat spinal cord, autoradiographic studies have revealed the predominance of CCK-B receptors. In contrast, a preponderance of CCK-A receptors was found in monkey and human spinal cord (Hill *et al.*, 1988; Woodruff *et al.*, 1991).

Recent research by Lena and colleagues (1997) has revealed the probable existence of two different CCK-B receptor subsites in the anterior nucleus accumbens.

These receptor subsites, designated as CCK-B_1 and CCK-B_2, were characterized by their different avidities for various agonists. The investigators used BC 197 [c(Boc-D.Asp-Tyr(SO_3H)-Nle-D.Lys)-Trp-Nle-Asp-Phe-NH_2] which is highly selective for the CCK-B_1 subsite, and BC 264 [Boc-Tyr(SO_3H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH_2] which possesses the same affinity for both the CCK-B_1 and CCK-B_2 subsites. Stimulation of the two different subsites was reported to exert opposite effects on dopamine release in the rodent nucleus accumbens (Lena *et al.*, 1997).

In summary, the cholecystokinin receptors, CCK-A and CCK-B, are widely distributed throughout the periphery and central nervous system. Like cholecystokinin peptides, the distribution of CCK receptors are tissue- and species-dependent. Evidence that cholecystokinin peptides and receptors are concentrated in the gastrointestinal tract, brain and spinal cord are consistent with findings which suggest that the cholecystokinergic system plays an important role in gallbladder contraction, pancreatic enzyme release, satiety, memory, anxiety and analgesia .

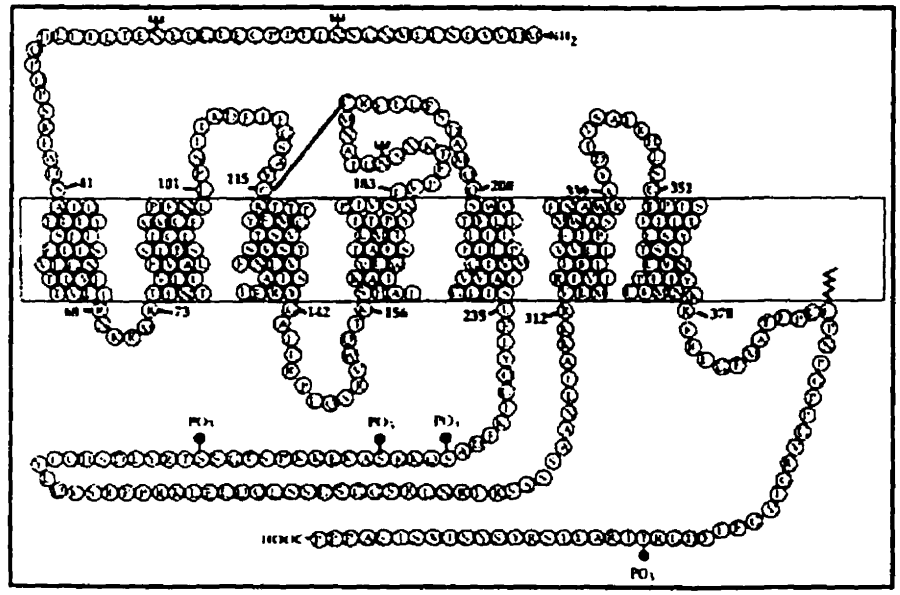

Figure 2. Diagram of the rat CCK-A receptor showing the postulated transmembrane topology, sites for putative NH_2-linked glycosylation, serine and threonine phosphorylation by PKC and protein kinase A (PO_3).
NH_2—N terminus; COOH—C terminus. Adapted from Noble *et al.*, 1999.

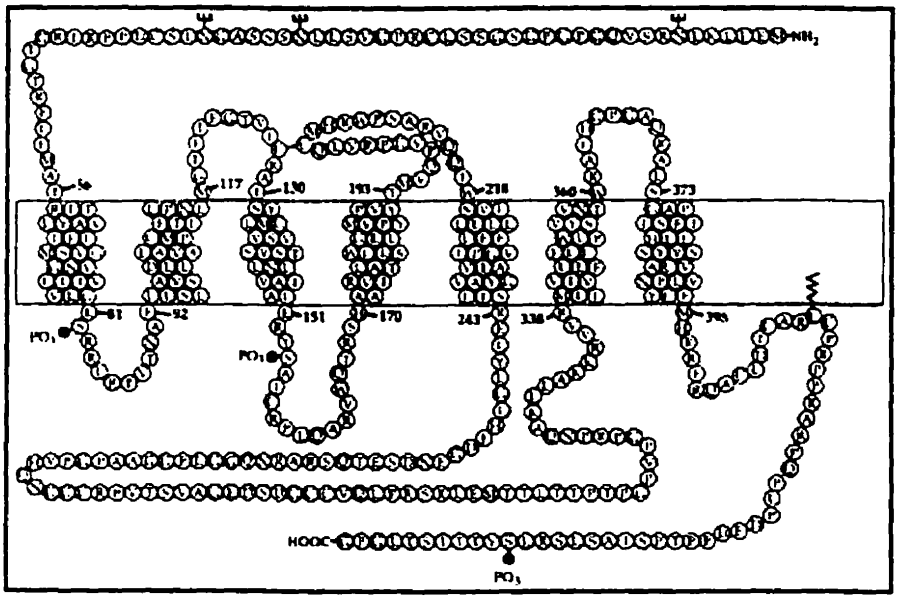

Figure 3. Diagram of the rat CCK-B receptor showing the postulated transmembrane topology, sites for putative NH_2-linked glycosylation, serine and threonine phosphorylation by PKC and protein kinase A (PO_3).
NH_2—N terminus; COOH—C terminus. Adapted from Noble *et al.*, 1999.

CHAPTER THREE

MECHANISMS OF CHOLECYSTOKININ RELEASE

CHAPTER THREE

MECHANISMS OF CHOLECYSTOKININ RELEASE

3.1 Cholecystokinin Release in the Gastrointestinal Tract

There has been considerable investigation into the modulation of cholecystokinin release in the gastrointestinal tract by various dietary factors, such as amino acids, proteins, fats and carbohydrates. CCK-like immunoreactivity in plasma is used as general measure of postprandial CCK stimulation, as plasma cholecystokinin is mainly derived from endocrine cells in the intestinal mucosa (Buchan *et al.*, 1978; Rehfeld, 1978). In contrast to the elevation of postprandial CCK observed in plasma, cholecystokinin levels in cerebrospinal fluid do not seem to be affected after eating (Geracioti *et al.*, 1993). A number of studies have shown that circulating levels of CCK are increased upon ingestion of a mixed-meal (Himeno *et al.*, 1983; Höcker *et al.*, 1992; Liddle *et al.*, 1985; Merani *et al.*, unpublished results, Fig. 12). In addition, the influence of individual dietary components on CCK release has been examined, including free amino acids, protein, fat and carbohydrates. In humans, cholecystokinin concentrations are reported to increase 2-8 fold over baseline fasted values, depending on the nutritional composition of the ingested meal. In general, CCK levels peak 10-45 minutes after the initiation of feeding, and remain elevated for up to 5 hours after eating (Cantor, 1986; Liddle *et al.*, 1985; Walsh *et al.*, 1982). The individual effects of free amino acids, protein, fat, carbohydrates, alcohol and bile on cholecystokinin release are discussed

below. The effect of caffeine on cholecystokinin levels is discussed in Chapter Nine, Section 9.4.

Free Amino Acids

The ability of free amino acids to stimulate cholecystokinin release is dependent upon the particular amino acid examined and its isoform. Species differences have also been observed (Backus *et al.*, 1995. Liddle *et al.*, 1986; Sharara *et al.*, 1993). In the human gastrointestinal tract, L-isomers of amino acids, particularly phenylalanine and tryptophan, are potent releasers of CCK. Oral administration of a mixture of L-amino acids has been shown to induce plasma cholecystokinin release (Liddle *et al.*, 1985). Another study reported that L-phenylalanine alone could increase plasma levels of CCK in human subjects. No significant increase in plasma CCK was observed following D-phenylalanine ingestion (Ballinger and Clark, 1994). Intraduodenal infusion of an amino acid mixture increased CCK levels in plasma (Himeno *et al.*, 1983). A moderate, but significant increase in plasma CCK has also been observed in the cat after oral administration of amino acids (Backus *et al.*, 1995). In contrast to felines and humans, ingestion of free amino acids does not effectively stimulate CCK in rats (Liddle *et al.*, 1986; Sharara *et al.*, 1993). These findings suggest that in some species, free amino acids can stimulate plasma cholecystokinin release, but the extent of this release varies with the type of amino acid, the particular isoform and the species.

Protein, Fat and Carbohydrates

In humans, the ingestion of protein has been confirmed as a potent stimulant of plasma cholecystokinin release (Liddle *et al.*, 1985). Moreover, ingestion of protein (50 grams) or fat (50 grams) exerted similar effects on CCK release, indicating that, in man, protein and fat may be equally effective as stimulators of cholecystokinin release (Hopman *et al.*, 1985). Augmentation of plasma CCK has been observed in the cat after oral administration of casein and whey protein (Backus *et al.*, 1995). Intact protein is the primary dietary stimulant of CCK release in the rat (Liddle *et al.*, 1986; Sharara *et al.*, 1993).

In humans, fat is another potent stimulator of CCK release. Intraduodenal infusion of oleate (Walsh *et al.*, 1982), soybean oil (Himeno *et al.*, 1983) and free fatty acids (Guimbaud *et al.*, 1997) increased CCK levels in human plasma. Oral fat, in the form of oleic and linoleic acids (Liddle *et al.*, 1985), or soybean oil (Hopman *et al.*, 1985) has been shown to stimulate plasma cholecystokinin release. Our own work has shown that the ingestion of a caffeinated milkshake, containing 25 grams of fat, caused a 4-8 fold increase in basal plasma cholecystokinin levels in healthy volunteers (Merani *et al.*, unpublished results, Chapter Nine, Section 9.4). In cats, oral ingestion of corn oil has been shown to induce CCK release (Backus *et al.*, 1995). Conversely, in rats, fat is a weak stimulant of cholecystokinin release (Liddle *et al.*, 1986; Sharara *et al.*, 1993).

In general, carbohydrates appear to be weaker stimulants of cholecystokinin release, as compared to fat and protein. In humans, oral glucose has been shown to affect plasma CCK in a dose-dependent manner. Low to moderate doses of glucose of 50-60

grams caused insignificant (Liddle *et al.*, 1988) or modest increases in plasma CCK (Hasegawa *et al.*, 1996); whereas higher doses of 75-100 grams glucose induced a greater response (Liddle *et al.*, 1985; Hasegawa *et al.*, 1996). Ingestion of starch has been shown to be virtually ineffective in releasing CCK (Hopman *et al.*, 1985). In rats, glucose was unable to stimulate cholecystokinin release (Sharara *et al.*, 1993).

As with free amino acids, the extent to which fat, protein and carbohydrates increase cholecystokinin levels depends on a number of factors, including the quantity and composition of the nutrient. In addition, cholecystokinin release by nutrients is also highly species dependent.

Alcohol

A number of studies have reported an interaction between cholecystokinin and alcohol (Carr *et al.*, 1993; Harro *et al.*, 1994; Weatherford *et al.*, 1993). For instance, CCK octapeptide has been shown to have an inhibitory effect on ethanol intake (Kulkosky *et al.*, 1989). These findings, combined with the discovery that both cholecystokinin and alcohol administration can induce pancreatitis in rats and in humans (Saluja *et al.*, 1997), instigated research on the effects of alcohol on CCK levels. Oral ethanol induced an increase in postprandial CCK secretion in patients with chronic pancreatitis, but not in healthy volunteers, suggesting that an interaction between CCK and ethanol may play a role in the pathology of this illness (Hirano *et al.*, 1996). Other studies have also found ethanol to be ineffective in stimulating cholecystokinin release in

humans (Chari *et al.*, 1996; Fried *et al.*, 1984). Studies on cholecystokinin release in rodents have produced conflicting results. For example, Liddle and co-workers (1984) found that intragastric instillation of ethanol to rodents resulted in a 15-fold elevation of plasma cholecystokinin levels. In comparison, Saluja *et al.*, (1997) reported less-pronounced effects following intravenous administration of ethanol; Yamada and colleagues (1998) determined that daily ingestion of ethanol for twelve days was without effects on plasma CCK concentration. Although a comprehensive review of the methodology of these studies is beyond the scope of this dissertation, it is likely that the confounding results are the result of differences in the dose of ethanol administered, the time-course of ethanol treatment and the manner by which ethanol was administered. Further, the methods by which researchers opt to measure cholecystokinin concentrations is frequently a cause of divergent results, as discussed in Chapter Six, Section 6.1 – 6.3.

Bile

Bile is thought to have an tonic inhibitory effect on both basal and meal-stimulated cholecystokinin release (Koop *et al.*, 1996). In most species, cholecystokinin release is inhibited in the presence of, and enhanced in the absence of, luminal bile acids (Koop, 1990). Administration of cholestyramine, a bile-salt binding agent, to canines has been shown to enhance CCK secretion (Gomez *et al.*, 1988). Oral cholestyramine also increased cholecystokinin levels in human subjects (Koop *et al.*, 1988). In rats, interruption of bile flow into the duodenum enhanced plasma CCK levels (Ohta *et al.*,

1990), as well as CCK concentrations in the intestinal mucosa (Miyasaka *et al.*, 1993). Likewise, chronic bile diversion increased fasted and fat-stimulated CCK levels in dogs, and oral administration bile attenuated fat-stimulated release of CCK (Takahashi *et al.*, 1996). In humans, basal and postprandial plasma cholecystokinin concentrations were augmented when bile was excluded from the intestine, while normal physiological bile flow normalized CCK values (Koide *et al.*, 1993). Taken in concert, these results suggest that endogenous bile salts can modulate the release of intestinal CCK.

Cholecystokinin Release: Summary

Cholecystokinin is a well-established and important mediator in gastrointestinal physiology. In rodents, intact protein is the primary dietary stimulant of CCK release. In humans, amino acids such as phenylalanine and tryptophan, intact protein and fat constitute potent dietary stimulants of CCK release. Carbohydrates can also induce cholecystokinin release, but to a lesser degree than other nutrients. Bile is thought to have an important regulatory role in the tonic inhibition of endogenous CCK, while the effects of alcohol are more tenuous. Although cholecystokinin is a potent stimulator of postprandial gallbladder contraction and pancreatic enzyme release, the precise mechanisms by which nutrients induce CCK release are not yet fully understood (Liddle, 1997).

3.2 CCK Release in the Central Nervous System

Depolarization

A number of studies have demonstrated the neuronal release of CCK-like immunoreactivity upon chemical and electrical depolarization. Cholecystokinin release evoked by depolarization has been reported to consist primarily of the sulfated octapeptide (Maidment *et al.*, 1991; Vallebuona *et al.*, 1993). Both *in vitro* and *in vivo* experiments have revealed that depolarizing stimuli capable of inducing CCK release include potassium, veratridine, 4-aminopyridine and electrical stimulation.

Potassium, which causes membrane depolarization by opening voltage dependent calcium channels, has been shown to evoke cholecystokinin release in a dose-dependent manner. Potassium-induced release of CCK has been observed in synaptosomal preparations from various brain regions. In synaptosomal fractions from the rat cerebral cortex, CCK was released after exposure to 15, 25 and 35 mM KCl. The introduction of a calcium-free medium significantly attenuated this release. This calcium-dependent release of CCK induced by potassium depolarization was also observed in the rat nucleus accumbens (Paudice and Raiteri, 1991). Using a transcerebral microdialysis technique, Vallebuona and colleagues (1993) performed extensive examination of depolarization-evoked release of CCK in vivo. The infusion of 75 mM and 100 mM potassium elicited a calcium dependent CCK overflow, as evaluated through a microdialysis probe inserted into the frontal cortex of freely moving, unrestrained rats. The release of cholecystokinin

by potassium has also been demonstrated by the infusion of 40 mM KCl into the rat spinal column (Yaksh *et al.*, 1982).

Veratridine causes depolarization by trapping tetrodotoxin (TTX)-sensitive sodium channels in the open state. Transcerebral microdialysis studies revealed that infusion of veratrine evoked calcium dependent CCK release in the rat frontal cortex. Interestingly, a second veratrine infusion within 200 minutes of the first application was unable to stimulate CCK release. This finding suggested that a single dose of veratrine could deplete the releasable pool of CCK and that more than 200 minutes are needed to replenish the vesicles containing CCK (Vallebuona *et al.*, 1993).

The drug 4-aminopyridine blocks potassium channels and opens TTX-sensitive sodium channels. The addition of 4-aminopyridine to rodent synaptosomal fractions evoked release of cholecystokinin (Verhage *et al.*, 1991). In addition, Vallebuona and co-workers (1993) applied their *in vivo* microdialysis method to show that 4-aminopyridine evoked a calcium dependent CCK release in the rat frontal cortex. The *in vivo* release of cholecystokinin has also been documented in the rat cerebral cortex. Analysis of cortical perfusate revealed that electrical stimulation (20 Hz) elevated CCK levels in anesthetized rats (Yaksh *et al.*, 1987).

Stress

The involvement of cholecystokinin in stress and anxiety is well-established. Several studies have demonstrated that stressful stimuli stimulate cholecystokinin release

in the central nervous system. By employing various behavioural paradigms, central CCK levels have been analyzed in rats subjected to anxiety-provoking conditions. The precise functional relationship between the central cholecystokinergic system and anxiety remains unclear. The evaluation of cholecystokinin levels in animal models of anxiety has been an important tool in examining the interplay between CCK and the stress-response. The following is an overview of the various types of stressors which can elicit cholecystokinin release in the central nervous system. The specific alterations of the cholecystokinergic system in anxiety is explored in Chapter Nine.

In a novel study by Pavlasevic and colleagues (1993), rats were exposed to a cloth which harboured the scent of a male cat. The researchers observed that the predatory scent caused the rats to adopt a freezing posture, which is indicative of anxiety. After a 15 minute exposure to the "cat-cloth", the rats were decapitated and cholecystokinin levels were measured. CCK-4 levels were increased in the olfactory bulb, frontal cortex, central cortex, dorsal striatum, ventral striatum, central amygdala and the nucleus of the solitary tract in the stressed rats, as compared to tetrapeptide levels in non-exposed rats. CCK-8 levels were also increased, but only in the ventral striatum. These findings suggest a differential release of CCK peptides in this particular animal model of anxiety. The authors speculated that a central release of cholecystokinin peptides is implicated in this model, as CCK-4 concentrations were below the limits of detection in plasma from control rats, as well as in plasma from animals exposed to the cat-cloth. Moreover, plasma levels of CCK-8 did not differ in rats subjected to the cat-cloth (Pavlasevic *et al.*, 1993).

The *in vivo* release of cholecystokinin has been assessed in the frontal cortex of freely moving rats exposed to various stressful stimuli. Using microdialysis, CCK-like immunoreactivity was measured in animals subjected to physical restraint. After 30 minutes of restraint, CCK-LI, of which approximately 70% was CCK-8s, was increased in these rats. Cholecystokinin levels were also elevated when a 2 minute exposure to diethyl ether was employed as the stressful stimuli (Nevo *et al.*, 1996). Short-term restraint stress has also been shown to stimulate CCK release. CCK-like immunoreactivity was increased in the periaqueductal grey of rats restrained for 1-minute (Rosen *et al.*, 1992).

Stress-induced release of cholecystokinin has been observed in rats exposed to acute shock. Animals were subjected to foot shock stress and immediately decapitated to permit CCK determination. Elevated concentrations of CCK were observed in the hypothalamic posterior arcuate nucleus following 60 minutes of exposure to the stressor (Siegal *et al.*, 1987).

In 1989, scientists discovered that cholecystokinin produced anxiety and panic-like behaviour in humans (de Montigny,1989). In the past two decades, a number of researchers, pioneered by Bradwejn and colleagues, have conducted dozens of innovative studies that involve the injection of CCK-4 to human subjects and the subsequent characterization of the ensuing panicogenic effects of cholecystokinin administration (Bradwejn *et al.*, 1990; 1991; 1992a; 1992b; 1994; Jerabek *et al.*, 1999; Koszycki *et al.*, 1998; Shlik *et al.*, 1997). These experiments, as well as other studies that focus on anxiety and the cholecystokinergic system, are reviewed in Chaper Nine..

CHAPTER FOUR

NEUROTRANSMISSION AND CO-MODULATION

CHAPTER FOUR

NEUROTRANSMISSION AND CO-MODULATION

4.1 Introduction

From its first discovery in the brain in 1975 (Vanderhaeghen *et al.*), cholecystokinin is now established as an important neurotransmitter and neuromodulator in the mammalian central nervous system (Crawley and Corwin, 1994). This is evidenced by a number of studies which report that cholecystokinin is synthesized in neurons (Ingram *et al.*, 1989), and stored in both neuronal cell bodies and nerve terminals (Lindefors *et al.*, 1991). Moreover, as with classical neurotransmitters, CCK is released by cellular depolarization (Raiteri *et al.*, 1993; Vallebuona *et al.*, 1993) and binds to specific receptors in the central nervous system (Innis and Snyder, 1980). Cholecystokinin can modulate the effects of other neurotransmitters (Itoh *et al.*, 1988; Reum *et al.*, 1997), and pharmacological studies have revealed that cholecystokinin antagonists can counteract the actions of CCK (Bradwejn *et al.*, 1992b; Harro and Vasar, 1991). Collectively, these findings validate the assertion that cholecystokinin is a critical neurotransmitter in the central nervous system (Bradwejn *et al.*, 1992a).

The neuronal co-localization of CCK with classical neurotransmitters, such as dopamine, serotonin, noradrenaline and gamma-aminobutyric acid (GABA), as well as endogenous opioids, establishes a fundamental anatomical basis for the functional interactions between these substances. The following is a brief description of each of

these neurochemical systems and their complex relationship with the cholecystokinergic system.

4.2 The Dopaminergic System

Dopamine is a catecholamine neurotransmitter derived from the amino acid tyrosine. The first of two enzymes required for the synthesis of dopamine is tyrosine hydroxylase, an oxidase which converts tyrosine to L-DOPA. Tyrosine hydroxylase is frequently used as a marker to indicate the presence of dopamine, since it is an essential synthetic enzyme. At least six dopamine receptors have been identified to date. These receptors, designated as D_1, D_{2a}, D_{2b}, D_3, D_4 and D_5, are further classified into two major categories based on cDNA cloning and signaling pathways. The D_1-like group includes D_1 and D_5 which result in the increase of adenylyl cyclase activity. The D_2-like receptor class includes $D_{2a,b}$, D_3 and D_4, which result in the inhibition of adenylyl cyclase activity.

The neuronal co-localization of cholecystokinin and dopamine was first demonstrated in the substantia nigra of the rat brain (Hökfelt *et al.*, 1980). Subsequent studies revealed that CCK-LI (Seroogy *et al.*, 1989a) is present in the majority of midbrain dopaminergic neurons. Both CCK mRNA and tyrosine hydroxylase mRNA have also been found in many of the dopamine cells in the substantia nigra (Kiyama *et al.*, 1992; Seroogy *et al.*, 1989b). The demonstration that dopamine and cholecystokinin co-exist in the central nervous system sparked interest in the role of endogenous CCK in the regulation of dopamine.

Effects of Cholecystokinin on the Dopaminergic System

The regulation of dopaminergic neuronal function by cholecystokinin is complex. The effects of CCK on dopamine transmission, various dopamine-mediated behaviours and receptor binding vary considerably depending on which region of the brain is examined, the concentration of CCK used, and the depolarization state of the tissue (Homer *et al.*, 1986; Lena *et al.*, 1997; Vaccarino and Rankin, 1989).

Functional studies have examined the role of CCK-8 on the firing rate of rodent midbrain dopaminergic neurons. The octapeptide form of cholecystokinin is generally used in examining interactions between CCK and other neurotransmitter systems, as it has been thus far been demonstrated that CCK-8s is the predominant form of cholecystokinin in the brain (Beinfeld *et al.*, 1981). Studies which involve direct application of cholecystokinin, i.e via microiontophoretic injection, have revealed that cholecystokinin: increases the firing rate of dopamine neurons in the substantia nigra and ventral tegmental area (Skirboll *et al.*, 1981), increases dopamine release in the substantia nigra via CCK-A receptors (You *et al.*, 1996) and potentiates the D_2 receptor mediated decrease in the firing rate of mesolimbic neurons (Stittsworth and Mueller, 1990).

CCK has also been shown to modulate the affinity of dopamine receptors. Administration of CCK-8, *in vitro* and *ex vivo*, strongly regulated the affinity of dopamine D_2 receptors for dopamine in the rat striatum, implicating a CCK/D_2 receptor-receptor interaction (Li *et al.*, 1995; Ferraro *et al.*, 1996).

The role of cholecystokinin in the regulation of the dopaminergic system has been explored by employing various behavioural paradigms in rats. The behavioural measures

of dopaminergic activity include exploratory locomotion, grooming and sniffing (Crawley *et al.*, 1992). Numerous studies have demonstrated that CCK modulates dopamine-mediated behaviour. Generally, dopamine-mediated responses are enhanced when CCK is injected into the posterior nucleus accumbens and inhibited when CCK is injected into the anterior nucleus accumbens. The posterior nucleus accumbens is innervated by neurons containing both dopamine and CCK, while the anterior nucleus accumbens is innervated separately by dopamine and CCK neurons (Crawley *et al.*, 1985; Vaccarino and Rankin, 1989).

Until recently, it was generally accepted that CCK enhanced potassium-stimulated release of dopamine in the rodent posterior nucleus accumbens via a CCK-A receptor mediated pathway, and inhibited dopamine release in the anterior nucleus accumbens via CCK-B receptors (Crawley and Corwin, 1994). However, more recent studies have demonstrated a CCK-B receptor-mediated *increase* in dopamine in the anterior part of nucleus accumbens (Reum *et al.*, 1997). A recent plenary investigation revealed that the CCK-B receptor agonist, BC 197, potentiated potassium-stimulated dopamine release in the anterior nucleus accumbens. However, another CCK-B agonist, BC 264, inhibited potassium-stimulated dopamine release at low concentrations (nM) and increased dopamine release at high concentrations (μM). These apparently conflicting findings can be reconciled by the existence of distinct CCK-B receptor subsites in the anterior nucleus accumbens which exert opposing effects on potassium-stimulated dopamine release. These receptor subsites, designated as CCK-B_1 and CCK-B_2, have different avidities for

the agonists examined: BC 197 is highly selective for the CCK-B_1 subsite, while BC 264 has the same affinity for both the CCK-B_1 and CCK-B_2 subsites (Lena *et al.*, 1997).

Alterations in the cholecystokinergic system have been implicated in the alteration of dopaminergic function and subsequent development of hypertension, as aberrations in CCK-induced release of dopamine have been noted in the pathogenesis of cardiovascular dysfunction (Kirouac and Ganguly, 1995). These observations are discussed further in Chapter Ten, Section 10.4.

Effects of Dopamine on the Cholecystokinergic System

A reciprocal relationship exists between the cholecystokinergic and dopaminergic systems. CCK has been shown to affect dopamine transmission and likewise, some evidence suggests that dopamine can modulate CCK function. For example, D_2 receptor agonists, including quinpirole LY-141,865 and LY-171,555 have been shown to attenuate the potassium-stimulated release of CCK-8 from rat midbrain slices (Beinfeld *et al.*, 1996).

The dopaminergic system has been shown to regulate the affinity and density of CCK receptors. In post-mortem human brain tissue, dopamine decreased the IC_{50} value of CCK-8s for ^{125}I CCK-8 binding in membranes from the caudate-putamen. However, in the nucleus accumbens, dopamine was ineffective in enhancing CCK receptor affinity binding (von Euler *et al.*, 1992). In the rat frontal cortex, administration of D_2 receptor

antagonists increased the number of CCK binding sites, or Bmax (Fukamauchi *et al.*, 1987).

As with the effects of CCK on dopamine transmission, a complex interaction also underlies the effects of dopamine on CCK release. In the aforementioned study by Beinfeld and colleagues (1986), LY-171,555 decreased the release of CCK at concentrations of 0.1 - 50 nM, but lost its inhibitory capability when used at a concentration of 100 nM. This suggests that D_2 receptor stimulation does indeed mediate CCK release in the posterior accumbens, but in a biphasic manner.

The dopaminergic system has been implicated in the modulation of cholecystokinin messenger RNA expression. In the rat, dopamine depletion caused by lesioning of the nigrostriatal pathway, via 6-hydroxydopamine injection, increased the level of expression of cholecystokinin messenger RNA in the ipsilateral striatum (Schiffmann and Vanderhaeghen, 1992).

In summary, several lines of evidence suggest that the cholecystokinergic and dopaminergic systems interact to regulate normal physiology and pathology. The mechanisms of this interaction, however, are extremely complex and have not yet been fully established.

4.3 The Serotonergic System

Serotonin, or 5-hydroxytryptamine (5-HT), is an indolamine neurotransmitter derived from the amino acid tryptophan. At least seven main types of serotonin receptors have been identified: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7. The 5-HT_1,

5-HT_2, 5-HT_4 and 5-HT_6 receptors are coupled to G-proteins, whereas 5-HT_3 are ligand-gated ion channels. The 5-HT_1 receptor class is further classified into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D}. The 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors are linked to the inhibition of adenylyl cyclase and the 5-HT_{1C} receptor to the stimulation of PI turnover. The co-localization of the cholecystokinergic and serotonergic systems in the brain, as well as the joint mediation by both substances of many physiological and behavioural processes, suggested a putative interaction between CCK and 5-HT in the central nervous system.

Effects of Cholecystokinin on the Serotonergic System

The direct administration of cholecystokinin peptides into the brain has been shown to influence serotonin release and metabolism. Injection of cholecystokinin octapeptide increased the concentration of 5-HT in the rat paraventricular hypothalamic nuclei (Esfahani *et al.*, 1995). Alternatively, intracerebroventricular administration of CCK-4 has been shown to stimulate the metabolism of 5-HT in the rat brain (Itoh *et al.*, 1988).

Much of the evidence to support the hypothesis that both CCK and 5-HT interact in the central nervous system is derived from studies in the field of anxiety research. In general, stimulation of the 5-HT system, as with the cholecystokinergic system, is anxiogenic, while 5-HT and CCK receptor antagonists are anxiolytic. Anxiety is frequently assessed in rodent models by using a variety of behavioural paradigms, including measures of exploration. In these studies, reduced exploratory behaviour is

generally indicative of high levels of anxiety. Stimulation of the serotonergic system by administration of the serotonin reuptake blocker citalopram has been shown to decrease exploration in rats. Interestingly, CCK-B receptor antagonists were able to diminish the anxiogenic effect of citalopram, while CCK-A receptor antagonists were ineffective, suggesting that the 5-HT regulation of anxiety is mediated by a CCK-B receptor-mediated pathway (Matto *et al.*, 1996).

The interaction between CCK and serotonin is well-documented in the mediation of satiety. In general, agents that activate the cholecystokinergic and/or the serotonergic system within certain neuronal pathways increase satiety, and antagonists of these systems evoke a feeding response in rats. There is some evidence to suggest that the co-suppression of CCK and serotonin function may be involved in overeating. For example, Voigt and colleagues (1995) found that CCK-8 administration diminishes the feeding response induced by 8-OH-DPAT (8-hydroxy-2-(di-*n*-propyl-amino)tetralin HBr), a 5-HT_{1A} agonist that reduces central serotonin synthesis and release. Another study reported that antagonists of the CCK-A receptor potentiate the feeding response elicited by raphe injection of 8-OH-DPAT (Currie and Coscina, 1995).

Effects of Serotonin on the Cholecystokinergic System

Administration of various serotonin agonists and antagonists has been shown to directly alter levels of cholecystokinin. Subcutaneous injections of 5-HT subtype-selective receptor agonists was able to modulate plasma CCK levels in rats. The 5-HT_{1A}

receptor agonist 8-OH-DPAT and 5-$HT_{2A/C}$ receptor agonist DOI (2,5-HT-dimethyoxy-4-iodoamphetamine HBr) decreased CCK levels, while the 5-HT_{1B} agonist TFMPP (N-(3-trifluoromethylphenyl) piperazine HCl) increased plasma CCK concentrations (Uvnas-Moberg *et al.*, 1996). Subchronic (14 day) treatment with clomipramine, a 5-HT reuptake inhibitor, increased CCK-like immunoreactivity in the rat ventral tegmental area, cingulate cortex and periaqueductal grey (Brodin *et al.*, 1994).

The discovery that a significant proportion of CCK-containing neurons express the 5-HT_3 receptor in the rat neocortex suggested that serotonin might regulate cholecystokinin neurotransmission via a 5-HT_3 receptor-mediated pathway. In an *in vitro* study of rat brain synaptosomes, 5-HT enhanced the potassium-evoked release of CCK in the cerebral cortex and nucleus accumbens via a 5-HT_3 receptor-mediated mechanism (Paudice and Raiteri, 1991). In vivo experiments using intracerebral microdialysis demonstrated that the 5-HT_3 receptor antagonists, ondansetron and tropisetron, attenuated the depolarization-dependent release of cholecystokinin from the rat frontal cortex (Raiteri *et al.*, 1993).

As previously described, putative CCK and 5-HT interactions have frequently been examined in animal and human models of anxiety. 5-HT receptor antagonists, particularly 5-HT_3 blockers have been shown to be anxiolytic. For example, Vasar and colleagues (1993) found that, in rodents, the anxiogenic effects of the CCK receptor agonist caerulein could be effectively antagonized by ondansetron administration.

In human subjects diagnosed with panic disorder, chronic treatment with imipramine, a serotonin reuptake inhibitor, reduced the number, duration and intensity of

panic symptoms induced by CCK-4 administration (Bradwejn and Koszycki, 1994). This marked reduction in the sensitivity of panic disorder patients to a CCK-4 challenge was replicated in a study using the serotonin reuptake inhibitor fluvoxamine (VanMegan *et al.*, 1997). Acute tryptophan depletion, which decreases central 5-HT concentrations, enhanced ACTH/cortisol and prolactin secretion elicited by CCK-4 administration. These findings implicate the 5-HT system in the modulation of the neuroendocrine effects of CCK-4 (Koszycki *et al.*, 1996). Further evidence for the involvement of the serotonergic system in anxiety comes from treatment studies. Serotonin reuptake inhibitors are commonly prescribed to alleviate symptoms of anxiety (Coplan *et al.*, 1992; Costall and Naylor, 1992).

In general, a reciprocal relationship exists between the cholecystokinergic and serotonergic systems: each system is capable of modulating the release, metabolism and action of the other system. Because the normal functioning and interaction between these systems appear to mediate various behavioural processes, a disruption in this interaction may be involved in the etiology of psychopathological disorders, such as eating disorders and anxiety. Accordingly, it would be prudent for future studies to focus on potential dysfunctions in the relationship between CCK and 5-HT, rather than to examine each system individually.

4.4 The Noradrenergic System

The catecholamine noradrenaline, or norepinephrine, is a neurotransmitter synthesized from the amino acid tyrosine. Noradrenergic pathways originate primarily in

the locus ceruleus and project throughout the cortex, cerebellum and spinal cord. The noradrenergic receptors, β_1, β_2, α_1 and α_2, constitute a superfamily of G-protein-coupled receptors. Both β receptors are positively coupled to adenylyl cyclase, while the α_2 receptor is negatively coupled to adenylyl cyclase. The α_1 receptor exerts its effects through phospholipase C mobilization of intracellular calcium.

Effects of Cholecystokinin on the Noradrenergic System

A limited number of studies have examined the effects of cholecystokinin administration on the noradrenergic system. Cholecystokinin has been documented to both potentiate and inhibit noradrenaline transmission in the central nervous system. In the supraoptic nucleus of the rat, intravenous injection of CCK enhanced the release of noradrenaline (Onaka *et al.*, 1995). Intravenous administration of CCK-8 also induced extracellular noradrenaline release in the paraventricular nucleus (Ueta *et al.*, 1993). Modulatory effects of cholecystokinin with respect to noradrenaline were investigated by examining the effect of CCK on potassium-evoked noradrenaline release from hypothalamic slices. CCK-8 was able to inhibit noradrenaline release, suggesting that CCK might have an inhibitory role in noradrenergic neurotransmission in the hypothalamus (Beresford *et al.*, 1988).

Recently, Jerabek and colleagues (1999) investigated the effects of CCK4 administration on the catecholaminergic system in human subjects. The researchers injected either CCK4 or placebo to sixteen healthy subjects on two separate occasions

and found that plasma noradrenaline and adrenaline were significantly elevated above baseline levels in the immediate post-CCK4 injection period.

Effects of Noradrenaline on the Cholecystokinergic System

Several lines of evidence suggest that the noradrenergic system might modulate cholecystokinergic function. For example, intraperitoneal administration of DSP-4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine], a neurotoxin that specifically destroys noradrenergic nerve terminals, showed parallel reduction of noradrenaline and elevation CCK receptor density in the rat hippocampus and frontal cortex (Harro *et al.*, 1992). Another study found increased levels of procholecystokinin mRNA in human neuroblastoma cell cells treated with noradrenaline or the β-adrenoceptor agonist isoproterenol, suggesting that expression of proCCK may be regulated by noradrenergic mechanisms (Monstein *et al.*, 1990). Finally, Nevo and colleagues (1996) reported that intraperitoneal administration of yohimbine, an α2 antagonist which acts at presynaptic autoreceptors to increase noradrenaline release, increased the release of CCK from cortical cholecystokinergic neurons within the rat frontal cortex. Interestingly, yohimbine has been documented to increase fear and anxiety in patients with panic disorder (Charney *et al.*, 1984; 1992). Taken together, these experiments provide provocative, though incomplete, evidence of regulation of the expression of CCK and CCK receptors by norepinephrine.

The panicogenic symptoms that are induced by CCK-4 injection are reported to be qualitatively and quantitatively similar to those evoked by CO_2 inhalation (Bradwejn and Koszycki, 1991; Koszycki *et al.*, 1991). This finding led to the speculation that the activation of the noradrenergic system may underlie the panicogenic potential of the two agents (Hamon, 1994). This is in line with theories that assert that central noradrenergic dysfunction is involved in fear and anxiety. Recently, Le Mellédo and co-workers (1998) demonstrated that pre-treatment with propranolol attenuated the panicogenic effects of CCK-4 injections in healthy volunteers.

A number of studies implicate an interaction between the cholecystokinergic and noradrenergic systems in fear and anxiety. Confirmation of this hypothesis, through further investigation, would facilitate the development of preventative and therapeutic strategies, which presumably might have target both systems simultaneously in order to be effective.

4.5 The GABAergic System

Gamma-aminobutyric acid, or GABA, is a major inhibitory neurotransmitter in the central nervous system. A primary function of GABA is to inhibit neurotransmitter release from axons via GABA receptor stimulation. GABA receptors are classified into two types: $GABA_A$ and $GABA_B$. $GABA_A$ receptors comprise a ligand-gated ion channel which, when activated, mediate post-synaptic inhibition by opening chloride channels. The consequent chloride influx initiates hyperpolarization and inhibits target cells. $GABA_B$ receptors inhibit neurotransmitter release by opening post-synaptic potassium

channels and blockade of pre-synaptic calcium channels via a G-protein coupled mechanism. Immunocytochemical studies have revealed that CCK and GABA are co-localized in cerebral cortical (Hendry *et al.*, 1984) and hippocampal neurons (Somogyi *et al.*, 1984).

Effects of Cholecystokinin on the Gabaergic System

CCK has been shown to facilitate *in vitro* striatal GABA release via a CCK-B receptor mediated mechanism (Pérez de la Mora *et al.*, 1993). In the rodent cerebral cortex and striatum, application of CCK octapeptide caused a dose-dependent increase in both basal and electrically-evoked release of GABA via the activation of CCK-B receptors located on GABAergic neurons (de Belleroche and Bandopadhyay, 1992; (Rakovska, 1995). Ferraro and co-workers demonstrated that local perfusion with CCK octapeptide caused an increase in GABA release in the central accumbens of the rat. However, the perfusion of CCK, together with a CCK-B antagonist, diminished this release. Further, the attenuation of GABA release was inhibited by the addition of a CCK-A antagonist. Accordingly, the authors speculated that, in the accumbens, cholecystokinin modulates GABA release by two different mechanisms: a dominant CCK-B receptor mediated excitatory effect and a secondary CCK-A receptor mediated inhibitory effect on GABA release. Accordingly, the CCK-induced increase of GABA release is mediated by CCK-B receptors — when these receptors are antagonized, the inhibitory effect of CCK-A receptors are unmasked (Ferraro *et al.*, 1996).

Effects of the Gabaergic System on the Cholecystokinergic System

It has been postulated that cholecystokinin release is under tonic control by the GABAergic system (Yaksh *et al.*, 1987). Potassium-evoked release of CCK can be inhibited by stimulation of $GABA_B$ receptors in the rat neostriatum (Conzelmann *et al.*, 1986). A typical $GABA_B$ agonist, baclofen, has been shown to diminish the inhibitory effect of cholecystokinin on food intake in rats (Ebenezer, 1996). This finding suggests that a $GABA_B$-receptor mediated mechanism may underlie the suppressant effect of exogenous CCK on food intake. Interestingly, baclofen has also been reported to possess anxiolytic properties (Andrews and File, 1993), leading to the speculation that the $GABA_B$ system may modulate the anxiogenic effects of CCK. Studies have revealed that baclofen can inhibit CCK release from rat striatum and spinal cord (Benoliel *et al.*, 1992). Baclofen also attenuated the release of CCK evoked by depolarization in human synaptosomes. Thus, it has been proposed that CCK-releasing nerve terminals possess $GABA_B$ receptors that mediate the inhibition of depolarization-evoked CCK release. However, the mechanisms by which these presynaptic $GABA_B$ heteroreceptors modulate CCK release remain unclear (Raiteri *et al.*, 1996).

Effects of GABA on Cholecystokinin: Indirect Evidence from Benzodiazepine Studies

Benzodiazepines constitute an effective and frequently prescribed class of drugs in the treatment of anxiety disorders. Benzodiazepines exert their therapeutic anxiolytic effects by directly enhancing GABAergic neural inhibition. These anti-anxiety drugs increase the affinity of the $GABA_A$ receptor for GABA, thus enhancing chloride conductance and hyperpolarization

A ground-breaking study of the effects of benzodiazepines on CCK-induced excitation of rat hippocampal pyramidal neurons instigated further neurobiological investigation into the role of CCK in anxiety. In 1984, Bradwejn and de Montigny demonstrated that both intravenous and microiontophoretic administration of benzodiazepines (diazepam, chlorodiazepoxide, lorazepam and flurazepam) antagonized CCK-8s induced neuronal activation. This novel discovery that CCK could induce neuronal excitation which in turn could be blocked by anxiolytic drugs indicated a putative role of CCK in anxiogenesis.

Further research on the interaction of benzodiazepines and CCK revealed that chronic treatment with benzodiazepines in rats reduced hippocampal neuronal reactivity to CCK (Bouthillier and de Montigny, 1988). Diazepam has been shown to prevent stress-induced enhancement of cholecystokinergic neurotransmission in the rodent frontal cortex, suggesting that the anxiolytic action of benzodiazepines may be partially due to their inhibitory effects on CCK cortical neurons (Nevo *et al.*, 1996).

These findings suggest that the GABA-benzodiazepine receptor complex has a modulatory effect on CCK in the central nervous system, though the exact mechanisms involved have not yet been established.

4.6 The Opioidergic System

There are three main classes of endogenous opioids: the enkephalins, the proopiomelanocortin (POMC) family and the dynorphins. Each family consists of a precursor molecule which gives rise to a number of smaller active fragments. The proenkephalin molecule generates met- and leu-enkephalin. The POMC precursor gives rise to various peptides, including beta-endorphin. Prodynorphin generates neoendorphin and dynorphin. Opioid peptides, as well as opiates such as morphine, bind to different subtypes of opiate receptors. There are three major receptor classes: mu, delta and kappa. Anatomical distribution of CCK and CCK receptors are similar to the pattern observed for endogenous opioids in the central nervous system (Gall *et al.*, 1987; Stengaard-Pedersen *et al.*, 1981). A recent study has reported that mu-opioid receptor-like immunoreactivity is present in approximately 66% of cholecystokinin-positive neurons in laminae I and II of rat spinal cord, and that 40.4% of mu-opioid receptor-positive neurons contain CCK-like immunoreactivity (Zhang *et al.*, 2000).

Generally, opposing functional effects have been noted for CCK and opioids. Studies have shown that CCK and opioids behave antagonistically in analgesia (Kellstein and Mayer, 1992), satiety (Bray, 1993), thermoregulation (Kapas *et al.*, 1989) and respiration (Gillis *et al.*, 1983).

Effects of Cholecystokinin on the Opioidergic System

The effect of cholecystokinin on opioid analgesia is complex, as both enhancement and reduction of pain have been reported. Initial experimentation on the effect of cholecystokinin on the opioid system revealed that CCK was capable of producing analgesia (Zetler *et al.*, 1980). However, this was soon followed by the demonstration that CCK also had anti-analgesic effects, in that it could antagonize the antinociception induced either by exogenously administered opioids or by the endogenous opioid system. Administration of CCK-8s diminished the analgesic response produced by morphine administration, as well analgesia caused by footshock-evoked endogenous opioid release in rodents (Itoh *et al.*, 1982; Faris, 1983). The effects of CCK were specific to the sulfated octapeptide, as experiments with comparable doses of the nonsulfated octapeptide were ineffective in inhibiting opioid analgesia (Faris, 1983).

Researchers have postulated that cholecystokinin possesses both analgesic and anti-analgesic properties and that these discordant effects are reflective of a biphasic dose response. A high "pharmacological" dose of CCK is thought to produce analgesia, while a more physiologically relevant dose antagonizes opioid analgesia (Kellstein and Mayer, 1992).

Numerous studies have shown that the administration of CCK antagonists enhance opioid analgesia (Klein *et al.*, 1992; O'Neil *et al.*, 1992; Price *et al.*, 1985; Watkins *et al.*, 1985). In rats, antagonists of the CCK-B receptor potentiated

antinociception by endogenous opioids (Xu *et al.*, 1997). In a clinical model of acute pain, low doses of proglumide, a CCK-B receptor antagonist, enhanced both the magnitude and the duration of morphine analgesia in human subjects experiencing post-operative pain (Lavigne *et al.*, 1989).

One obstacle in cholecystokinin-opioid research is the fact that in the monkey and human spinal cord, CCK-A receptors predominate (Hill *et al.*, 1988). In rodents, the experimental animal in which the majority of pain research has thus far been conducted, CCK receptors are primarily of the CCK-B subtype (Hill and Woodruff, 1990). Hence, it is difficult to generalize rodent experimental results to primates. The limited studies performed with primates revealed that the CCK-A antagonist, devazepide, enhanced both the peak and duration of the analgesic effect of morphine (O'Neil *et al.*, 1992; Klein *et al.*, 1992). Interestingly, proglumide also enhanced morphine analgesia (Price *et al.*, 1985). This is consistent with the finding that multiple sites of CCK-opioid action exist and that opioid analgesia occurs at both the spinal and supraspinal level.

Effects of Opioids on the Cholecystokinergic System

Though the exact mechanisms remain unclear, it has been postulated that stimulation of the opioidergic system potentiates cholecystokinin release. Morphine injected to rats, as well as *in vitro* application of opioids to spinal tissue, increased levels of cholecystokinin in the dorsal horn (Watkins *et al.*, 1985; Benoliel *et al.*, 1992). A single injection of morphine increased CCK mRNA in the rat hypothalamus and spinal

cord. Chronic administration of morphine resulted in elevated CCK mRNA and peptide levels in the hypothalamus, spinal cord and brain stem (Ding and Bayer, 1993). The increased CCK gene expression following repeated injections of morphine implicates enhanced CCK activity in opioid tolerance (Pu *et al.*, 1994).

One promising model that describes the interaction between the opioidergic and cholecystokinergic system has been proposed by Kellstein and Mayer (1992). At the level of the spinal cord, opioids increase the release of CCK, whether the opioids are applied exogenously or released endogenously. The endogenously-released CCK then binds to cholecystokinin receptors, which are adjacent to opioid receptors, and hinders the binding of opioids to opioid receptors. This interference may be a function of steric hindrance or conformational change. In this manner, CCK can antagonize opioid analgesia. Other possible mechanisms by which cholecystokinin may exert its anti-analgesic effects include the direct binding of CCK to opioid receptors or post-receptor modulation via second messenger systems (Han, 1992; Scheafer *et al.*, 1998).

There is some controversy regarding the involvement of specific opioid receptors in CCK-mediated opioid analgesia. A recent study has revealed that stimulation of delta 2-opioid receptors enhances analgesia through an increase in endogenous CCK, while activation of delta 1-opioid receptors has an inhibitory effect (Noble *et al.*, 1996). One study has reported that mu and delta receptors mediate opposite effects by morphine of the spinal release of cholecystokinin (Benoliel *et al.*, 1994). Although the significance of the delta and kappa receptor have been debated, the mu receptor has been identified as an important site for CCK action in most reports (Dourish *et al.*, 1992; Noble *et al.*, 1995).

Because the mechanisms underlying the relationship between the cholecystokinergic and opiodergic system is undoubtedly complex, further studies are necessary to unravel the intricacies of this interaction. Such studies would likely prove quite beneficial to those scientists involved with the arduous undertaking of developing efficacious and safe analgesics.

CHAPTER FIVE

SECOND MESSENGER SYSTEMS

CHAPTER FIVE

SECOND MESSENGER SYSTEMS

5.1 Introduction

It is generally agreed that CCK exerts its biological effects by activating specific second messenger systems; more speculative is the concept that these signal transduction pathways mediate the regulation of cholecystokinin release. Both the adenylyl cyclase pathway and phosphoinositide cascade have been implicated in the modulation of CCK secretion and signal transduction via guanine-nucleotide regulatory protein (G-protein) coupled mechanisms. These effector systems have been extensively described in various tissues and cell types in the mammalian periphery and central nervous system.

5.2 Regulation of CCK Secretion

Adenylyl Cyclase

The precise signaling pathways involved in CCK secretion are as of yet undetermined (Liddle, 1997). Beinfeld and colleagues conducted a series of experiments investigating the role of cyclic adenosine monophosphate (cAMP) as a putative intracellular mediator of CCK release. A combined treatment of forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was employed to elevate intracellular cAMP, and in rat prefrontal cortical slices, this treatment enhanced potassium-evoked CCK release. The mechanism underlying this release has been

postulated to be the phosphorylation of GTPases involved in secretion (Beinfeld, 1996). Forskolin and IBMX also potentiated CCK secretion from various endocrine cells including mouse pituitary tumour, rat insulinoma (Beinfeld, 1992), mouse intestinal tumour (Yoon and Beinfeld, 1994) and rat thyroid carcinoma cells (Beinfeld *et al.*, 1992).

The Phosphoinositide Pathway

Increased intracellular calcium concentrations seem to have an important role in CCK secretion. This increase in intracellular calcium can be elicited by 1,4,5-inositol triphosphate, cAMP, or a presently unidentified pathway which directly activates the opening of Ca^{2+} channels (Liddle, 1997). Some CCK-secreting cells possess G-protein-coupled receptors which generate 1,4,5-inositol triphosphate via the phosphoinositide cascade. The consequent increase in intracellular Ca^{2+} is then believed to induce CCK cellular release (Snow *et al.*, 1994). Studies which demonstrate the ability of calcium channel blockers to diminish CCK secretion underscore the importance of an extracellular calcium influx in cholecystokinin secretion (Mangel *et al.*, 1995).

5.3 CCK Signal Transduction

Mediation by G-Proteins

In a study of the bovine gallbladder, guanine nucleotide-binding proteins were determined to be important regulators of CCK-A receptor function. It has been proposed that CCK activates the traditional pathway which includes G-protein-receptor association,

phospholipase C activation, phosphatidyl-inositol hydrolysis and protein kinase C activation, in sequence. Interestingly, the GTP analogues Gpp(NH)p and GTPγS were able to convert high affinity binding sites for CCK to a low affinity state. These findings demonstrated the presence of two interconvertable affinity states for the gallbladder CCK receptor which is regulated by a guanine nucleotide-binding protein (Molero and Miller, 1991).

The importance of G-proteins has also been demonstrated in CCK-B receptor function. CCK-B receptors, located on GH_3 cells, a cell line derived from a rat anterior pituitary, demonstrate high affinity for CCK agonists and antagonists. The binding of CCK-8 to GH_3 membranes was inhibited by GTP and its stable analogues GppNHp and GTPγS, indicating the coupling of CCK-B receptors to G-proteins (Kuwahara *et al.*, 1993). Similar findings have also been reported for cerebral CCK-B receptors (Wennogle *et al.*, 1988). Recent cloning studies have confirmed that CCK-A and CCK-B receptors are both members of the seven transmembrane domain G-protein-coupled receptor superfamily (Wank, 1992).

The Phosphoinositide Cascade

A generalized model of cholecystokinin signal transduction has been proposed based on CCK pancreatic receptors in the stimulation of amylase secretion. Occupation of the CCK receptor activates a guanine-nucleotide regulatory protein, which, in turn, activates phospholipase-C. Phospholipase-C then stimulates phosphatidyl-inositol 4,5-

biphosphate hydrolysis to form diacylglycerol and 1,4,5-inositol triphosphate. Diacylglycerol activates protein kinase C and 1,4,5-inositol triphosphate releases calcium from intracellular stores. Protein kinase C and calcium then act through phosphorylation of proteins to mediate enzyme secretion (Louie, 1994). Several lines of evidence affirm the plausibility of this model.

In the human T Jurkat cell line, a model for T lymphocytes, the application of CCK-8 dose-dependently increased calcium concentrations. This increase was neither affected by the addition of extracellular Ca^{2+} to the medium nor by the addition of diltiazem, a calcium channel blocker. These findings revealed that CCK increased calcium levels via the mobilization of intracellular stores, and not by extracellular Ca^{2+} influx through the opening of membrane Ca^{2+} channels. Moreover, the ability of L-365,260, a specific CCK-B antagonist, to inhibit this increase confirmed the involvement of the CCK-B receptor (Dornand *et al.*, 1995). CCK-B receptors have also been linked to an intracellular calcium signaling pathway in small lung carcinoma (Sethi and Rosengurt, 1992), parietal cells (Chew and Brown, 1986), synaptosomes (Gala *et al.*, 1992) and human peripheral blood mononuclear cells (McMillen *et al.*, 1995).

It has been shown that CCK-8, via CCK-B receptors, excites rat neostriatal neurons by the stimulation of cationic conductance mediated by G-proteins. The coupling mechanism via G-proteins has been postulated to involve the production of 1,4,5-inositol triphosphate, which in turn induces Ca^{2+} release and the opening of non-selective cation channels (Wu and Wang, 1996). Similar findings were observed in substantia nigra dopaminergic neurons (Wu and Wang, 1994).

In summary, numerous studies have implicated the adenylyl cyclase pathway and phosphoinositide cascade in the mediation of CCK secretion and signal transduction via G-protein-coupled mechanisms. Further developments in this field will likely shed light on the identification of and mechanism underlying different affinity states of cholecystokinin receptors.

CHAPTER SIX

CHOLECYSTOKININ ASSAYS

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CHOLECYSTOKININ ASSAYS

6.1 Introduction

An accurate and reliable measurement of endogenous cholecystokinin is essential to progress in identifying the role of CCK in various normal and disease states. The quantification of CCK in plasma has been particularly difficult for several reasons. First, basal concentrations of cholecystokinin in plasma are low — usually in the picomolar range (Cantor, 1986; Höcker *et al.*, 1992; Merani *et al.*, 1997). Assays which are capable of measuring CCK must be sensitive enough to detect physiological concentrations of the peptide. Second, CCK exists in a number of heterogeneous molecular forms (Cantor, 1989; Maton *et al.*, 1982; Merani *et al.*, 1997; Rehfeld, 1984). Antibodies directed at a specific portion of one molecular form may not recognize the other molecular forms present in plasma. Similarly, an antibody may not recognize all the molecular forms with the same degree of avidity. Third, because the carboxy terminal pentapeptide of cholecystokinin is identical to that found in gastrin, antisera raised to detect CCK usually show a high degree of cross-reactivity with gastrins. Because levels of gastrin may be significantly higher than concentrations of cholecystokinin in plasma, any contribution of gastrin to total levels of immunoreactive material reported must be reconciled (Cantor, 1986; Rehfeld, 1984). In any assay designed to measure cholecystokinin, the aforementioned obstacles must be addressed. In addition, the universal difficulties that

are inherent to the radioimmunological measurement of all peptides must also be surmounted. These include the availability of high titer and high avidity specific antisera, a labeling technique which does not interfere with antibody binding, sufficient amounts of pure antigen available for tracer and standard preparation, elimination of non-specific interference by other plasma proteins and prevention of both peptide and tracer degradation. Finally, the assay should be able to be performed in a manner consistent with the resources available to many laboratories; in other words, it should be reasonably fast, convenient and inexpensive (Rehfeld, 1984).

The assays employed thus far to measure cholecystokinin can be classified into 2 major groups, bioassays and radioimmunoassays. The development of radioimmunoassays can further be subdivided into three distinct phases, discussed in greater detail below.

6.2 Biological Assays

Historically, the first measurements of cholecystokinin were carried out in biological assay systems, or bioassays. In 1928, Ivy and Oldberg discovered that an intestinal hormone, which they termed cholecystokinin, caused gallbladder contraction. In 1948, Harper and Raper demonstrated that pancreozymin, which was in fact CCK, stimulated pancreatic enzyme secretion. Modern bioassays exploit both of these two main physiological properties of CCK — the cholecystokinetic and pancreozyminic functions (Mutt, 1980).

Bioassays: Cholecystokinetic Activity

The first *in vivo* bioassay for cholecystokinin can be traced back to Ivy and Oldberg in 1928. This pair of researchers explored canine and feline physiology by clamping the cystic duct and cannulating the dome of the gallbladder. Upon injection of cholecystokinin into a thin glass rod which was connected to the cannula, the pressure in the gallbladder, and subsequently the volume of bile in the tube, increased in a dose-related manner. The amount of cholecystokinin activity that increased the intra-gallbladder pressure by one centimeter of bile was termed the "Ivy dog unit." Historically, and perhaps arbitrarily, pure CCK-33 was defined as possessing 3 IDU units per microgram and was used as the ultimate reference to compare the activity of sample preparations (Debas and Grossman, 1973; Mutt, 1980).

The measurement of intragastric pressure in rats is another method by which some investigators monitor CCK activity (Raybould and Reeve Jr., 1996; Raybould *et al.*, 1987). Catheters are placed in the body of the stomach of anesthetized rats through the pylorus and intraluminal pressure is recorded using a transducer and chart recorder. Various standard doses of cholecystokinin are administered by i.v. injections through a catheter in the jugular vein. Responses are expressed as changes in intragastric pressure from values taken prior to CCK injection. A dose-response curve is generated and samples containing unknown concentrations of CCK can be analyzed from the curve (Raybould and Reeve Jr., 1996).

Preparations of gallbladder strips have generally been used to examine gallbladder contraction as an *in vitro* assay of cholecystokinin activity (Yau *et al.*, 1973; Von Schrenck *et al.*, 1988). Gallbladder strips, usually from the guinea pig, are incubated in an organ bath and connected to isometric transducers, which in turn are connected to a recorder. Values obtained with unknown samples are then determined by comparison to the percentage of increase in the recorded tension caused by a known concentration of CCK (Yau *et al.*, 1973; Von Schrenck *et al.*, 1988). Cholecystokinin-induced contractions of the guinea pig gallbladder *in situ* recorded by strain gauge transducers have also been used to measure CCK activity (Ljungberg, 1969).

Bioassays: Pancreozyminic Activity

The measurement of cholecystokinin's pancreozyminic function is another method with which to determine CCK activity. This method is based on the ability of bioactive CCK fragments to stimulate amylase release of isolated pancreatic acini (Liddle *et al.*, 1985). The general methodology involved in a bioassay based on pancreatic enzyme release begins with the removal of rat pancreas and subsequent exposure to various collagenase treatments, dissociation and separation methods to obtain purified acini. Dispersed acini from guinea pig or mouse are also commonly used (Vinayek *et al.*, 1987). Aliquots of acini suspensions are then incubated with sample plasma extracts or standard known concentrations of CCK. Amylase release is directly assayed in the incubation medium. The concentration of amylase is compared with a dose-response

curve for CCK-8 in order to calculate the CCK content of test plasma samples. These values are expressed as CCK-8 equivalents (Liddle *et al.*, 1985).

In 1992, Höcker and colleagues developed an automated bioassay system which used small volumes of acini (0.25 ml) and measured up to 100 plasma samples in a single assay. This bioassay is based on the ability of cholecystokinin to release lipase from pancreatic acini. A similar method to that described for amylase release is used. After incubation of plasma extracts with the isolated pancreatic acini, lipase secretion is determined upon complete cell disruption by sonication (Höcker *et al.*, 1992).

Von Schrenck and colleagues (1988) compared the ability of cholecystokinin to stimulate gallbladder contraction and pancreatic enzyme secretion. They demonstrated that the dose-response curve of CCK octapeptide for gallbladder contraction of guinea pig muscle strips and stimulation of pancreatic enzyme extended over the same concentration range (10^{-10} to 10^{-6} M). By supporting their bioactivity data with binding studies, they concluded that both tissues possessed the same subclass of CCK-A receptors. Though many laboratories today use either the cholecystokinetic or pancreozyminic bioassay to characterize and quantify cholecystokinin, many of the bioassays are not sensitive enough to detect the minute concentrations CCK present in human plasma. Further, bioassays frequently reflect the sum or total amount of cholecystokinin present in an given sample and are unable to provide measurements of specific cholecystokinin peptides without further separation and purification techniques (Liddle *et al.*, 1985; Raybould and Reeve, Jr., 1996). Accordingly, the radioimmunoassay is preferable when precise and specific

quantification of individual CCK peptides is required (Bloom and Long, 1982; Eng and Yalow, 1989).

6.3 Radioimmunogical Methods

Historically, the first phase in the development of radioimmunoassays occurred in the late 1960's and early 70's (Go *et al.*, 1971; Harvey *et al.*, 1973; Young *et al.*, 1969). These original assays were limited by access to pure antigen, as the synthesis of sulfated CCK-33 was not yet readily available (Rehfeld, 1984). The immunization process was also restricted by the lack of accessible cholecystokinin. A limited number of animals could be immunized, and then only with small concentrations of antigen. Further, the harsh oxidative method used in the isotope labeling process resulted in unstable tracer preparations. Today, the results obtained from these preliminary assays are considered controversial and difficult to evaluate (Rehfeld, 1984).

The second stage in the development of cholecystokinin assays consisted of "subtraction" assays. Antibody "A" was designed to detect both cholecystokinin and gastrin equally by producing the antibody against the common C-terminus of both peptides. Antibody "B" was produced only against that portion of gastrin which was not homologous to cholecystokinin. Every sample was then assayed separately with each antibody. The concentration of CCK was calculated by subtracting the concentration measured by antibody B, the gastric-specific antiserum, from the concentration measured by antibody A, the antiserum that detects both CCK and gastrin. Theoretically, this type of subtraction assay system seemed to be a logical and effective method to measure CCK.

However, in practice, this system had a number of inherent drawbacks. In order to obtain an accurate estimate of cholecystokinin, the two different antibodies had to measure all the molecular forms of gastrins with identical potency. This was not an easy task and the production of antisera that exactly measured all gastrins equally was rarely achieved (Rehfeld, 1984). Moreover, the use of two assays for every sample increased the coefficients of variation. Since the concentration of gastrin is greater than that of cholecystokinin in plasma, the magnitude of error was frequently similar to or larger than the actual CCK concentration (Rehfeld, 1984). As with the first class of assays, results from the subtraction assay systems are considered to be inconclusive and the methodology is now obsolete (Cantor, 1989; Rehfeld, 1984).

A third method of measuring cholecystokinin involves the production of antiserum which does not cross-react with gastrin. This procedure requires the development of a specific antibody directed against sequences of cholecystokinin which are not homologous to that found in gastrin. In order to develop such an antibody and still retain the capacity to detect CCK octapeptide, antiserum against sequence 26-30 of the CCK molecule was required. Though some laboratories have succeeded in producing such antisera, they have reported that this method, though not impossible, is quite difficult (Rehfeld, 1984). A significant drawback to producing an antibody against sequence 26-30 is that detection of the cholecystokinin sequence 29-33 —CCK tetrapeptide— becomes impossible.

The Relevance of CCK-8 and CCK-4 Quantification

As discussed in greater detail above, cholecystokinin octapeptide is the most abundant form of cholecystokinin in the central nervous system (Beinfeld, 1981). This peptide has been characterized as a neurotransmitter and neuromodulator, with effects upon the serotonergic, dopaminergic, noradrenergic and opioid systems. Recent studies have implicated CCK-8 in a number of normal and pathological behaviors including satiety (Ballenger and Clark, 1994; Lydiard *et al.*, 1993), memory (Lemaire *et al.*, 1994), analgesia (Han, 1995) and anxiety (Lydiard *et al.*, 1992; Harro *et al.*, 1993).

The seventh tyrosine of cholecystokinin octapeptide may be sulfated (CCK-8s) or nonsulfated (CCK-8ns). The two forms of CCK-8 can differ with respect to biological activity and metabolism. The presence of a sulfated tyrosine has been reported to influence binding affinity to the CCK-A receptor (Steigerwalt and Williams, 1981). A bioassay based on gallbladder stimulation demonstrated that CCK-8s is more potent than CCK-8ns (Tokunga *et al.*, 1993). Degradation studies revealed that sulfated octapeptide has a longer half-life in both human and rat plasma, as compared to CCK-8ns (Koulischer *et al.*, 1982). Alternatively, *in vitro* studies have indicated that sulfation may actually enhance cholecystokinin degradation by certain enzymes (Pauwels *et al.*, 1989). Differential metabolic clearance rates of endogenous CCK-8s and CCK-8ns may modulate the bioavailability of the two peptides. This, in turn, may affect the duration of each peptide's stimulatory period and consequently influence the relative pharmacological importance of each peptide *in vivo*. Thus the ability to separate and

quantify both CCK-8s and CCK-8ns was an important consideration in the development of our assay.

In recent years, scientists have focused on the role of cholecystokinin tetrapeptide in the central nervous system. Quantification of CCK tetrapeptide is essential in determining the true role that cholecystokinin plays in various psychopathological illnesses. These illnesses, primarily Eating Disorders, Premenstrual Dysphoric Disorder and Panic Disorder, are discussed at length in Chapters Seven, Eight and Nine respectively. For instance, a recent line of research has implicated CCK-4 in anxiety disorders. Intravenous administration of CCK-4 has been reported to induce panic attacks in healthy volunteers and patients with panic disorder, with the latter group showing an enhanced sensitivity to the challenge (Bradwejn *et al.*, 1991a; 1991b). Two separate studies have reported diminished levels of CCK octapeptide in the cerebrospinal fluid of CSF of patients with panic disorder (Lydiard *et al.*, 1992) and the eating disorder, bulimia nervosa, (Lydiard *et al.*, 1993). These findings have led to speculation that an elevated tetrapeptide concentration may play an important role in the etiology of these disorders (Lydiard *et al.*, 1992; 1993).

The putative involvement of either CCK-4, or an aberrant CCK-8: CCK-4 ratio in psychopathology prompted our research team to develop an assay system which could measure both peptides with precision and accuracy. We developed a three-system which involved the extraction of CCK fragments from plasma, the separation of peptides by reverse phase high performance liquid chromatography (HPLC) and the quantification of CCK-4, CCK-8s and CCK-8ns by radioimmunoassay (Merani *et al.*, 1997).

The following Section highlights the rationale behind and development of our assay system, as well as a brief comparison to other similar systems. The technical aspects of this novel system are outlined in detail in the manuscript following this Section and will not be reiterated here (please see the manuscript entitled *Development of a Sensitive and Specific Assay System for Cholecystokinin Tetrapeptide* by Merani *et al.*, 1997, Section 6.4).

In addition to our own laboratory, an independent team of investigators in Sweden also recognized the importance of measuring both CCK-4 and CCK-8 (Qureshi *et al.*, 1993). They developed an assay system which encompassed reversed-phase HPLC combined with electrochemical detection. Electrochemical detection may be used to measure CCK-4 and CCK-8 since both peptides contain tryptophan residues which are electrochemically oxidizable. However, the researchers reported quantification of only CCK-4 and sulfated CCK-8. The concentration of nonsulfated CCK-8 was not measured presumably because the optimal conditions required to quantify both CCK-4 and CCK-8s could not simultaneously measure CCK-8ns. Moreover, although the electrochemical detection method was sufficiently sensitive to measure levels of cholecystokinin in the brain, concentrations of CCK in human plasma may be below the lower limit of detection. Qureshi and colleagues report that the detection limit for their system is 2.0 pmol and 10.0 pmol for CCK-4 and CCK-8s respectively (Qureshi *et al.*, 1993). Our research has determined the mean levels of plasma cholecystokinin to be 1.0 ± 0.2 pM, 3.4 ± 0.8 pM and 1.9 ± 0.4 pM for CCK-4, CCK-8s and CCK-8ns respectively. Therefore, an assay system which is designed to accurately analyze blood samples from

human subjects would have to be capable of detecting CCK in the low pM range (1-2 pmol/L; 1-2 fmol/ml). Even assuming that one could obtain a relatively large volume of plasma in clinical studies (eg., 100 ml), the assay would still need to be sensitive enough to detect 0.1 pmol, or 100 fmol — a value *twenty times* less than that which is can be measured by the system designed by Qureshi and co-workers. It is important to note that, recently, the same team of scientists have reported the development of an assay which can detect 0.1 pmol/l for CCK-4 and 0.5 pmol/l for CCK-8s; however, the methodology and data supporting these values remain unpublished (Gunnarsson *et al.*, 1997).

Some early attempts to characterize large and small forms of cholecystokinin in human plasma utilized high pressure liquid chromatography combined with radioimmunoassay. In 1982, Maton and colleagues, interested in gut physiology and disease, sought to measure the different forms of circulating CCK in blood. Their system involved two HPLC separations followed by two radioimmunoassays — one for cholecystokinin and one for gastrin. The concentration of gastrin was then subtracted from the CCK values, and as with other subtraction assays, was subject to the same limitations as described above. The detection limit of the radioimmunoassay was 3 pM for CCK-8; CCK-4 in human plasma failed to be detected by this method (Maton *et al.*, 1982). In 1986, Miller and co-workers recognized that "a sensitive and specific method to quantify the molecular species of CCK in tissue and biological fluids does not exist." They also attempted to establish a method to measure CCK using reverse-phase HPLC and radioimmunoassay (Miller *et al.*, 1986). The antiserum used in the assay recognized the pentapeptide amide common to CCK and gastrin and was able to detect both CCK

tetrapeptide and octapeptide, though not with equimolar potency. Gastrin-17 was used as standard and iodinated gastrin-17 as tracer. The detection limit of the assay was 20 pg/ml gastrin-17 equivalents, which, as our experimentation revealed, is not sufficiently sensitive to quantify physiological concentrations of cholecystokinin in human plasma (Merani *et al.*, 1997).

Antibody Production

Young and colleagues were the first group of investigators to report a radioimmunoassay for cholecystokinin (Young *et al.*, 1969). Initial studies reported values in the range of 100-1000 pM cholecystokinin in human plasma (Reeder *et al.*, 1973; Schlegel *et al.*, 1977; Young *et al.*, 1969). As a consequence of technical improvements, it is now know that concentrations of cholecystokinin in human plasma are substantially lower than those first reported. Today, basal values of <20 pM are generally documented for total CCK-like immunoreactivity in plasma (Cantor, 1986; Höcker *et al.*, 1992; Merani *et al.*, 1997). The high concentrations described in these early studies have been attributed, in part, to the lack of specific CCK antisera (Cantor, 1989). Since synthetic cholecystokinin was not readily available, partially purified preparations of CCK-33 were generally used to immunize animals such as rabbits, guinea pigs and goats (Go *et al.*, 1971; Rehfeld, 1978; Young *et al.*, 1969). Some of the antisera produced in these studies reacted with both CCK and gastrin, while other antibodies recognized various forms of cholecystokinin. The variability in antisera has been and

continues to be a significant factor in the wide range of cholecystokinin concentrations reported to occur in human plasma (Cantor, 1989).

In order to develop a radioimmunoassay for specific cholecystokinin peptides, our first task was to raise antisera which could recognize all of the relevant peptides. Our specific aim was to produce a high-titer antibody which recognized CCK-4, CCK-8s and CCK-8ns with equimolar potency. With such an antibody, we would be able to develop a radioimmunoassay which could reliably quantify all three peptides. As a pilot study, we injected three New Zealand White rabbits with cholecystokinin tetrapeptide conjugated to thyroglobulin. Fortunately, two of the three rabbits produced antibodies against cholecystokinin-derived peptides. One animal in particular, rabbit 785X, produced an exceptionally high titer antibody which recognized CCK-4, CCK-8s and CCK-8ns equally. The plasma from this animal served as the source of antibodies for all the studies reported here.

Purification and Concentration

Many biological fluids and tissue homogenates cannot be applied directly onto the an HPLC column. Biological samples frequently contain large particles which can clog the column and disrupt the elution profile of the desired peptide (Miller *et al.*, 1986). The use of octadecylsilicane-packed cartridges is one effective method to eliminate such extraneous tissue factors. However, when using such cartridges, it is necessary to optimize the sample, washing and elution solvents in order to ensure that all relevant

cholecystokinin peptides are adsorbed and eluted in a reproducible manner and with high yields. In our laboratory, samples were extracted with Sep-Pak™ C_{18} octadecylsilicane-packed cartridges. Immediately prior to use, each cartridge was activated with acetonitrile and washed with distilled water (dH_2O). Samples were applied to cartridges, and allowed to flow through the matrix at a rate of 1 ml per minute, in order to allow sufficient time for adsorption. The cartridges were subsequently washed with dH_2O and eluted with 70% ethanol in dH2O. Upon elution of peptides by ethanol, the eluant was immediately lyophilized to ensure the stability of the cholecystokinin peptides. Lyophilization was also required in order to reconstitute the sample in appropriate solvents designed for either direct radioimmunoassay or injection onto HPLC.

In line with our own findings, Imamura and co-workers (1993) have reported that extraction of cholecystokinin peptides by ethanol is an effective method to elute smaller cholecystokinin fragments while removing non-specific interfering factors found in human plasma. Although ethanol extraction of plasma eliminates the majority of non-specific interference, the extracts can still contain some interfering factors (Cantor, 1989). To confirm that our extracted plasma was free of any interfering substances, radioimmunoassay curves were prepared in both buffer and plasma. We added known amounts of standard preparations of cholecystokinin to equal volumes of buffer or plasma and simultaneously incubated and assayed both sample sets. The finding that both curves were not only statistically similar, but nearly superimposable, indicates that plasma extracted by our method did not contain substances which could interfere with our assay (Fig. 5).

Finally, in addition to providing an ideal method for the purification of cholecystokinin peptides, extraction by Sep-Pak™ was also an efficient technique by which cholecystokinin could be concentrated. Due to the low levels of individual CCK peptides in human plasma, samples were routinely concentrated ten-fold prior to analysis (Merani *et al.*, 1997).

Degradation

Cholecystokinin is highly susceptible to proteolytic cleavage by various peptidases (Camus *et al.*, 1989; Cantor, 1986; Eberlein *et al.*, 1987; Koulischer *et al.*, 1982). In order to avoid artifactual values caused by *in vitro* degradation of larger cholecystokinin forms, enzyme inhibitors were consistently used to protect CCK. For example, blood samples were drawn with syringes rather than into vacutainer tubes. Though somewhat more laborious, the use of syringes permitted us to minimize *in vitro* degradation because we were able to pre-prepare each syringe with an inhibitor cocktail prior to blood withdrawal. The enzyme inhibitors used by our laboratory consisted of bestatin, an aminopeptidase B and leucine aminopeptidase inhibitor; phenylmethanesulphonyl fluoride (PMSF), a serine peptidase inhibitor; and aprotinin, an inhibitor of trypsin, chymotrypsin, plasmin, kallikrein and fibrinolytic activity in blood samples. The use of these inhibitors had previously been reported by others to effectively inhibit the degradation of cholecystokinin, in particular cholecystokinin octapeptide and tetrapeptide (Camus *et al.*, 1989; Cantor, 1986; Koulischer *et al.*, 1982). The bacteriostat

sodium azide was used throughout radioimmunoassay sampling to prevent proteolysis by bacterial contamination. Non-specific loss of cholecystokinin was prevented by using bovine serum albumin (BSA) in all assay tubes. Albumin inhibits adsorption to and denaturation of peptides on plastic and glass surfaces (Solomon *et al.*, 1984).

As degradation of cholecystokinin has been reported to be temperature-dependent (Cantor, 1986), precautions were taken to ensure that all samples were kept as cold as possible. For instance, blood was drawn in 4°C syringes which contained the inhibitor cocktail described above and then instantly dispensed into chilled EDTA tubes and placed on ice. Blood tubes were immediately centrifuged at 4°C and plasma was stored at -70°C until use or immediately processed for analysis. Tubes used in the radioimmunoassay protocol were consistently kept refrigerated or on ice-baths (Merani *et al.*, 1997).

Tracer Preparation

Radioiodination of cholecystokinin requires a particularly mild labeling technique. Conventional iodination methods, such as using chloramine-T, are generally too harsh as oxidation of the methionine residues typically occurs (Cantor, 1989; Rehfeld, 1978). CCK-33 contains three methionine residues, two of which are present in the C-terminal octapeptide fragment, and one in the tetrapeptide. Our own experience with iodination of CCK octapeptide by chloramine-T resulted in unsuccessful labeling of the peptide. When labeling did occur, the tracer gave erratic and unreproducible results in radioimmunoassays and degraded rapidly (Merani *et al.*, unpublished observations). To

circumvent the oxidation of cholecystokinin, CCK-8ns was iodinated using a modified technique based on a method designed particularly to obtain maximal tracer yield (Tower *et al.*, 1977). By employing a combination of lactoperoxidase and glucose oxidase, stable, immunoreactive ^{125}I-CCK-8ns was routinely prepared in our laboratory.

The labeling of cholecystokinin tetrapeptide posed a more difficult barrier. Indeed, one of the major obstacles in the development of our assay was the generation of radioiodinated CCK-4. To our knowledge, the radioiodination of CCK-4 had never before been successfully performed. CCK-4 does not possess an amino acid residue capable of being iodinated. Consequently, CCK-4 had to be conjugated to hydroxyphenylpropionic-succinimide ester, or Bolton-Hunter reagent. In 1973, Bolton and Hunter developed a method to iodinate peptides for which direct substitution of ^{125}I into tyrosine residues was not possible. This method may be used for proteins which are particularly susceptible to iodination damage and those peptides which lack tyrosine, such as cholecystokinin tetrapeptide (Bolton and Hunter, 1973). By combining the methods of Bolton and Hunter with those of Tower *et al.* (1977), we developed a technique of iodinating Bolton-Hunter cholecystokinin tetrapeptide, or BH CCK-4. This novel immunoreactive tracer was then used as the label in our radioimmunoassay (Merani *et al.*, 1997).

Separation of Cholecystokinin Peptides

The occurrence in plasma of multiple forms of cholecystokinin which exhibit cross-reactivity to antisera was another major obstacle in individually quantifying CCK-4, CCK-8s and CCK-8ns. Since our antibody was specific for the C-terminal sequence Trp-Met-Asp-Phe-NH_2, a method was required which could separate CCK-4 and CCK-8 from other forms of cholecystokinins and gastrins which contained this sequence. We developed a system which employed analytical reverse phase HPLC fractionation of extracted plasma in order to separate these immunogenic peptides (Merani *et al.*, 1997). Chromatographic characteristics, such as the solvent, flow rate and gradient, were optimized to obtain a high resolution and reproducible separation of CCK-4, CCK-8s and CCK-8ns (Fig. 7). Each fraction containing the specific cholecystokinin peptide could then be quantified by radioimmunoassay.

Radioimmunoassay

Though a number of biological assays are available to measure cholecystokinin, we chose to employ radioimmunoassay because it possesses a number of advantages over other assay systems. First, radioimmunoassays are rapid. Our assay was designed to generate the exact concentration of cholecystokinin in any tube within 72 hours. Second, radioimmunoassays are sensitive. We were able to detect levels of cholecystokinin as low as 2.7 fmol in any given sample. Third, multiple samples can be analyzed. Our laboratory routinely processed 300 samples without difficulty. Finally,

radioimmunoassays can be designed to measure either sum levels of CCK-like immunoreactivity or individual peptides. Our radioimmunoassay was constructed to measure both total CCK-LI when plasma extracts were directly assayed, and to specifically detect CCK-4, CCK-8 and CCK-8ns upon HPLC fractionation.

In summary, subsequent to the production of a highly sensitive antiserum raised against the CCK tetrapeptide and the production of labeled Bolton Hunter CCK-4, we used the combined techniques of peptide extraction, high performance liquid chromatography and radioimmunoassay to discriminate among circulating levels of cholecystokinin and gastrin in human plasma. The development of this novel 3-step assay system represents the crux of my doctoral research and may now be used as an important tool to investigate the role of cholecystokinin in normal and pathological human physiology and behaviour.

6.4 Manuscript—Development of a Sensitive and Specific Assay System for Cholecystokinin Tetrapeptide

Development of a Sensitive and Specific Assay System for Cholecystokinin Tetrapeptide

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ABSTRACT

Cholecystokinin is a gastrointestinal and neuropeptide which has been implicated in a wide range of physiological and behavioural processes. We have developed a sensitive and specific assay system to measure the various forms of cholecystokinin (CCK) in human plasma. This 3-step system involves i) extraction of CCK fragments from plasma using reverse phase chromatography; ii) separation of peptides by high performance liquid chromatography; and iii) detection and quantification of peptides with a double-antibody radioimmunoassay, using an antibody raised against cholecystokinin tetrapeptide (CCK-4) coupled to thyroglobulin and ^{125}I Bolton-Hunter CCK-4 as tracer. The antibody detects CCK-4, sulfated CCK-8 (CCK-8s) and nonsulfated CCK-8 (CCK-8ns) with equal affinity. The lower limit of detection is 2.7 fmol, with an ED_{50} of 10.6 ± 2.2 fmol. Mean CCK-like immunoreactivity (CCK-LI) in the plasma of 12 healthy subjects was determined to be 12.9 ± 2.1 pM CCK-4 equivalents. Concentrations of each individual peptide in plasma were determined to be 1.0 ± 0.2 pM, 3.4 ± 0.8 pM and 1.9 ± 0.4 pM for CCK-4, CCK-8s and CCK-8ns respectively.

Key words: ***Cholecystokinin; CCK-4; CCK-8s; CCK-8ns; Radioimmunoassay; RIA; HPLC; Healthy Volunteers; Bolton-Hunter; Plasma Levels; Anxiety; Panic Attacks***

INTRODUCTION

Cholecystokinin (CCK) is a gastrointestinal and neuropeptide that is involved in the regulation of physiological and behavioural processes. CCK was first isolated from the intestine (Mutt and Jorpes, 1971) and its ability to stimulate gallbladder contraction (Ivy and Oldberg, 1928) and pancreatic enzyme release (Harper, 1943) has long been established. CCK is also one of the most abundant peptides in the central nervous system and is a putative neurotransmitter and neuromodulator (Beinfeld *et al.*, 1981). CCK has been implicated in such behaviours as satiety (Ballenger *et al.*, 1994; Lieverse *et al.*, 1994), memory (Lemaire *et al.*, 1992; 1994), analgesia (Han 1995; Noble *et al.*, 1994; Suh *et al.*, 1995) and anxiety (Bradwejn *et al.*, 1992; Harro *et al.*, 1993; Lydiard 1994).

CCK is a heterogeneous protein that exists in a number of molecular forms derived from a propeptide precursor. The human mRNA translation product of CCK comprises 115 amino acids (Takahishi *et al.*, 1985) and undergoes a variety of post-translational modifications, including sulfation of tyrosine, amidation of the C-terminal phenylalanine and a series of cleavage steps which generate multiple CCK fragments (Eberlein *et al.*, 1992). The C-terminal octapeptide is highly conserved in mammals, while the remaining sequences vary among species (Cantor, 1989). It has been reported that CCK octapeptide (CCK-8) is the predominant form in the central nervous system (Miller *et al.*, 1994), while CCK-12 is perhaps the major form in cerebrospinal fluid (Geracioti *et al.*, 1993). CCK-8, -22, -33, -39 and/or CCK-58 have all been detected in human plasma (Cantor and Rehfeld, 1987; Eberlein *et al.*, 1987; Jansen and Lamers

1980; Liddle *et al.*, 1985). To our knowledge, the simultaneous discrimination and quantification of plasma CCK-4, CCK-8s (sulfated) and CCK-8ns (nonsulfated) has not been reported, in part due to the lack of a sensitive and specific assay for the tetrapeptide.

CCK tetrapeptide is of particular interest due its ability to induce panic attacks in healthy volunteers and panic patients, with the latter group showing an enhanced sensitivity to the challenge (Bradwejn *et al.*, 1990; 1991a; 1991b). In addition, reports of decreased CSF levels of CCK octapeptide in certain psychiatric patient populations has led to speculation that an altered metabolism may lead to an increased tetrapeptide concentration (Lydiard *et al.*, 1992; 1993). An evaluation of this hypothesis requires an assay which reliably separates CCK-4 and CCK-8. The ability to discriminate between the sulfated and nonsulfated forms of the octapeptide is also important, since the two differ with respect to biological activity (Tokunga *et al.*, 1993; Vinayek *et al.*, 1987). Accordingly, an assay system which could accurately quantify and compare endogenous levels of CCK-4, CCK-8s and CCK-8ns would greatly advance investigations into the role of cholecystokinin in health and pathology.

The following study outlines the development of a specific and sensitive assay which can reliably differentiate and quantify plasma levels of CCK-4 and the sulfated and nonsulfated forms of CCK-8. Using the combined techniques of peptide extraction, high performance liquid chromatography (HPLC) and radioimmunoassay, we also present preliminary qualitative and quantitative profiles of CCK-like immunoreactivity (CCK-LI) in the plasma of healthy control subjects.

MATERIALS AND METHODS

Materials

CCK-4, sulfated CCK-8 (CCK-8s), nonsulfated CCK-8 (CCK-8ns) and human gastrin 17-I (G-17-I) were obtained from Peptides International, Louisville, KY; Thyroglobulin, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (CDI), lactoperoxidase, sodium azide, bovine serum albumin (BSA), triton-X, bestatin, aprotinin, and PMSF were from Sigma Chemical Company, St. Louis, MO; Freund's adjuvant was purchased from DIFCO, Detroit, MI; *Bordetella pertussis* vaccine was from Eli Lily Co. Indianapolis, IN; 1,3-diisopropyl-carbodiimide (DIC), CH_2Cl_2, diisopropylethylamine, thioanisole, dimethyl sulfide, ether and glacial acetic acid were from Aldrich, Milwaukee, WI; Bolton-Hunter (BH) reagent and trifluoroacetic acid (TFA) and were obtained from Pierce, Rockford, IL; 1-hydroxybenzotriazole (HOBt) was from Flukachemie, Switzerland; Acetonitrile and polyethylenglycol were purchased from BDH Inc., Toronto, ON; Glucose oxidase was obtained from Boehringer Mannheim, Laval, PQ; Sodium phosphate was obtained from Fisher, Montreal, PQ; ^{125}I was from Amersham, Oakville, ON; NaCl was from Biopharm, Laval, PQ; Normal rabbit serum and Goat anti-rabbit γ-globulin were purchased from Immunocorp, Montreal, PQ.

Antisera Preparation

CCK-4 was conjugated to thyroglobulin by the carbodiimide method (Skowsky and Fisher 1972). Twenty-five mg of thyroglobulin in 0.5 ml of distilled water (dH_2O) was added to 5 mg of CCK-4 dissolved in 0.5 ml of distilled water and adjusted to pH 5.5. Five mg of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide-HCl (CDI) in 0.4 ml of dH_2O was then added dropwise with constant mixing. After an overnight incubation at 4°C, 35 mg of CDI was added and mixed at room temperature for 2 hours. The removal of excess coupling agent was accomplished by a 24 hour dialysis against 2 L of 0.001 M phosphate buffer (pH 7.4) containing 0.9% NaCl. The dialyzed conjugate was stored at -70°C until immunization.

Immunization Procedure

The antigen solution (100 μg/ml saline) was emulsified in complete Freund's adjuvant by repeated aspiration and expulsion of the mixture through a 20-gauge needle. New Zealand White rabbits were injected with the emulsified antigen intradermally on the back at 50 or more sites. Intra-muscular injections of 0.5 ml *Bordetella pertussis* vaccine were administered as a non-specific stimulus of the immune system. Booster injections of 50 μg of the antigen emulsified in incomplete Freund's adjuvant were given every 4 weeks for 8 months (Vaitukaitis *et al.*, 1971).

Synthesis Of Bolton Hunter CCK-4

The synthesis of Bolton Hunter CCK-4 (BH CCK-4) was performed by the manual solid-phase technique using a Merrifield resin (1% cross-linked, 200-400 mesh,

0.69 mmol p-methylbenzhydrylamine HCl/g resin) obtained from USB, Cleveland, Ohio. Peptides were assembled using BOC-protected amino acids and 1,3-diisopropylcarbodiimide (DIC) as coupling agent. The β-carboxylic group of aspartic acid was protected by benzyl ester. The following steps were performed in each cycle:

1. addition of BOC amino acid in CH_2Cl_2 (2.5 eqiv.)
2. addition of DIC (2.5 equiv.) and mixing 1-2 hr
3. washing with CH_2Cl_2 (3 x 1 min)
4. washing with EtOH (1 min)
5. monitoring completion of the reaction with the ninhydrin test
6. BOC deprotection with 50%(v/v) TFA in CH_2Cl_2 (30 min)
7. washing with CH_2Cl_2 (5 x 1 min)
8. neutralization with 10% (v/v) N,N-diisopropylethylamine in CH_2Cl_2 (2 x 5 min)
9. washing with CH_2Cl_2 (5 x 1 min)

Coupling of 3-(4-hydroxyphenyl) propionic acid was then performed according to the protocol described above. DIC and 1-hydroxybenzotriazole (HOBt) (2.5 equiv.) were used as coupling agents. After final coupling, the resin was washed with CH_2Cl_2 (3 x 1 min) and EtOH (3 x 1 min) and dried in a dessicator. The peptide was cleaved from the resin by HF treatment with the addition of thioanisole and dimethyl sulfide in the cleavage mixture for 120 min at 0°C (20 ml HF plus 1 ml of thioanisole and 1 ml of dimethyl sulfide/g resin). After evaporation of HF, the resin was washed three times with ethyl ether and extracted three times with glacial acetic acid. The crude peptide was obtained in solid form through lyophilization of the acetic acid extract. The peptide was purified by reverse-phase chromatography on an octadecasilyl silica column (Vydac, cat. 218TP1022) with a linear gradient of 20-50% ACN with 0.1% TFA. Molecular weight was confirmed by FAB-MS.

Tracer Preparation

Bolton Hunter CCK-4 was iodinated using a previously described method designed to achieve high tracer yield (Tower *et al.*, 1977). Briefly, a solution of 5 µg BH CCK-4 in 40 µl phosphate buffer (0.05 M, pH 7.4), 40 µl ^{125}I (1 mCi) in phosphate buffer, 10 µl lactoperoxidase (0.1mg/ml dH_2O) and 10 µl glucose oxidase (5 µg/ml phosphate buffer) were added to an Eppendorf tube at 25°C. The reaction was initiated by the addition of 5 µl glucose (0.5% in dH_2O) and terminated after 20 minutes by the addition of 100 µl phosphate buffer with 0.1% sodium azide. The labeled peptide was purified by HPLC (Shimadzu) with a C_{18} µBondapak column (Waters 10µm, 125 Å, cat. WAT052860) using a 1 ml/ min gradient of 20-50% acetonitrile and dH_2O, each containing 0.1% TFA. Five fractions of 1 ml were collected while the gradient stabilized at 20% acetonitrile prior to the collection of 60 fractions of 1 ml with the gradient. A 10 µl aliquot of each tube was counted to determine the position of the labeled peptide. 100 µl of 0.2% BSA and 1 ml of EtOH was added to the fraction containing the first peak following the elution of free iodine and stored at -20°C until use. The tracer was immunoreactively stable for 6-7 weeks.

Subjects And Sample Collection

Six healthy male volunteers (age 30.2 ± 2.3 years; range: 24-34) and 6 healthy female volunteers (age 26.3 ± 1.4 years; range: 24-28) participated in this study after giving informed consent. Subjects arrived at the Allen Memorial Institute, Montréal, Québec between 07:00 - 09:00 after an overnight fast of 12-15 hours. A 19-gauge

butterfly catheter was inserted in the antecubital vein and 100 mls of blood were collected in chilled syringes containing the enzyme inhibitors bestatin (0.03 mM), aprotinin (1000 IU/ml) and PMSF (10^{-5} M). The samples were immediately dispensed into chilled EDTA tubes on ice and centrifuged at 3000 rpm at 4°C for 15 minutes. Plasma was stored at -70°C until use or immediately extracted. The study was approved by the AMI, Royal Victoria Hospital Research Ethics Board.

Sample Extraction

Samples were extracted using Sep-Pak C_{18} cartridges (Waters). Cartridges were activated with 8 mls acetonitrile and washed with 8 mls dH_2O. Samples were applied to cartridges, washed with 5 mls dH_2O and eluted with 3 mls 70% EtOH. The eluant was dried in a speed-vac (Savant) overnight. Each sample was directly assayed for total CCK levels and applied to HPLC to measure individual CCK concentrations.

Peptide Recovery

To assess extraction recovery of CCK-4, 25, 000 cpm of ^{125}IBH CCK-4 was added to 1 ml of plasma or assay buffer and processed by Sep-Pak C_{18} cartridges as outlined above. Recovery of peptides from HPLC was determined by the application of known quantities of standard preparations of CCK-4, CCK-8s and CCK-8ns and quantification of each peptide by radioimmunoassay subsequent to fractionation. Recovery of endogenous plasma CCK was determined by radioimmunoassay quantification prior to

HPLC application (to determine sum total injected) and subsequent to fractionation (to determine sum total recovery).

HPLC Fractionation

Reverse phase high performance liquid chromatography on a C_{18} μBondapak column (Waters) using a 20-50% gradient of ACN and dH_2O, each containing 0.1% TFA, was used to separate standard preparations of CCK-4, CCK-8s, CCK-8ns and human gastrin 17-I, with a flow rate of 1 ml/min. All solutions were filtered prior to use and degassed continuously with helium during chromatography. Thirty fractions of 1 ml each were collected and placed in a speed-vac overnight. Lyophilized plasma samples were reconstituted in 20% ACN and applied to HPLC in a similar manner.

Radioimmunoassay

100 μl of standards in the range of 0.4 - 200 pg CCK-4 /100ul assay buffer (0.1M NaCl, 1% BSA, 1% Triton X-100, 0.1% sodium azide in 0.1M phosphate buffer, pH 7.4) or unknown sample were added in duplicate to polystyrene tubes at 4°C. This assay buffer was used for all subsequent dilutions. Standards were prepared by 1:2 serial dilutions in buffer, as well as in hormone-free plasma to asses non-specific interference. 200 μl or 100 μl of assay buffer was added to tubes to test for non-specific binding (NSB) or total binding respectively. 100 μl of antiserum (diluted 1:10 000) was added to each tube except the NSB tubes and incubated at 4°C for 24 hours. 100 μl of tracer (6000-8000 cpm) was added to each tube and incubated as above. Tubes to determine total counts

were also prepared. 100 µl of normal rabbit serum (1:35) and 100 µl goat anti-rabbit γ-globulin (1:50) were added and incubated at 25°C for 2 hours. Tubes were centrifuged at 3000 rpm at 4°C for 20 minutes after the addition of 1 ml of polyethylenglycol. Upon aspiration of the supernatant, the radioactivity in the precipitate was counted (Packard Cobra II Auto-gamma counter).

Statistical Analysis

Statistical evaluation was performed with a 2 x 3 ANOVA for repeated measures followed by Tukey's test for multiple comparisons. The level of significance was set at 5% for all analyses. Results were expressed as mean ± SEM.

RESULTS

Antibody Characterization

The antibody recognizes the carboxy terminal portion of cholecystokinin and is highly specific for CCK-4. The antiserum cross-reacts with equimolar potency with both the sulfated and nonsulfated forms of CCK-8, as well as with G-17-I (Fig. 4).

Radioimmunoassay Results

The range of the standard curve was determined to be 0.7 - 336 fmol CCK-4 equivalents/tube (0.4 - 200 pg CCK-4/tube). Data are presented as a logit/log transformation of percent binding (B/T) vs dose. Parallelism was observed in curves performed in plasma extracts (Fig. 5). From 5 separate radioimmunoassays, intra-assay

(≥ 10 replicates per assay) and interassay coefficients of variation were calculated as 8 and 9% respectively. Half-maximal displacement (ED_{50}) of 10.6 ± 2.2 fmol was observed. Binding of tracer in the absence of the standard (zero binding) was 27.9 ± 2.1 % at a 1:10 000 antisera dilution. Non-specific binding was calculated as 1.7 ± 0.1 %. Specific activity of the monoiodinated tracer, comprising the first peak following elution of free iodine (Fig. 6), was ~ 1025 Ci/mmol.

Peptide Recovery

Recovery of iodinated BH CCK-4 from assay buffer and human plasma, respectively, were determined to be 89% and 87%. HPLC recoveries of standard preparations of CCK-4, CCK-8s and CCK-8ns were calculated as 92%, 89% and 90% respectively, resulting in a mean methodological loss of ~10%. HPLC recovery of endogenous CCK from plasma was 81% (n=12), indicating that ~90% [(81%/90%) x 100] of CCK -LI can be accounted for subsequent to fractionation.

HPLC Results

Fractionation by HPLC resulted in the reliable separation of CCK-4, CCK-8s and CCK-8ns. As determined by radioimmunoassay, CCK-4 was eluted at an acetonitrile concentration of 32%, corresponding to a retention time of 12 min; CCK-8s at 41%, 21 min; CCK-8ns at 44%, 24 min. Human gastrin 17-I eluted at 47% and had a retention time of 27 min (Fig. 7).

Plasma Analysis

The assay system was able to discriminate among individual cholecystokinin peptides and quantify levels in plasma. HPLC analysis of plasma extracts from 12 individual healthy subjects revealed that plasma CCK-4 eluted at an acetonitrile concentration in the range of 31-34%, CCK-8s at 41-43% and CCK-8ns at 44-45%. Retention times ranged from 11-14 min, 21-23 min and 24-25 min for endogenous CCK-4, CCK-8s and CCK-8ns respectively. Profiles were similar for male and female subjects (Fig. 8). Total baseline CCK levels (CCK-LI) from 12 healthy volunteers averaged 12.9 ± 2.1 pM CCK-4 equivalents. Concentrations of each individual peptide in plasma (n=12) were determined to be 1.0 ± 0.2 pM, 3.4 ± 0.8 pM and 1.9 ± 0.4 pM for CCK-4, CCK-8s and CCK-8ns respectively (Fig. 9). A 2 x 3 ANOVA for repeated measures revealed a global peptide effect, F (2, 20) = 7.3, p = 0.004. Multiple comparisons analysis showed that mean CCK-8s levels were significantly higher than that of CCK-8ns and CCK-4 (Fig. 10). There was no effect of gender on total or individual concentrations of CCK.

CONCLUSIONS

The present study reports the development of a novel assay system which reliably separates and quantifies CCK-4, CCK-8s and CCK-8ns in human plasma. This 3-step system involves i) extraction of CCK fragments from plasma by Sep-Pak C_{18} cartridges ii) separation of peptides by reverse phase high performance liquid chromatography iii)

detection and quantification of peptides with a double-antibody radioimmunoassay, using ^{125}I Bolton-Hunter CCK-4 as tracer.

Although radioimmunoassays have previously been employed to measure CCK, a wide range of plasma levels has been reported (Höcker *et al.*, 1992; Lemaire *et al.*, 1992; Walsh *et al.*, 1982). This may be due, in part, to a number of technical difficulties inherent to CCK determination in plasma. First, fasted circulating CCK levels in human plasma are quite low, especially if each form is measured individually, thus requiring some method of concentration. The initial step of our assay system utilizes ethanol extraction on Sep-Pak cartridges not only to concentrate the samples, but also to remove any non-specific interference derived from plasma proteins. It is also important to note that CCK is subject to rapid proteolytic cleavage if preventative measures are not taken (Koulischer *et al.*, 1982). Accordingly, protease inhibitors, anti-bacterial agents and low temperatures were employed throughout sample preparation and processing. Second, multiple forms of CCK in plasma share the common carboxy terminal sequence of Trp-Met-Asp-Phe-NH_2, and thus all forms may exhibit cross-reactivity to antisera directed against the C-terminus. In addition, circulating forms of human gastrin also contain this identical sequence. Therefore, a direct radioimmunoassay on plasma samples would not be able to discriminate among the various forms of CCK and gastrin. Analytical HPLC fractionation of extracted plasma is used in our system in order to separate these immunogenic peptides. A radioimmunoassay is the final technique used in our 3-step system. The production of a sensitive antibody which detects CCK-4, sulfated CCK-8 and nonsulfated CCK-8 with equipotency and the iodination of Bolton Hunter CCK-4,

accomplished by the conjugation of 3-(p-hydroxyphenyl)propionic acid to the CCK tetrapeptide, represent two critical components of this radioimmunoassay. This results in a sensitive radioimmunoassay capable of measuring CCK levels as low as 0.7 fmol per sample.

An analysis of plasma CCK in 12 healthy control subjects revealed that mean fasted levels of CCK-8s were significantly higher than those of CCK-8ns and CCK-4. The mean ratio of CCK-4: CCK-8S: CCK-8NS was approximately 1:3:2. However, the proportion of each peptide, particularly for the tetrapeptide, was quite variable, ranging from 6.4 - 40.4% of total CCK. Similar HPLC profiles were observed for both male and female subjects. There was no effect of gender on total or individual levels of CCK. A more complete evaluation of the ranges and distributions of CCK-like immunoreactivity in normal individuals, as well as in psychiatric samples, will require a considerably expanded population sample.

The assay system described herein will be a fundamental tool in any study which requires quantification of cholecystokinin tetrapeptide and octapeptide in human plasma. Preliminary studies in our laboratory also suggest that that CCK levels in CSF can be measured using minor modifications of this methodology. Although the evaluation of normal values and variability is important, this assay system will be of even greater interest in examining the significance of CCK in pathology. The discovery of CCK-4 as an anxiogenic peptide (Bradwejn *et al.*, 1991a; de Montigny 1989) and the recent report that central CCK levels are elevated in experimental animals subjected to threatening stimuli (Pavlasevic *et al.*, 1993) warrant an investigation of circulating tetrapeptide levels

in patients with anxiety disorders. Moreover, the somewhat unexpected reports of decreased CSF CCK-8s levels in patients with panic disorder may potentially be reconciled by the determination of tetrapeptide levels, as it has been suggested that increased CCK-4 levels — with a compensatory decrease in the octapeptide — may be the primary disparity between the control and patient groups (Lydiard *et al.*, 1992). Accordingly, a method which can reliably quantify endogenous levels of each peptide may enhance current research into the role of CCK in psychiatric disorders. CCK has also been implicated in a number of other pathologies which may also benefit by this assay. These include diabetes (Miyasaka *et al.*, 1994; Wisniewska and Wisniewska, 1982), gastrointestinal diseases (Cantor, 1987) and cardiomyopathy (Merani *et al.*, 1996).

The discrimination between CCK-8s and CCK-8ns will also comprise an important application of this assay. The two forms of the octapeptide differ with respect to bioactivity and metabolism. A bioassay based on gallbladder stimulation showed that CCK-8s is more potent than CCK-8ns (Tokunga *et al.*, 1993). Degradation studies demonstrate that CCK-8s has a longer half-life in both human and rat plasma, as compared to CCK-8ns (Koulischer *et al.*, 1982). Alternatively, *in vitro* studies reveal that sulfation may enhance cholecystokinin degradation by certain enzymes (Pauwels *et al.*, 1989). Differential metabolic clearance rates of endogenous CCK-8s and CCK-8ns may influence the bioavailability of the peptides which, in turn, may modulate their pharmacological effects *in vivo*.

Through the production of a highly sensitive antiserum raised against the CCK tetrapeptide and the synthesis of labeled Bolton Hunter CCK-4, we were able to combine

the techniques of peptide extraction, high performance liquid chromatography and radioimmunoassay to discriminate among circulating cholecystokinin and gastrin levels in plasma. This 3-step assay system, which can accurately detect and quantify CCK-4, CCK-8s and CCK-8ns, will be preliminary to further studies on the role of cholecystokinin in normal and pathological human physiology and behaviour.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Peter Schiller, in whose laboratory the BH CCK-4 synthesis was performed and Dr. Bernard Roques, for the initial preparation of BH CCK-4. We also wish to thank Dr. Suhayla Mukaddam-Daher for her invaluable guidance, as well Céline Coderre, Nathalie Charron and Dr. Jose Ma Mejia for skilled technical assistance, and Marc Dumont for statistical analysis. This research was supported by a Group Grant from the Medical Research Council of Canada and by the Réseau Santé Mentale of the Fonds de Récherche de Santé du Québec.

REFERENCES

References for this manuscript are integrated in the References Section provided at the end of this dissertation.

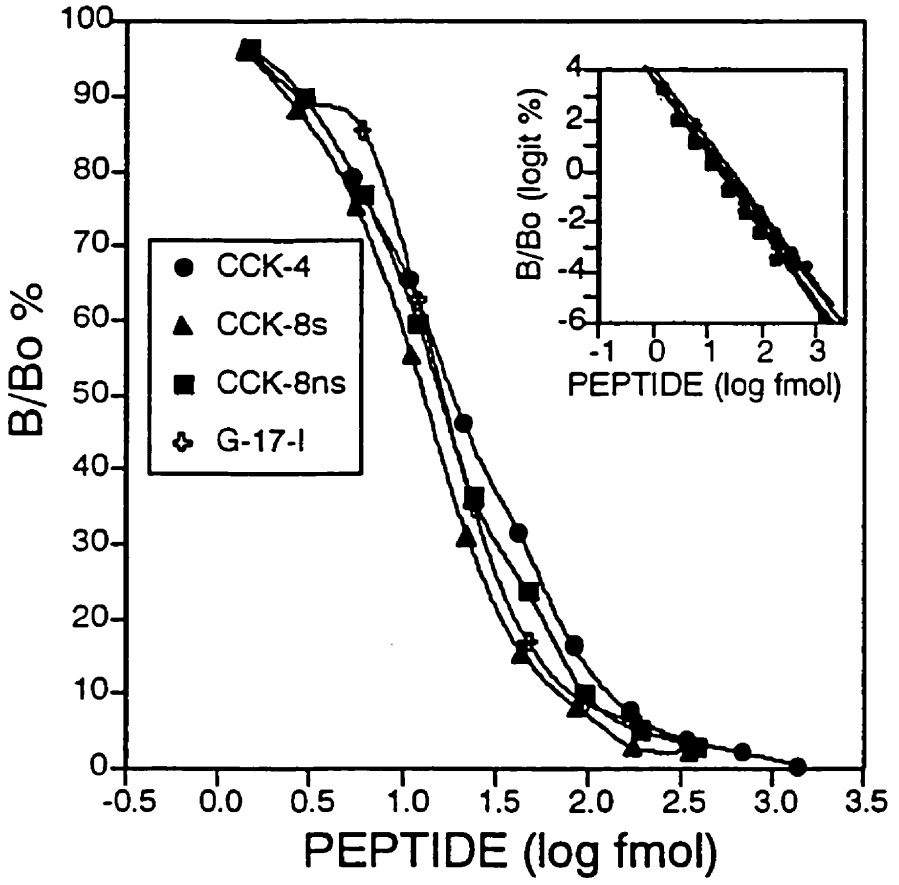

Figure 4. Cross-reactivity curves for CCK-4, CCK-8s, CCK-8ns and G-17-I. Radioimmunoassay curves using ^{125}I BH CCK-4 as tracer shows that the antibody recognizes each peptide with equipotency. The insert depicts a linear transformation of the data.

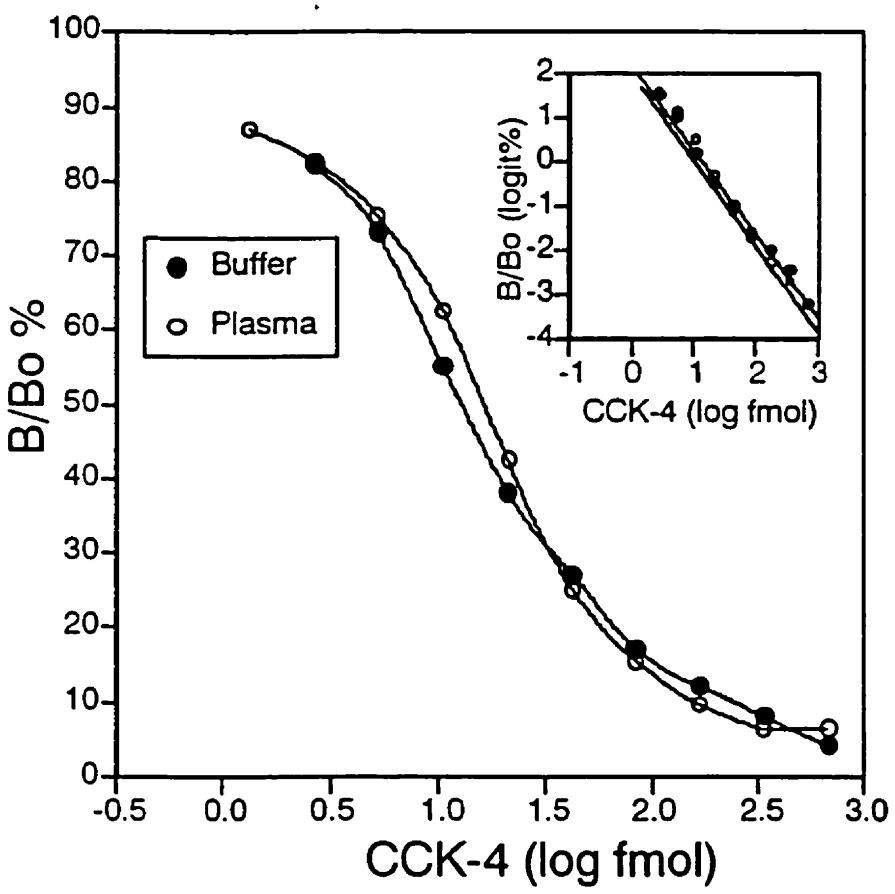

Figure 5. Standard curve of the CCK-4 radioimmunoassay. Serial dilution of plasma extracts showed parallelism to the CCK-4 standard curve. The insert depicts a linear transformation of the data.

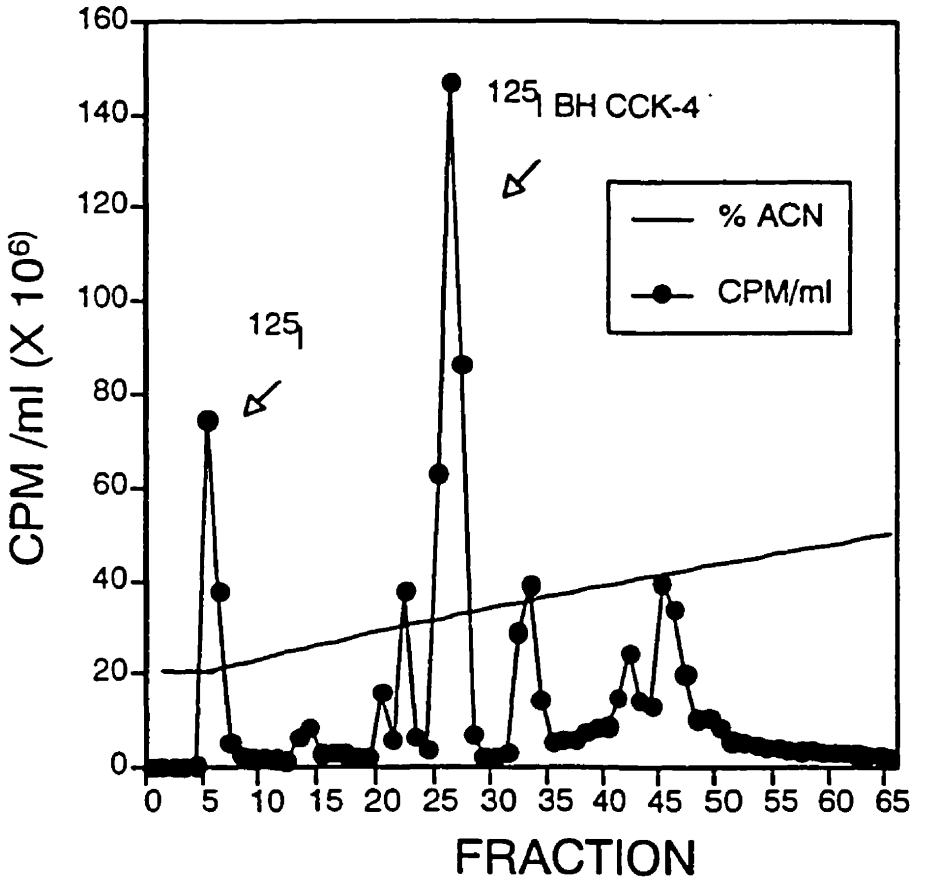

Figure 6. Purification of ^{125}I BH CCK-4 by HPLC. The first peak following elution of free iodine (^{125}I) comprises the monoiodinated tracer.

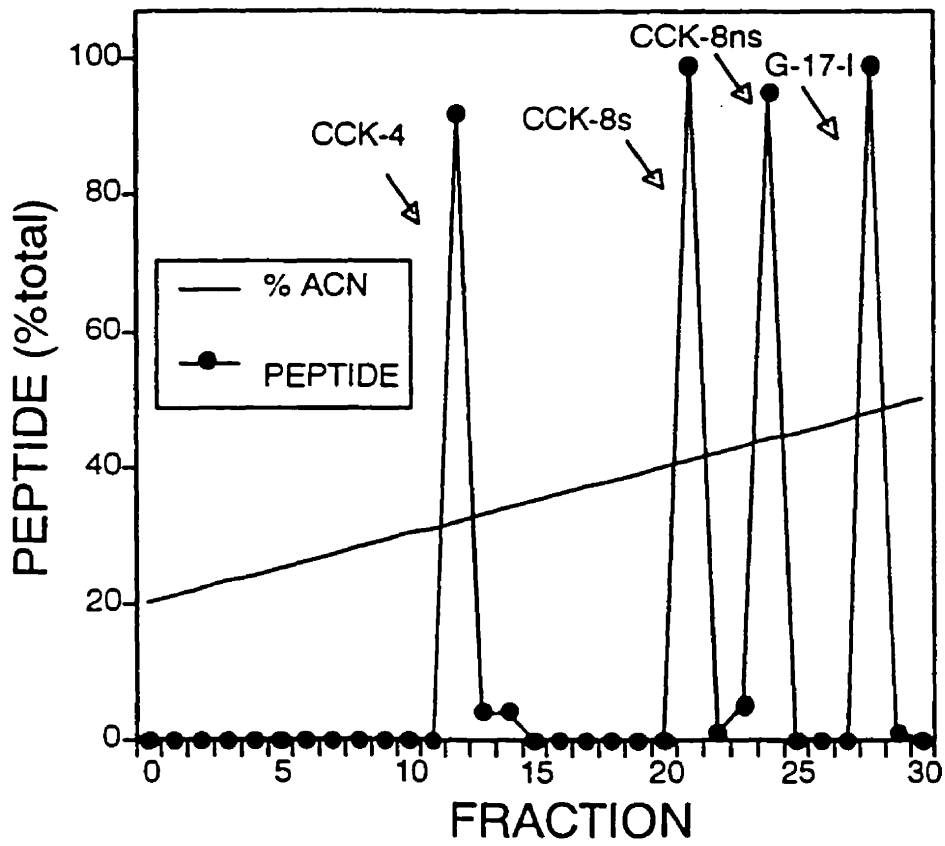

Figure 7. Separation of standard preparations of CCK-4, CCK-8s, CCK-8ns and G-17-I by HPLC using a 20 - 50% acetonitrile (ACN) gradient. Arrows indicate elution of individual peptides.

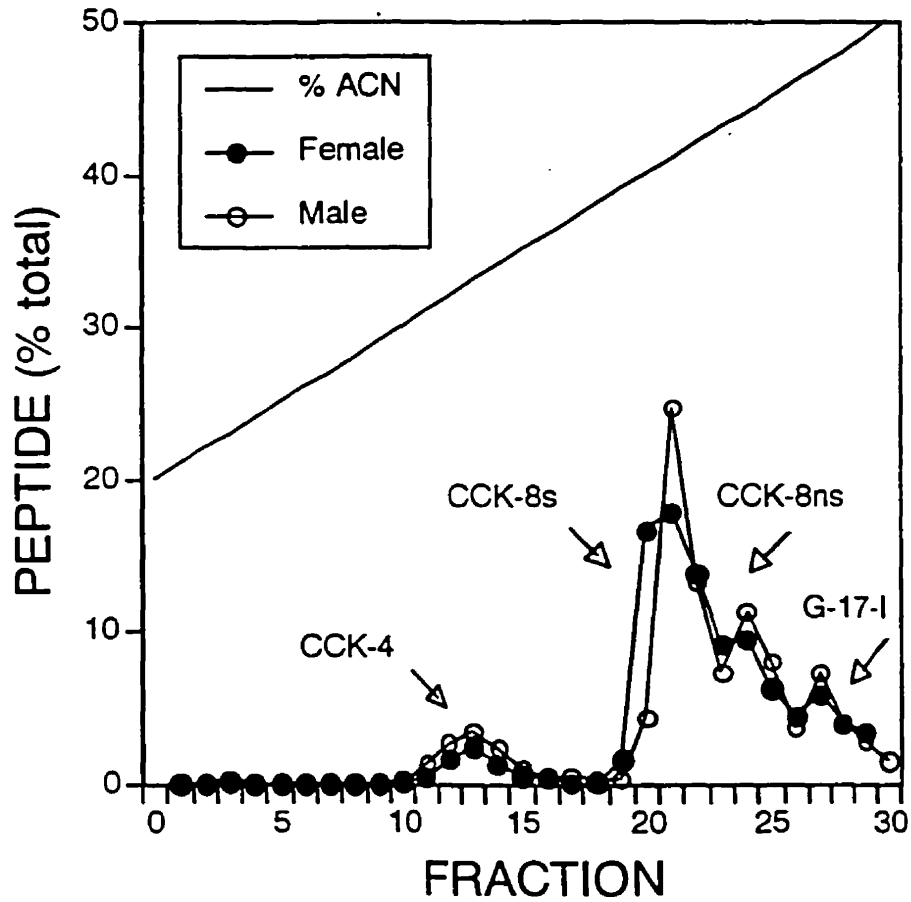

Figure 8. Separation of CCK-4, CCK-8s, CCK-8ns and G-17-I in the plasma of 6 healthy female and 6 healthy male volunteers by HPLC using a 20 - 50% acetonitrile (ACN) gradient. Arrows indicate elution of individual peptides.

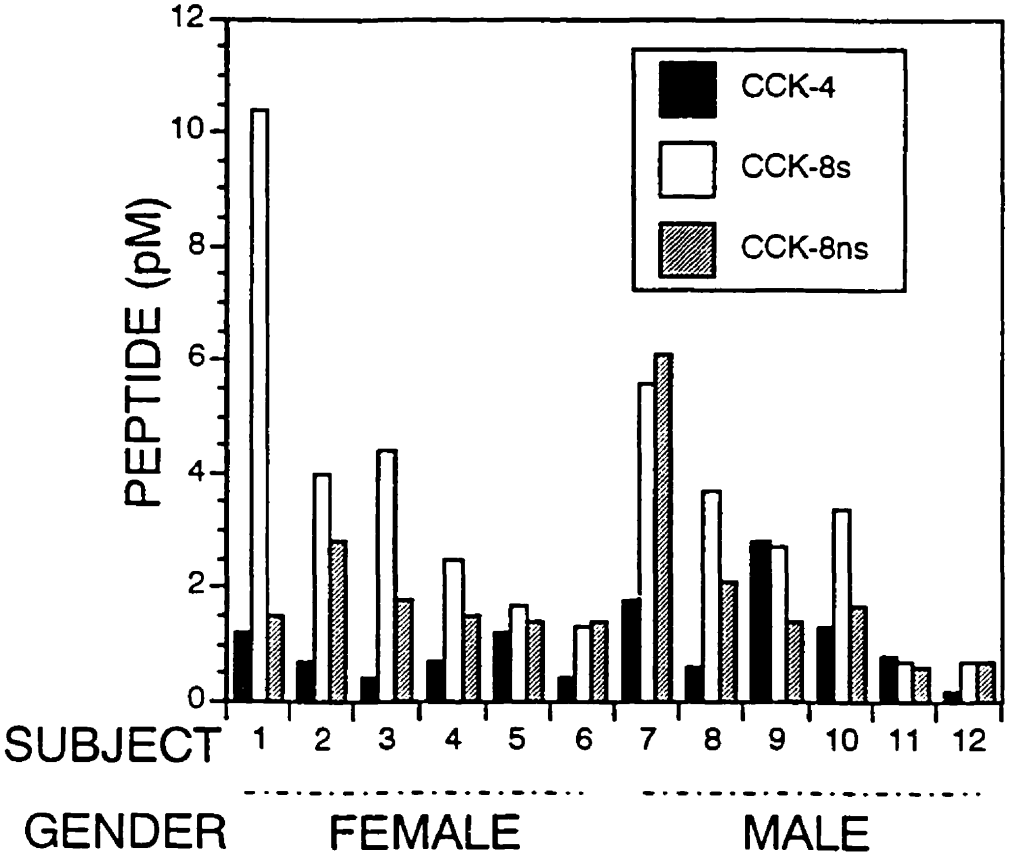

Figure 9. Individual plasma levels of CCK-4, CCK-8s and CCK-8ns from 6 healthy females (subjects 1-6) and 6 healthy males (subjects 7-12).

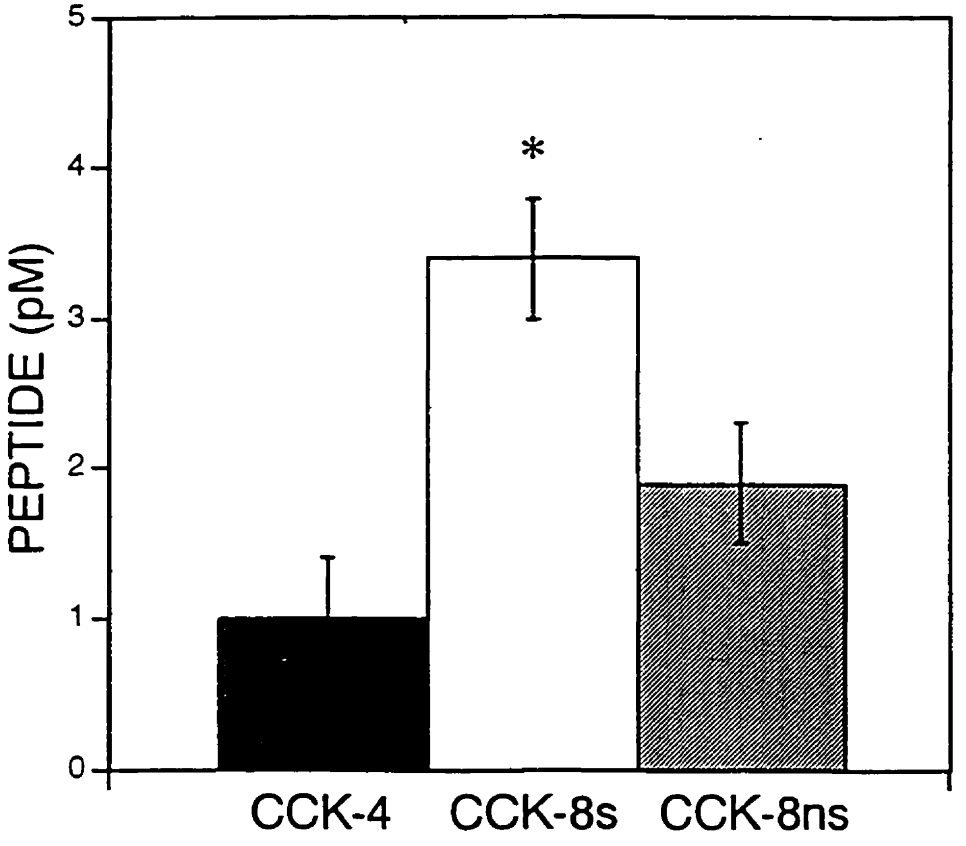

Figure 10. Mean plasma levels of CCK-4. CCK-8s and CCK-8ns in 12 healthy volunteers. Values reported as mean ± SEM. *CCK-8s > CCK-8ns, CCK-8s > CCK-4 at $p < 0.05$.

6.5 Cholecystokinin Assays—A Critical Analysis

It is undisputed that the cholecystokinergic system plays an important role in the regulation of various gastrointestinal and neurobiological processes. In order to fully understand the effects of this system in normal physiology and in pathology, scientists must be able to accurately quantify and characterize cholecystokinin peptides in healthy control subjects and in affected patient populations. Accordingly, the development of an assay system that is able to reliably quantify the different forms of cholecystokinin peptides found in plasma, cerebrospinal fluid and other tissues is critical to any study which aims to investigate the role of cholecystokinin in physiology and behaviour.

The assay system developed in our laboratory, which involved the extraction of CCK fragments using reverse phase chromatography, the separation of peptides by high performance liquid chromatography and the detection and quantification of peptides with a double-antibody radioimmunoassay, is capable of detecting and discriminating among physiological concentrations of CCK-4, CCK-8s and CCK-8. Although the development of our assay system represents a great advance over currently available techniques (as critiqued above in Sections 6.2 - 6.3), our system is also subject to a number of limitations.

Although our assay system permits identification of individual peptide levels, a large volume of blood is required (approximately 200 mls) to establish a complete profile of these peptides. This limitation makes it difficult to obtain multiple samples at short time intervals, thus renderingdetailed evaluation of time-course studies in human subjects

extremely difficult. Moreover, the large volume of plasma needed also restricts studies on small mammals, especially rodents. Future refinements to our assay system should include techniques which yield reliable results with smaller sample volumes.

Further, although our results revealed no effect of gender on plasma cholecystokinin levels, future studies must evaluate cholecystokinin levels in a larger population size. Future studies should also include individuals of diverse ages and ethnicities in order to uncover any possible effects of age or race on cholecystokinin levels. Such comprehensive studies would serve to establish cholecystokinin levels in a healthy population and, through comparative analysis, would greatly facilitate investigations into the role of the cholecystokinergic system in pathology.

Finally, like most scientific accomplishments, major advances in the field of cholecystokinin research will certainly be predicated upon the contributions of hundreds of different researchers and laboratories around the world. In order to fully take advantage of this international data, it is essential that the tools and techniques used to quantify and characterize cholecystokinin levels be standardized. The dearth of standardized protocols continues to be a significant obstacle in cholecystokinin research; that is, it has been unduly challenging to extrapolate cholecystokinin data from one study to the next because different laboratories are measuring different forms of cholecystokinin depending on the methods they are employing. In the interest of effective and efficient scientific pursuit, laboratories committed to cholecystokinin research should use a standardized approach when measuring cholecystokinin concentrations. Despite its limitations, our assay system represents the most accurate and reliable technique currently

available to quantify and characterize cholecystokinin peptides, and it would behoove cholecystokinin laboratories, both nationally and internationally, to adopt our assay system, or some improved modified version thereof.

CHAPTER SEVEN

CHOLECYSTOKININ AND EATING DISORDERS

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7.1 Introduction

The cholecystokinergic system plays an important role in the integration of various digestive processes. By coordinating the contraction of the gallbladder, relaxation of the sphincter of Oddi, release of bile and stimulation of pancreatic enzymes, cholecystokinin is essential in the digestion, mobilization and metabolism of nutrients (Cantor, 1989; Crawley and Corwin, 1993; Louie, 1994). Several studies have demonstrated that cholecystokinin serves as a biological mediator of feeding behaviour. Systemic or central administration of cholecystokinin has been shown to reduce food consumption in several animal species (Della Ferra and Baile, 1981; Sills and Vaccarino, 1991; Voigt *et al.*, 1995), including human subjects (Ballinger and Clark, 1994; Tamai *et al.*, 1993; Lieverse *et al.*, 1994). Cholecystokinin is released into the bloodstream by various nutrients (Himeno *et al.*, 1983; Höcker *et al.*, 1992; Liddle *et al.*, 1985), and postprandial endogenous CCK release has been associated with subjective ratings of satiety (Ballinger and Clark, 1994; Holt *et al.*, 1992). In light of substantial evidence implicating cholecystokinin in satiety, a number of researchers have hypothesized that a dysregulation of cholecystokinergic system is involved in the pathology of eating disorders (Brambilla *et al.*, 1995a; Devlin *et al.*, 1997; Lydiard *et al.*, 1993).

Anorexia nervosa and bulimia nervosa are the two primary eating disorders which are described in the Diagnostic and Statistical Manual (DSM-IV, American Psychiatric Association, 1994). A limited number of studies have examined either basal or meal-stimulated cholecystokinin levels in these illnesses to test the theory that a dysregulation of cholecystokinin exists in anorexia or bulimia. Our laboratory is currently participating in a study involving the analysis of plasma cholecystokinin levels in bulimic women during various stages of their pathology.

7.2 Clinical Features of Eating Disorders

Anorexia Nervosa

Anorexia nervosa is an eating disorder which is characterized by a refusal to maintain a minimally normal body weight, accompanied by a fear of gaining weight and a distorted perception of the size or shape of one's body. The illness typically appears in early to middle adolescence (Table 1). According to a Harvard Mental Health Report (1997), the prevalence of anorexia nervosa in the United States is estimated at 0.1% to 0.6% in the general population, with the rate being several times higher in adolescent girls. Approximately 90% - 95% of subjects affected with anorexia are female (Mauri *et al.*, 1996). According to the diagnostic manual of the American Psychiatric Association (Diagnostic and Statistical Manual of Mental Disorders, or DSM-IV, 1994), a clinical diagnosis of anorexia is made when an individual's weight has decreased to 15% below the normal range, he or she exhibits an intense fear of gaining weight, has a disturbed

perception of his or her body image and for the female subject, has amenorrhea for at least 3 months (Table 1). Other symptoms may include lethargy, withdrawal, anemia, hypercholesterolemia, edema and dry skin. Anorectic women exhibit a marked reduction in female hormones and may exhibit a delay in sexual development. Decreased heart rate and blood pressure may also occur. Occasionally, irregular heart rhythms result from a potassium deficit. Osteoporosis and kidney damage are observed in some patients. Mortality in anorexia nervosa is estimated at 5% - 10% of female cases. The main causes of death are infections or cardiac failure. Family studies indicate that a genetic component exists in anorexia nervosa (DSM-IV, 1994).

Bulimia Nervosa

Bulimia nervosa, as defined by the DSM-IV, is an eating disorder characterized by two or more episodes of binge eating every week for at least three months. Binge eating is the rapid consumption of a large amount of food accompanied by a feeling of loss of control. After an episode of binge eating, the bulimic patient frequently engages in vomiting or purging (use of laxatives or diuretics). This cycle may also include periods of compulsive exercise and fasting (Table 2). Bulimia is at least two or three times as common as anorexia nervosa. According to the DSM-IV, the prevalence of bulimia among adolescent and young adult females is estimated to be as high as 3%. At least 90% of bulimic patients are women. The symptoms of bulimia nervosa usually develop in late adolescence to early adulthood. Physical symptoms of bulimia include

fatigue, edema, electrolyte imbalance, enlarged salivary glands, erosion of dental enamel and sore throat from vomiting. Prolonged use of laxatives may result in gastrointestinal discomfort and other digestive difficulties. Dehydration, potassium depletion, and tearing of the esophagus may also occur. The long-term outcome of bulimia is not well established. A genetic component has been postulated in the illness (DSM-IV, 1994).

Table 1. Diagnostic criteria for anorexia nervosa. Adapted from the Diagnostic and Statistical Manual (American Psychiatric Association, 1994).

A. Refusal to maintain body weight at or above a minimally normal weight
for age and height (e.g., weight loss leading to maintenance of body weight less than 85% of that expected; or failure to make expected weight gain during period of growth, leading to body weight less than 85% of that expected).

B. Intense fear of gaining weight or becoming fat, even though underweight.

C. Disturbance in the way in which one's body weight or shape is experienced, undue influence of body weight or shape on self-evaluation, or denial of the seriousness of the current low body weight.

D. In postmenarcheal females, amenorrhea, i.e., the absence of at least three consecutive menstrual cycles. (A woman is considered to have amenorrhea if her periods occur only following hormone, e.g., estrogen,
administration.)

Specific type:

* Restricting Type: During the current episode of Anorexia Nervosa, the person has not regularly engaged in binge-eating or purging behaviour (i.e., self-induced vomiting or the misuse of laxatives, diuretics, or enemas)

* Binge-Eating/Purging Type: During the current episode of Anorexia Nervosa, the person has regularly engaged in binge-eating or purging behaviour (i.e., self-induced vomiting or the misuse of laxatives, diuretics, or enemas)

Table 2. Diagnostic criteria for bulimia nervosa. Adapted from the Diagnostic and Statistical Manual (American Psychiatric Association, 1994).

A. Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following:
(1) eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than most people would eat during a similar period of time and under similar circumstances
(2) a sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating)

B. Recurrent inappropriate compensatory behaviour in order to prevent weight gain, such as self-induced vomiting; misuse of laxatives, diuretics, enemas, or other medications; fasting; or excessive exercise.

C. The binge eating and inappropriate compensatory behaviours both occur,
on average, at least twice a week for 3 months.

D. Self-evaluation is unduly influenced by body shape and weight.

E. The disturbance does not occur exclusively during episodes of Anorexia
Nervosa.

Specific type:

* Purging Type: during the current episode of Bulimia Nervosa, the person has regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics, or enemas

* Nonpurging Type: during the current episode of Bulimia Nervosa, the person has used other inappropriate compensatory behaviours, such as fasting or excessive exercise, but has not regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics, or enemas

7.3 Evidence of Cholecystokinin as an Endogenous Satiety Hormone

Several lines of evidence support the contention that cholecystokinin plays an important role in satiety. Administration of exogenous cholecystokinin has been shown to decrease food intake in a variety of species (Baldwin, 1992; Della Ferra and Baile, 1981; Gibbs *et al.*, 1973; Sills and Vaccarino, 1991; Voigt *et al.*, 1995). In humans, CCK infusions have elicited a decrease in subsequent caloric intake, as well as an increase in subjective ratings of satiety (Ballinger and Clark, 1994; Holt *et al.*, 1992; Lieverse *et al.*, 1994). For example, infusions of CCK-33 in both lean and obese subjects caused significant decreases in feelings of hunger and desire to eat as compared to saline infusions. Subjects also consumed fewer calories if they had received CCK, rather than saline, fifteen minutes prior to being presented a test meal (Lieverse *et al.*, 1994).

To determine whether *endogenous* cholecystokinin acts as a true satiety hormone, Ballinger and Clark (1994) manipulated the release of endogenous CCK by stimulating its release *in vivo*. Healthy volunteers were orally administered L-phenylalanine, a potent stimulator of cholecystokinin release, D-phenylalanine (a weak CCK stimulant), or placebo. Twenty minutes later, when endogenous CCK levels were at peak concentrations, the subjects were presented with a standard test meal. Analysis of plasma cholecystokinin revealed that only L-phenylalanine significantly increased CCK levels from basal values at twenty minutes after administration. Subjects who had ingested L-phenylalanine prior to the test meal consumed significantly fewer calories (1,089 ± 86 kcal) as compared to those in the D-phenylalanine (1,492 ± 126 kcal) or placebo (1,587 ± 174) groups. The reduction in caloric consumption after L-phenylalanine also coincided

with a greater sensation of satiety. Although this experiment would be strengthened by replication with a specific CCK antagonist, these results nonetheless suggest that endogenous cholecystokinin is a major satiety hormone in humans (Ballinger and Clark, 1994).

7.4 Dysregulation of the Cholecystokinergic System in Eating Disorders

Anorexia nervosa and bulimia nervosa are characterized by abnormal eating patterns and dysregulated satiety. Because cholecystokinin is implicated in the regulation of satiety and feeding behaviour, many researchers, including our own research team, have postulated that the cholecystokinergic system is involved in the pathophysiology of these diseases. The etiology of anorexia and bulimia is not well understood (Brambilla *et al.*, 1995a; Brambilla *et al.*, 1995b; Devlin *et al.*, 1997; Geary, 1998). Alterations in various neurotransmitters, neuropeptides and peripheral hormones have been implicated in the biological basis of eating disorders, though a concrete causal connection has yet to be established (Brambilla *et al.*, 1995a; 1995b; Kaye *et al.*, 2000).

In a study designed to determine whether plasma levels of cholecystokinin were abnormal in women with anorexia nervosa, Tamai and co-workers (1993) discovered that basal concentrations of plasma CCK were significantly higher in patients than in age-matched, healthy female subjects. The authors postulated that elevated basal CCK levels, which normally signal satiety to the hypothalamus, result in premature satiety in anorexic patients. The same researchers also tested cholecystokinin levels after the anorexic patients underwent nutritional and cognitive therapy. After partial restoration of body

weight, the mean basal concentrations of cholecystokinin approached that of the control group (Tamai *et al.*, 1993). This finding seems to suggest that abnormal cholecystokinin levels are a consequence of the disorder (i.e. a state marker), rather than an indicator of genetic vulnerability (i.e. a trait marker), and may be involved in the perpetuation of anorexia rather than its pathogenesis.

In addition to analysis of cholecystokinin levels in plasma, CCK concentrations have also been examined in T-lymphocytes, peripheral cells which have been reported as reflecting brain neuronal secretion and regulation (Brambilla *et al.*, 1995b). Basal cholecystokinin octapeptide concentrations in T-lymphocytes were significantly lower in patients with anorexia nervosa than in healthy subjects (Brambilla *et al.*, 1995a). In a complementary study, the same authors found that CCK-8 concentrations were also lower in women with bulimia nervosa (Brambilla *et al.*, 1995b). After four months of treatment with fluvoxamine, a drug with serotonergic stimulating activity, CCK-8 values in bulimic patients approached normal levels. Interestingly, treatment with amineptine, a drug with dopaminergic stimulating activity, had no effect on CCK-8 levels (Brambilla *et al.*, 1995b). This finding suggests that the cholecystokinergic and serotonergic systems may interactively influence the pathology of bulimia. For a review on the complex modes of interaction between these two systems, see Chapter Four, Section 4.3.

Another study examined the central functioning of the cholecystokinergic system by analyzing CCK levels in cerebrospinal fluid. Lydiard and colleagues discovered that levels of CCK-8 in cerebrospinal fluid were significantly lower in women with bulimia nervosa as compared to healthy subjects (Lydiard *et al.*, 1993).

In order to test the theory that patients with eating disorders have an abnormal cholecystokinergic response after the ingestion of a meal, a few researchers have measured postprandial CCK levels. Devlin and colleagues examined plasma cholecystokinin levels and gastric emptying after bulimic and control women consumed radiolabeled liquid meals. Patients with bulimia nervosa demonstrated a blunted postprandial cholecystokinin release and delayed gastric emptying, as compared to healthy volunteers (Devlin *et al.*, 1997). In another study, postprandial plasma cholecystokinin levels were measured in bulimic and anorexic women. After ingestion of a protein- and fat-rich test meal, CCK-8s plasma levels were elevated in anorexic and control women, but not in patients with bulimia (Pirke *et al.*, 1994). Geracioti and co-workers demonstrated that patients with bulimia had impaired postprandial cholecystokinin secretion which was correlated with a reduced sense of subjective fullness. Amelioration in this blunted cholecystokinin response, as well as subjective reports of satiety, were observed after treatment with tricyclic antidepressants. This latter finding suggests that cholecystokinin interacts with other neurotransmitters in the maintenance and progression of the disorder (Geracioti *et al.*, 1989).

A Pilot Study: Preliminary Findings

In a preliminary study of patients with bulimia nervosa, we compared cholecystokinin-like immunoreactivity in four women with bulimia nervosa and eight healthy volunteers. We observed that fasted plasma CCK-LI levels were approximately

five fold lower in bulimic woman as compared to healthy control subjects (Table 3). However, at the post-binge stage, CCK levels in bulimic women were similar to those found in control women, tested postprandially. These initial data suggest that abnormal cholecystokinin levels may contribute to overeating in bulimia nervosa (McConaha *et al.*, 1998). Although provocative, these findings must obviously be replicated in a larger sample of patients and controls prior to publication.

7.5 Conclusions and Critical Analysis

Our results indicated that fasted plasma CCK-LI levels were nearly five times lower in bulimic women as compared to healthy control subjects. However, postprandially, (i.e., after bingeing), cholecystokinin levels in bulimic women normalized to levels comparable to that of control women. These preliminary findings indicate that cholecystokinin may specifically contribute to the overeating phase in bulimia nervosa {contribute how? do you mean that failure of the normal cck satiety effect could underlie overeating?}.

Because our research into the role of the cholecystokinergic system in bulimia nervosa was a pilot study, an evaluation of a larger population of bulimic and healthy patients is essential. Although 90% of bulmic patients are female, a truly comprehensive study of bulimia nervosa should include not only a larger population sample, but also a comparison of CCK levels in male bulimic subjects. Studies which include affected males may help illuminate the large gender discrepancy in this disease.

Future studies should also include a comparison study in patients with anorexia nervosa. As opposed to patients with bulimia nervosa, patients with anorexia nervosa do not maintain a sufficient caloric intake. Because cholecystokinin has long been established as a potent satiety hormone, it would be interesting to determine whether elevated levels of cholecystokinin are observed in anorexic patients. It would also be intriguing to examine whether, after eating, cholecystokinin levels in anorectics also normalized.

It is important to note that the binge phase in this study was designed to simulate the physiological and psychological aspects of the over-eating phase in bulimia as accurately as possible. However, certain psychological and social factors that frequently accompany the binge phase could not be replicated due to the constraints of the clinical setting. For example, binge eating typically occurs in private because the binge eater is embarrassed and attempts to conceal his or her symptoms (Clark 1986, DSM-IV, 1994). Further, binges may be initiated by feelings of depression, anxiety, boredom or loneliness (Clark 1986). It remains unclear whether cholecystokinin levels observed in a simulated laboratory binge are identical to CCK levels occurring during a natural binge. Future research in this field should include a comprehensive psychological study that is conducted in parallel to the biological and pharmacological studies.

Finally, although it is uncontroverted that the cholecystokinergic system plays an important role in satiety, the precise role of CCK in eating disorders has not yet been established. There is some evidence that, in eating disorders, basal values of cholecystokinin are abnormal; other studies indicate that the CCK response after

ingestion of a meal may be aberrant. Conflicting results in this field of study may be attributed to the fact that CCK concentrations may differ depending on whether the source of the measured peptide was central (lymphocytes or cerebrospinal fluid) or peripheral (plasma). Moreover, various researchers have employed different methods of quantifying cholecystokinin levels. While some researchers may have been measuring total cholecystokinin-like immunoreactivity, other laboratories may only have been measuring specific fragments, depending on the antibody used to detect the peptide. Moreover, analytic methods which do not employ a reliable method of separation such as HPLC, may lead to inaccurate and misleading measurements of cholecystokinin levels (for further discussion, see Chapter Six, Section 6.1 – 6.3).

Although total cholecystokinin-like immunoreactivity was measured in our preliminary study of bulimia nervosa, it is imperative that future studies measure individual CCK peptide levels in plasma. The method described by Merani *et al.*, 1997 will provide a more accurate assessment of the role of cholecystokinin in eating disorders and other illnesses because our combined radioimmunoassay and HPLC method permits the quantification of CCK-4, CCK-8s and CCK-8ns. Our method is preferable because it permits the investigation into possible abnormalities of the ratio between CCK-4 and CCK-8, facilitating the evaluation of a possible reciprocal relationship between the two peptides.

Table 3. Plasma cholecystokinin levels (CCK-LI) in women with bulimia nervosa and control women. Fasted, 5 Minutes Post-Meal and 10 Minutes Post-Meal values shown for controls; Fasted, Post-Binge and Post-Purge values shown for bulimic group.

Stage	Control Women CCK-LI (pg/ml)	Bulimic Women CCK-LI (pg/ml	p-value
Fasted Baseline	12.09	2.5	0.01
+ 5 Minutes/ Binge	29.99	22.38	ns
+ 30 Minutes / Purge	30.33	16.75	ns

CHAPTER EIGHT

CHOLECYSTOKININ AND PREMENSTRUAL DYSPHORIC DISORDER

CHAPTER EIGHT

CHOLECYSTOKININ AND PREMENSTRUAL DYSPHORIC DISORDER

8.1 Introduction

The American Psychiatric Association recently recognized premenstrual dysphoric disorder (PMDD) as a "depressive disorder not otherwise specified," and included it in the appendix of the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 1994). Premenstrual dysphoric disorder replaces the previously named late luteal phase dysphoric disorder (LLPDD). PMDD is characterized as a pattern of severe, recurrent somatic and emotional symptoms that occur during the last week of the menstrual cycle and significantly interfere with daily living. These symptoms typically remit within a few days following onset of menstruation. Various physical and psychological complaints, such as depression, anxiety and mood swings, are frequently reported by young women during the premenstrual phase. Approximately 3-5% of this population experiences these symptoms in a manner severe enough to be diagnosed with premenstrual dysphoric disorder (Yonkers, 1997). Criteria for PMDD are listed in Table 4.

It has been postulated that premenstrual symptoms are an expression of a diverse set of vulnerability traits that surface in response to a trigger (Halbreich, 1997). The overlap of clinical features in PMDD and anxiety has led to speculation that some predictors of vulnerability to premenstrual syndromes may also increase vulnerability to

generalized anxiety disorders (Halbreich, 1997). These traits could include personality, cognitive function, and neurotransmitter function. Because stress and anxiety are salient features of premenstrual dysphoric disorder, a potential association between anxiety disorders and PMDD has been suggested (Halbreich, 1997; Hurt *et al.*, 1992; Yonkers, 1997). Women with PMDD have a higher lifetime prevalence of psychiatric illness, in which past histories of anxiety disorders are common, further implicating an underlying common etiology between the two illnesses (Yonkers, 1997).

An association between anxiety disorders and PMDD may also be deduced from reports which indicate that treatments which are effective for anxiety disorders are also efficacious in PMDD (Yonkers, 1997). These treatments include pharmacological therapies such as serotonin reuptake inhibitors, which increase the availability of serotonin in the brain. Serotonin reuptake inhibitors such as fluoxetine and paroxetine, have been efficacious in reducing premenstrual dysphoric symptoms such as irritability, depressed mood, increase in appetite, and anxiety in women affected by PMDD (Sundblad *et al.*, 1997; Steiner *et al.*, 1995). A pilot study with fluvoxamine, another a serotonin reuptake inhibitor, showed similar promising results (Freeman *et al.*, 1996). These findings have led to the hypothesis that an underlying serotonergic dysregulation is common to both anxiety disorders and PMDD (Steiner *et al.*, 1995).

Although premenstrual dysphoric disorder has only recently been recognized by in the DSM-IV, a number of studies establish a link between PMDD and anxiety and also support the involvement of serotonergic involvement. However, as discussed in the

following section (Section 8.2), there has been relatively little research into the putative role of cholecystokinin in PMDD.

Table 4. DSM-IV Criteria for Premenstrual Dysphoric Disorder. Adapted from the Diagnostic and Statistical Manual (American Psychiatric Association, 1994).

A. In most menstrual cycles during the past year, five (or more) of the following symptoms were present during the last week of the luteal phase and remitted within a few days after the onset of the follicular phase‡, and were absent in the week postmenses, with at least one of the symptoms being either (1), (2), (3), (4) below.

1. Markedly depressed mood, feelings of hopelessness, or self-deprecating thoughts
2. Marked anxiety, tension, feelings of being "keyed up" or "on edge"
3. Marked affective lability, e.g., feeling suddenly sad, tearful, or increased sensitivity to rejection
4. Persistent and marked anger or irritability or increased interpersonal conflicts
5. Decreased interest in usual activities, e.g., work, school, friends, hobbies
6. Subjective sense of difficulty in concentrating
7. Lethargy, easy fatigability or marked lack of energy
8. Marked change in appetite, overeating, or specific food cravings
9. Hypersomnia or insomnia
10. Subjective sense of being overwhelmed or out of control
11. Other physical symptoms, such as breast tenderness or swelling, headaches, joint or muscle pain, a sensation of "bloating," weight gain

B. The disturbance markedly interferes with work or school or with usual social activities and relationships with others, e.g., avoidance of social activities, decreased productivity and efficiency at work or school

C. The disturbance is not merely an exacerbation of the symptoms of another disorder, such as major depression, panic disorder, dysthymia (chronic mild depression), or a personality disorder (although it may be superimposed on any of these disorders)

E. Criteria A, B, and C must be confirmed by prospective daily self-ratings during at least two consecutive symptomatic cycles. (This diagnosis may be made provisionally prior to this confirmation.)

‡ In menstruating females, the luteal phase corresponds to the period between ovulation and the onset of menses, and the follicular phase begins with menses. In non-menstruating females (e.g., those who have had a hysterectomy), the timing of luteal and follicular phases may require measurement of circulating reproductive hormones (DSM-IV, 1994).

8.2 The Role of Cholecystokinin in PMDD

In light of the overwhelming evidence which establishes a close relationship between the cholecystokinergic and serotonergic systems (see Chapter Four, Section 4.3), there is a surprising paucity of studies which examine the role of the cholecystokinergic system in premenstrual dysphoric disorder. This void in the literature, combined with evidence that links PMDD with anxiety, and anxiety with cholecystokinin (see Chapter Nine), prompted our investigation of the cholecystokinergic system in PMDD

Nonetheless, a few studies have suggested that, like patients with panic disorder (Bradwejn *et al.*, 1991), women with PMDD exhibit enhanced sensitivity to panicogenic challenges. Both sodium lactate infusions and 35% CO_2 inhalation caused greater anxiety and panic symptoms in PMDD women than in control subjects (Harrison *et al.*, 1989; Sandberg *et al.*, 1993). In the article included at the end of this Chapter (*Sensitivity to CCK-4 in Women with and without Premenstrual Dysphoric Disorder (PMDD) During Their Follicular and Luteal Phases*, Le Mellédo *et al.*, 1998, Section 8.3), we have expanded upon preliminary observations by Le Mellédo and colleagues (1995) which indicated that PMDD women also exhibited heightened sensitivity to an intravenous administration of CCK-4. Moreover, in order to conduct a comprehensive investigation into the putative role of the cholecystokinergic system in premenstrual dysphoric disorder, we decided to measure cholecystokinin plasma levels in PMDD women as compared to healthy control subjects.

In order to assess the effect of the phase of the menstrual cycle, CCK-4 was administered during both the follicular and luteal phase. Endogenous fasted plasma

cholecystokinin levels were also measured during both phases. Cholecystokinin-like immunoreactivity was determined, as the volume of plasma was insufficient to assess the concentration of individual peptides (CCK-4, sulfated CCK-8s and nonsulfated CCK-8). The results of our study are described at length in the article following this section (Le Mellédo *et al.*, 1998). Briefly, we demonstrated that women with PMDD exhibited a heightened sensitivity to CCK-4 administration during both the follicular and luteal phases of the menstrual cycle. Following CCK-4 injections, PMDD patients had higher rates of panic, elevated anxiety scores, and longer durations of panic symptoms. A comparison of the menstrual phases demonstrated that PMDD women were more sensitive to CCK-4 during the late luteal phase than during mid-follicular phase. PMDD women displayed a greater anxiety and cardiovascular response in the luteal phase. This is consistent with the clinical course of the illness in which PMDD symptoms are evident during the late luteal phase and remit soon after onset of menses (DSM-IV, 1994).

We did not observe an effect of either diagnosis or phase on plasma cholecystokinin levels. However, independent of phase or diagnosis, basal CCK-LI concentrations were higher in women during their first visit to the clinic during which they received their first CCK-4 injection, than during their subsequent visit to receive their second CCK-4 injection. This significant "visit" effect may be explained by a surge in plasma cholecystokinin as a consequence of anticipatory anxiety. Uncertainty about the effects of the initial administration of CCK-4 presumably presented a novel and stressful event which may have led to increased arousal and apprehension. This heightened

affective state may have, in turn, been reflected in higher circulating endogenous cholecystokinin levels.

Our findings are relevant for investigators conducting clinical studies in the field of cholecystokinin research, particularly with respect to the methodology employed in future experiments. The finding that plasma cholecystokinin levels do not vary with menstrual phase will be important to researchers who were previously uncertain about the potential confounding effects of menstrual phase in the interpretation of cholecystokinin data. In addition, this finding will provide a basis for scientists to reconsider the typical exclusion of female subjects from their studies on grounds that menstrual phase may affect cholecystokinin levels. Finally, our observation that anticipatory anxiety can affect plasma cholecystokinin levels must be considered as a confounding variable in studies which undertake to evaluate cholecystokinin plasma levels in human subjects.

8.3 Manuscript—Sensitivity To CCK-4 In Women With and Without Premenstrual Dysphoric Disorder (PMDD) During Their Follicular and Luteal Phases

Sensitivity To CCK-4 In Women With and Without Premenstrual Dysphoric Disorder (PMDD) During Their Follicular and Luteal Phases

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ABSTRACT

The authors determined whether women with premenstrual dysphoric disorder (PMDD) exhibit a heightened sensitivity to the panicogenic effects of CCK-4 administration and whether this enhanced sensitivity to CCK-4 would vary with the phase of the menstrual cycle at the time of CCK-4 injection. Twenty-one normal controls and 18 PMDD women were randomly assigned to receive the first and second CCK-4 injection during the follicular phase and the luteal phase or vice versa. Results: PMDD women showed a greater anxiety and panic response to CCK-4. These preliminary results suggest that the CCK-B system may play a role in the pathophysiology of PMDD.

Key words: ***Premenstrual; Cholecystokinin; Anxiety; Panic; Women***

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INTRODUCTION

Premenstrual dysphoric disorder (PMDD) is a clinical syndrome characterized by significant mood, behavioural, and somatic symptoms. These symptoms regularly occur in the late luteal phase (LP) of the menstrual cycle, begin to remit after the onset of menstrual bleeding, and are typically absent in the week following menses. Anxiety appears to be a salient characteristic of the disorder (Hurt *et al.*, 1992). Stein *et al.*, (1989)

reported that women with premenstrual disturbances who were free of any concurrent psychiatric disorder exhibited marked increases in self-rated anxiety in the premenstrual phase of the cycle. Other investigators have found that a substantial proportion of women diagnosed with severe premenstrual symptoms suffer from one or more anxiety disorders (Fava *et al.*, 1992; Veeninga *et al.*, 1994). The menstrual cycle (MC) also seems to contribute to the exacerbation of pathological anxiety in women suffering from panic disorder (Cameron *et al.*, 1986; Cameron *et al.*, 1988; Breier *et al.*, 1986, Kaspi *et al.*, 1994; Sanberg *et al.*, 1986). McLeod *et al.*, (1993) reported that women with both generalized anxiety disorder and PMDD experienced more severe anxiety symptoms than did women with generalized anxiety disorder alone. These findings suggest that the presence of PMDD may have an adverse effect on the clinical course of anxiety disorders.

Although the cause of PMDD remains unknown, the symptomatic overlap between anxiety and PMDD reported in studies raises questions as to shared underlying biological abnormalities. Available data to date appear to support this proposal. For instance, pharmacological data indicate that alprazolam, a high potency GABA-A receptor agonist, and antidepressant agents which strongly inhibit serotonin reuptake (e.g., fluoxetine, clomipramine) are clinically effective in treating symptoms of both anxiety disorders (Ballenger *et al.*, 1988; Gorman *et al.*, 1987; Modigh *et al.*, 1993) and PMDD (Harrison *et al.*, 1990; Freeman *et al.*, 1995; Steiner *et al.*, 1995; Sundblad *et al.*, 1993). As with anxiety disorder patients, women with PMDD also show a heightened sensitivity to panicogenic challenges. For instance, sodium lactate infusion (Fachinetti *et al.*, 1992; Sandberg *et al.*, 1993) and 35% CO_2 inhalation (Harrison *et al.*, 1989) have

been found to elicit significantly greater anxiety and clinical manifestations of panic in subjects with PMDD than in control women.

The main objective of the present study was to further investigate the relationship between PMDD and anxiety. We assessed the potential contribution of the cholecystokinin (CCK) system to the expression of anxiety in PMDD. The putative role of CCK in the expression of fear and anxiety has been confirmed by a number of animal and human studies (for review, see Bradwejn and Vasar 1995). Human studies have revealed that systemic administration of central CCK-B receptor agonists (i.e., CCK-4, pentagastrin) are profoundly panicogenic, provoking significant somatic, affective and cognitive symptoms of panic as well as concomitant increases in heart rate and blood pressure (Bradwejn *et al.*, 1991; Abelson and Nesse 1994). Further, the effectiveness of these CCK-B agonists in provoking symptoms of panic anxiety is more evident in patients with panic disorder (Bradwejn *et al.*, 1991, Abelson and Nesse 1994), generalized anxiety disorder (Brawman-Mintzer *et al.*, 1997) and social phobia (McCann *et al.*, 1995) than in healthy controls. Concentrations of CCK-8, a mixed CCK-A and -B receptor agonist, have been found to be lower in CSF and lymphocytes of panic disorder patients compared to healthy volunteers (Lydiard *et al.*, 1992, Brambilla *et al.*, 1993). In light of evidence which suggests a link between PMDD and anxiety and between anxiety and CCK-4, we opted to determine whether PMDD women would also exhibit a heightened sensitivity to the panicogenic effects of the CCK receptor agonist, CCK-4.

We also decided to investigate whether a difference in sensitivity to CCK exists only during the LP or also during the follicular phase (FP). Such information could help

elucidate whether a difference in sensitivity to a panicogenic agent is "state" or "trait" related. In the only study (Harrison *et al.*, 1989) where a panicogenic agent was administered both in the FP and in the LP, vulnerability to developing a panic attack following CO_2 inhalation was increased in PMDD women relative to controls in both the FP and LP of the MC. However, because of the small number of PMDD patients who agreed to inhale CO_2 in both phases, the authors were unable to draw any conclusions regarding a phase-related difference in reactivity to panicogenic agents in PMDD women. In order to assess any putative phase effects, CCK-4 was administered to women during both the FP and LP.

Another objective of this study was to examine whether the behavioural effects provoked by CCK receptor activation are altered by the MC phases and their concomitant gonadal hormonal changes. Changes in CCK plasma concentrations have been detected during the MC in humans and during the estrous cycle in rats. For example, in healthy women, plasma CCK concentrations are more elevated during the LP of the menstrual cycle relative to the FP (Frick *et al.*, 1990). In female rats, CCK binding, CCK availability and the number of CCK-immunoreactive neurons in different central sites, vary according to the estrous cycle and following administration of estradiol (Goldman *et al.*, 1984; Akesson *et al.*, 1987; Micevych *et al.*, 1988; Oro *et al.*, 1988). For example, proestrus female rats show a greater number of cholecystokinin immunoreactive cells in the posterodorsal nucleus of the amygdala (Oro *et al.*, 1988). Recent experiments in rats have revealed that the pharmacological manipulation of female gonadal hormones alters the effects of CCK on food intake and lordosis (Geary *et al.*, 1994; Ulibarri and

Micevych 1993; Mendelson and Gorzalka 1984; Wagner-Srdar *et al.*, 1987). Interestingly, the CCK content (CCKi) of the amygdala, a central neuroanatomical structure involved in anxiety response, varies during the estrous cycle and is maximal during estrogen peaks of the cycle (see Micevych and Ulibarri, 1992 for review). Likewise, the panicogenic effects of CCK agonists might be expected to vary with hormonal changes. Likewise, the panicogenic effects of CCK agonists might be expected to vary with hormonal change. Preliminary results related to portions of this study have been published elsewhere in the form of a letter (Le Mellédo *et al.*, 1995).

SUBJECTS AND METHODS

Subjects

Eighteen women with DSM-IV PMDD (mean age: 31 ± 7 years) and 21 control women (mean age: 27 ± 7 years) who responded to newspaper advertisements participated in the study after providing oral and written informed consent. All subjects were physically healthy as determined by medical history, physical examination, electrocardiogram and routine laboratory tests. Subjects were evaluated with the Structured Clinical Interview for DSM-III-R for non patients (SCID-NP). Women with PMDD were free of any current Axis I psychiatric diagnoses and a lifetime history of bipolar disorder, psychotic disorders, anxiety disorders, somatoform disorders. We cannot report any data on the co-prevalence of these disorders with PMDD since the exclusion took place before PMDD was diagnosed with prospective monitoring of premenstrual symptoms. Because of the high prevalence of depression in PMDD, women

with a history of major depression were included in the study provided that their last episode remitted at least two years prior to the screen visit. None of the control women had a current or lifetime history of Axis I psychiatric disorders as determined by the SCID-NP. No subjects had a history of panic attacks or a first-degree relative with panic disorder. Subjects were excluded from the study if they: a) had serious medical disorders; b) were taking any medication; c) smoked more than 15 cigarettes a day; d) drank more than 5 cups of coffee a day; e) were pregnant or lactating; f) had given birth in the previous 6 months; g) had an abortion in the previous 3 months; h) had irregular menstrual cycles; i) had an average menstrual cycle length greater than 35 days or less than 24 days; and j) had used or discontinued hormonally based contraceptives in the previous 3 months.

The presence or absence of PMDD was ascertained by the prospective monitoring of at least four completed MCs using a modified Prospective Record of the Impact and the Severity of Menstrual Symptomatology (PRISM) (Reid and Robinson 1985) and 100 mm visual analogical scales (VAS). Our modification of the PRISM calendar consisted in replacing the item "restlessness" (described by many experts in the field as being not very informative) by the item "overwhelmed" (a new DSM-IV item not included in the pre-DSM-IV PRISM calendar). The range of scoring of every item was rated between 1 (not present) and 7 (very severe) on the version of the PRISM available at the time of the study. Subjects completed the PRISM calendar every day throughout the MC. Two VAS were completed during each MC, the first one was completed seven to ten days after the onset of menses and the second one to five days before the onset of the next menses

which was prospectively calculated according to the length of each woman's MC. Our instructions to the subjects were to complete their second VAS between two and five days before the expected date of menses. Unfortunately several shorter than expected MCs resulted in several VAS completed only 1 day prior the onset of menses. We assessed the presence of DSM-IV PMDD criteria based on the PRISM calendar (which ensured that premenstrual emotional symptoms were not limited to the day of the LP VAS completion). The menstrual cyclicity and severity of "mood symptoms" were objectively verified by comparing the ratings of the VAS ratings during the FP and the LP. Affect cyclicity was ensured by a within-cycle (FP to LP) increase of at least 50% in three menstrually-related mood symptoms (tension, dysphoria, mood swings and irritability) or a 100% increase in the severity of one of these symptoms. For the increase in severity to be considered clinically significant, the severity of menstrual symptoms had to be greater than 40 mm on the VAS scale during the LP. This 40 mm cut-off score is still accepted in the new National Institute of Mental Health (NIMH) guidelines (NIMH task force on PMDD, 1997). These requirements had to have be present for at least half of the MCs monitored in each subject. We did not use a maximal cut-off for the FP ratings because the NIMH guidelines for PMDD research in effect at the time we designed this study did not recommend a FP cut-off score. Recently, a FP cut-off score has been added to the NIMH guidelines to exclude women who present significant emotional symptoms during their FP. In our study, women with consistent severe emotional symptoms during the FP would have been excluded for current depressive episode or current anxiety disorder

following the performance of the Structure Clinical Interview (SCID) which was systematically administered during the FP, 5 to 10 days after the onset of the menses.

The NIMH guidelines for premenstrual syndrome (National Institute of Mental Health, 1983) were applied retrospectively to the PRISM ratings. These guidelines require that the sum of the LP ratings during the 6 days prior to menses "should be" 30% greater than the sum of the FP ratings between day 5 and 10 after the onset of menses. All the PMDD women included in this study met NIMH criteria for premenstrual syndrome which confirmed the severity and the cyclicity of their premenstrual symptoms.

Group assignment had not been made at the time of the placebo injection. The group assignment was performed during the third visit, following the monitoring of two complete MCs.

Design

The study employed a placebo-controlled cross-over design. Each subject participated in three sessions and received one placebo injection and two CCK-4 injections. All subjects received placebo first, which coincided with the LP of the MC. The two CCK-4 injections corresponded to the FP (7 to 10 days after the onset of menses) and LP (1 to 5 days prior to menses). The order of menstrual phase (i.e., whether CCK-4 was administered first during the FP or the LP) was counterbalanced across subjects. The use of placebo on the first test day allowed subjects to accommodate to the experimental procedure and reduced the probability of drop outs between the two CCK-4 sessions (to 0). Subjects were blind to the number of placebo and CCK-4

injections they received and to the menstrual phase order in which placebo and CCK-4 were administered. Menstrual phase was confirmed using a urine luteinizing hormone (LH) detection kit (Clearplan Easy, CIBA Unipath Ltd.). This test was intended to help the scheduling of the LP visit by ensuring that the LP injection was performed after the LH peak. We cannot state the study days with respect to the day of the LH surge because we found at several occasions discrepancies between absence of LH peak (suggesting an absence of ovulation) and high progesterone levels during the LP (suggesting that ovulation took place). The study was approved by the St Mary's Hospital Ethics Committee.

ASSESSMENT

Behavioural Analyses

A DSM-III-R-derived Panic Symptom Scale (PSS) (Bradwejn *et al.*, 1991) was used to characterize behavioural responses to the CCK-4 (and placebo) challenge. The panic symptoms on this scale were the following: "feeling short of breath/and or smothering sensation", "dizziness", "unsteady feeling", "faintness", "palpitations and/or rapid heart", "trembling and/or shaking", "sweating", "choking feeling", "nausea", "abdominal distress", "feeling unreal and/or detached from your body", "numbness and/or tinglings in part of your body, "hot flashes and/or cold chills", "chest pain and/or discomfort", "anxiety, fear and/or apprehension", "fear of dying", "fear of loosing control", "fear of going crazy". As in their previous studies, to avoid interference between subjects' basal states and post-injection ratings, this research group

systematically instructed subjects, in a standardized fashion, to rate these items with respect to the change from the way they were feeling before the injection. Subjects were directed to rate the severity of these 18 panic symptoms as either absent (0), mild (1), moderate (2), severe (3) or extremely severe (4). Two separate scores were obtained from this scale: (a) a sum intensity score (i.e., the sum of the intensity ratings); and (b) a score reflecting the total number of symptoms reported (i.e., number of symptoms with scores >1). The occurrence of panic attacks following CCK-4 administration was determined based on the DSM-IV criteria for panic attacks and based on a score of 2 or more on the PSS "anxiety, fear, apprehension" item. At the end of the study, subjects were asked to compare their response to the two CCK-4 injections. The duration of the panic symptoms was determined based on the subjects indication and timed by the blind rater (in this study an increase of 30 seconds represents an increase of approximately 1/3 of the total duration of the symptoms).

Cardiovascular Analyses

Heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded using an automatic sphingomanometer (every 20 seconds for the first 5 minutes following the injections).

Biochemical Analyses

Blood sampling for measurement of: CCK, estradiol (E), progesterone (P), luteinizing hormone (LH), and follicular hormone (FSH) took place 1 hour before injection and 45 minutes after IV installation.

Methods for Total CCK-Like Immunoreactivity (CCK-LI) Measurements:

Antisera and Tracer Preparation: Plasma cholecystokinin levels were analyzed by a radioimmunoassay using antisera directed against the cholecystokinin tetrapeptide (Merani *et al.*, 1997). Antiserum against CCK-4 was prepared by conjugation to thyroglobulin by the carbodiimide method (Vaitukaitis *et al.*, 1971). Briefly, 25 mg of thyroglobulin in 0.5 ml of distilled water (dH_2O) was added to 5 mg of CCK-4 dissolved in 0.5 ml of distilled water and adjusted to pH 5.5. Five mg of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide-HCL (CDI) in 0.4 ml of dH_2O was added dropwise with constant mixing and incubated overnight at 4°C. 35 mg of CDI was added and mixed at room temperature for 2 hours. Excess coupling agent was removed by 24 hour dialysis against 2 L of 0.001 M phosphate buffer (pH 7.4) containing 0.9% NaCl. The antigenic solution (100 mg/ml saline) was emulsified in complete Freund's adjuvant and injected intradermally to New Zealand White rabbits. Intra-muscular injections of 0.5 ml Bordetella pertussis vaccine were administered as non-specific stimulus. Booster injections of 50 μg of the antigen emulsified in incomplete Freund's adjuvant were given every 4 weeks for 8 months (Skowsky and Fisher, 1972). Bolton Hunter CCK-4 was iodinated using a previously described method designed to achieve high tracer yield (Tower *et al.*, 1972). 5 μg BH CCK-4 in 40 μl phosphate buffer (0.05

M, pH 7.4), 40 µl ^{125}I (1 mCi) in phosphate buffer, 10 ml lactoperoxidase (0.1mg/ml dH_2O) and 10 µl glucose oxidase (5 µg/ml phosphate buffer) were prepared at 25°C. The reaction was initiated by the addition of 5 ml glucose (0.5% in dH_2O) and terminated after 20 minutes with 0.1% sodium azide in 100 µl phosphate buffer. The labelled peptide was purified by HPLC using a 1 ml/ min gradient of 20-50% acetonitrile and dH_2O, each containing 0.1% TFA. 100 ml of 0.2% BSA and 1 ml of EtOH was added to the fraction containing the first peak following the elution of free iodine and stored at -20°C until use. The tracer was immunoreactively stable for 6 to7 weeks.

Sample Extraction and Radioimmunoassay: Plasma was extracted using Sep-Pak C18 cartridges (Waters). Cartridges were activated with 8 mls acetonitrile and washed with 8 mls dH_2O. Samples were applied to cartridges, washed with 5 mls dH_2O and eluted with 3 mls 70% EtOH. The eluant was dried in a speed-vac (Savant) overnight. Lyophilized samples were reconstituted in assay buffer (0.1M NaCl, 1% BSA, 1% Triton X-100, 0.1% sodium azide in 0.1M phosphate buffer, pH 7.4) and were added in duplicate 100 µl aliquots to polystyrene tubes at 4°C. This assay buffer was used for all subsequent dilutions. The standard curve consisted of 100 ml duplicates of CCK-4 in the range of 0.4 - 400 pg prepared by 1:2 serial dilutions in buffer. 100 µl or 200 µl of assay buffer was added to tubes to test for total binding or non-specific binding (NSB) or respectively. 100 µl of antiserum (diluted 1:10 000) was added to each tube except the NSB tubes and incubated at 4°C for 24 hours. 100 µl of tracer (6000-8000 cpm) was then added to each tube and incubated as above. 100 µl of normal rabbit serum (1:35) and 100 µl goat anti-rabbit γ-globulin (1:50) were added and incubated at 25°C for 2 hours. Tubes were

centrifuged at 3000 rpm at 4°C for 20 minutes after the addition of 1 ml of polyethylenglycol. Precipitate radioactivity was counted upon aspiration of supernatant.

Results: The antibody employed in the immunoassay was raised against the CCK tetrapeptide and is equipotent for the CCK tetrapeptide and octapeptide (Merani *et al.*, 1997). Thus the CCK values reported in this manuscript represent these total CCK measurements. The range of the standard curve was determined to be 0.4 - 400 pg CCK-4/tube (0.7 - 672 fmol CCK-4 equivalents/tube). Intra-assay and interassay coefficients of variation were determined to be 8% and 9% respectively (average value characteristic for this particular radioimmunoassay at approximately 30% binding). However, for this particular study, all samples were analysed in one assay, hence negating any inter-assay variation. Specific activity of iodinated BH CCK-4 was calculated as 1025 Ci/mmol. Binding of tracer in absence of standard (zero binding) was 15.3 ± 0.2 % at a 1:10 000 antisera dilution, with half-maximal displacement (ED_{50}) at 38.5 ±1.4 fmol. Non-specific binding was calculated as 1.4 ± 0.1 %.

Methods for Measurement of E, P, LH and FSH: E, P, LH and FSH were measured in the department of Clinical Biochemistry at the Hôpital Ste-Justine, Université de Montréal. Total serum E and P were measured with solid-phase double antibody immunofluorometric assays. The intra- and interassay coefficients of variation were, 3.0% and 5.0% at 0.9 nmol/L and 2.4% and 2.9% at 10.9 nmol/L respectively. LH and FSH were measured with solid-phase two-site fluorometric assays in which two mouse monoclonal antibodies are directed against two separate epitopes (Wallac Canada). The

intra- and interassay coefficients of variation for LH were respectively 1.6% and 2.3% at 7 I.U./L and, 0.8% and 3.0% for a FSH concentration of 6.7 I.U./L. In the aforementioned assays, AutoDelfia technology was used to measure the fluorescence generated by the tracer Europium™ (Wallac Canada, Kirkland, Quebec).

PROCEDURE

This study consisted of 5 visits (V1,V2,V3,V4,V5). V1 involved psychological and physical screening; an explanation of how to complete the PRISM calendar and associated VAS; and the planning of V2. V2 included the LP placebo injection. Randomization of subjects to the phase order of the CCK-4 injections took place during V3, when at least 2 complete MCs had elapsed since study enrollment. A list for randomization was generated in blocks of 6. The procedures of V2 (placebo injection), V4 (first CCK-4 injection) and V5 (second CCK-4 injection) were identical. Shortly after arrival, subjects sat on a reclining chair and an IV catheter was installed into their antecubital vein through which a NaCl 0.9% solution was slowly dripped. Bolus placebo and CCK-4 were administered through the catheter at least 1.5 hrs post catheterisation.

MATERIAL

Cholecystokinin-Tetrapeptide: CCK-4 was purchased from Peninsula Inc., Ltd. (California, U.S.A.) and a sterile solution was prepared by GIS Médicament (Nantes, France) according to previous protocols (Bradwejn *et al.*, 1991). The use of CCK-4 was

approved by the Health Protection Branch of Health and Welfare Canada. The placebo consisted of an identical volume (1.75 ml) of 0.9% NaCl solution.

DATA ANALYSIS

To compare the effects of CCK-4 and placebo on the behavioural, physiological and biochemical outcome variables, we used the data from the LP phase only, since placebo was only given during that phase, and we performed a 3-way analysis of the variance model with repeated measurements on one factor (Winer 1971). The repeated factor was treatment (placebo vs CCK-4), and the two between-subject factors were diagnosis (control vs PMDD) and sequence (CCK-4 injection during phase LP at visit 4 vs at visit 5). All the interactions were included in the model. For the binary outcome "panic" we used the generalized estimating equations (GEE) approach (Zeger and Liang, 1986; Carr & Chi, 1992) with the same model as for the continuous variables. To compare the effects of the FP versus the LP on CCK-4 injection response, we used a linear model for cross-over design (Jones and Kenward 1989). In this model, the main effects of interest were: I) diagnosis (control vs. PMDD); ii) sequence in which the subjects received the CCK-4 injections (i.e., FP-LP vs. LP-FP); iii) phase (FP vs. LP); iv) and visit (V4 vs V5). We also included in the model all the double interactions with the diagnosis factor. The same model was applied for the analysis of the binary outcome "panic" using the GEE method. Finally, for the categorical variable "phase preference", a chi-square test was performed to test the hypothesis of no phase preference for both groups separately. We considered as statistically significant p-values less than 5%. All

statistical analyses were conducted using SAS statistical software version 6.12 for Windows 95.

RESULTS

Eight women received a placebo injection (V2) and did not receive CCK-4 injections (V4). Because of their early drop out, these eight women were not adequately monitored which would have allowed us to clearly identify their assignment group. We are describing below their most probable diagnosis as well the reasons why they did not receive the first CCK-4 injection. Five women who contacted the unit and were screened as healthy volunteers were unable to return for the following visits; one woman who contacted the unit as a PMDD woman and had a first PRISM calendar compatible with the diagnosis of PMDD became depressed; one PMDD woman was "turned-off" of the study because we could not install the IV catheter to proceed with the first CCK-4 injection; and one likely healthy volunteer showed poor compliance in completing the PRISM calendars.

Randomization of the CCK-4 injection phase order resulted in 9 of the 18 PMDD women receiving their first CCK-4 injection during the FP and 9 receiving their first CCK-4 injection during the LP (9 FP-LP, 9 LP-FP). Among the 21 controls, 12 received their first CCK-4 injection during the FP and 9 during the LP (12 FP-LP, 9 LP-FP). Despite the unpleasant experience following the first CCK-4 injection (V4), all women agreed to return for the next and last visit (V5).

The means and standard deviations of the behavioural and physiological outcome variables for each diagnosis, phase and sequence are reported in Table 5. The panic rates are shown in Fig. 11.

Comparison Between the Effects of CCK-4 and Placebo During the LP

Table 6 presents the results of the 3-way analysis of variance with repeated measurements on one factor. For all outcome variables considered, there is a highly statistically significant increase in the response to CCK-4 compared to placebo. All other effects and interactions are not statistically significant except for the symptom "anxiety, fear, apprehension" where there is a diagnosis main effect and an interaction diagnosis by treatment (p-values = 0.02 and 0.01 respectively) indicating a significantly greater response to CCK-4 in PMDD women for that specific symptom. Figure 11 shows that no women experienced a panic attack during the placebo injection, contrasting with 67% in the PMDD group and 38% in the control group during the LP CCK-4 injection. This difference between panic rates in the two groups was only marginally significant (p = 0.08).

Comparison Between the 2 CCK-4 Injections

Table 7 illustrates the results of the linear modeling analysis for the cross-over trial.

***Differences between PMDD and Controls*:** There are statistically significant higher levels of: "anxiety, fear, apprehension"; duration of panic symptoms; and panic rates in PMDD women ($p = 0.011$, 0.049 and 0.002 respectively) (see Table 5).

***Menstrual Cycle Phase Effects on the Response to the CCK-4 Effects in PMDD Women and Controls*:** Although only marginally significant, the results of this analysis suggest, after adjusting for the possible confounding visit and sequence effects, a greater response during the LP for the "anxiety, fear, apprehension" and the PSS SI scores (estimate of the phase effect LP-FP were 0.37, $p = 0.077$ and 2.17, $p = 0.106$ respectively). Similarly, the p-value for diagnosis by phase of 0.075 for ΔHR reveals a trend towards a higher increase in heart rate in PMDD women during the LP. Analysis of the subjective impression (phase preference) for the effects of CCK-4 injections revealed that more PMDD women found the LP CCK-4 injection more intense ($x^2(1) = 6.33$, $p = 0.04$). 61% (11) of PMDD women found the LP CCK-4 injection to be more intense; 22% (4) found the FP injection to be more intense; and 17% (3) found no difference between the 2 injections. Among controls, 48% (10) found the LP CCK-4 injection to be more intense, 38% (8) found the FP injection to be worse and 14% (3) found no difference at all. The phase preference for control subjects did not reach significance ($x^2 = 3.71$, $p = 0.16$).

***Baseline CCK-LI Measurements*:** We found significantly lower levels of CCK-LI at V5 compared to V4 (0.050) but no other main effect (Table 8).

Hormones: No differences in E, P, FSH, LH plasma levels were found between diagnoses and there were no correlations between concentrations of these hormones and the response to CCK-4.

DISCUSSION

Our results demonstrate that women with PMDD exhibit a greater anxiety and panic response to CCK-4 as compared to control women during both the FP and the LP. They also suggest that an enhanced general behavioural sensitivity to CCK-4 exists in PMDD women. Indeed, in our PMDD women the duration of panic symptoms was greater (with statistical significance), the PSS SI and number of panic symptoms were greater (showing both marginal significance and nonsignificance in the 2 analysis performed). Our behavioural results are in accordance with those of previous studies which suggested that women with severe premenstrual symptoms have an enhanced vulnerability to other panicogenic agents, sodium lactate (Fachinetti *et al.*, 1992, Sandberg *et al.*, 1993) and CO_2 (Harrison *et al.*, 1989).

The absolute value of the outcome variable means favors a greater reactivity to CCK-4 during the LP than the FP in PMDD, but a strict statistical significance was found only for the subjective impression of worst injection. The phase effect was only marginally significant for other variables such as anxiety and PSS SI score. This apparent (although inconclusive) greater reactivity to CCK-4 during the LP of PMDD lends clinical relevance to our findings since the mid-FP is, by definition, symptom-free. The enhanced CCK-4 sensitivity of PMDD women during the non-symptomatic mid-FP

is therefore not a surprising find. It is consistent with the results obtained in other biological challenge studies of PMDD women (Bancroft *et al.*, 1991, Harrison *et al.*, 1989) and leads us to the state-versus-trait issue (Bancroft 1993) which needs to be addressed in future investigations. The differences in behavioural and physiological responses between placebo and CCK-4 injections in healthy volunteers are consistent with those already discussed in many studies (Bradwejn *et al.*, 1991, De Montigny 1989, Jerabek *et al.*, 1995).

CCK-LI plasma levels, independent of diagnosis or phase, were higher before the first CCK-4 injection than before the second CCK-4 injection. Based on increased blood-borne CCK described in sportsmen before a competitive marathon run, compared with control conditions (Philipp *et al.*, 1992), the difference that we observed could be explained by the fact that prior to a perceived stressful event subjects were in a different affective or arousal state. This state might have been different if women had known what to expect from an earlier experience with CCK-4 (i.e., before the second CCK-4 injection) compared to when they faced an unknown event (i.e., before the first CCK-4 injection). The novelty of the situation might have induced an increased arousal and/or apprehension which translated in greater CCK-LI plasma levels. The lack of phase effect that we observed on the CCK-LI plasma levels is at odds with Frick and co-workers' (1990) findings of increased CCK-LI plasma levels during the LP vs. the FP. In our study, this phase influence might have been overshadowed by the stronger "affective state effect" of potentially anticipatory anxiety, related to the visit effect.

Our baseline CCK-LI levels contribute little to the discussion of the still controversial mechanism of action involved in the anxiogenic activity of peripherally administered CCK-4. Indeed, it has never been demonstrated that CCK-4 crosses the blood-brain barrier. The lack of diagnosis x phase effect for CCK-LI levels suggests, however, that CCK-LI plasma level changes are not associated with the usual increase in baseline anxiety observed in the LP of PMDD women (Stein *et al.*, 1989).

In summary, our preliminary results show that the anxious and panic response to CCK-4 is greater in PMDD women than in controls. Our findings also suggest that an enhanced sensitivity to CCK-4 exists in PMDD women during the LP. This hypothesis, however, requires further testing with a greater sample size as few variables demonstrated strict statistical significance. Our preliminary results are consistent with a specific biological reactivity of PMDD women to CCK-4. These results call for additional studies on the cause of this hypersensitivity to CCK in PMDD women in which the physiology of CCK and the interaction between the CCK system and other neurotransmitter systems can be investigated in detail. The study of the potential therapeutic benefits of CCK-B antagonists and CCK-A agonists (due to their putative CCK-B antagonist activity) in PMDD seems worthwhile. Our results also suggest that when conducting panic challenge studies and clinical trials one should consider controlling for women with PMDD and menstrual phase. Furthermore, our findings suggest that the anxiety component of PMDD is as relevant as the depressive component.

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REFERENCES

References for this manuscript are integrated in the References Section provided at the end of this dissertation.

Table 5. PSS SI Score, Duration, Number of Panic Symptoms and Physiological Changes Following Placebo and CCK-4 Injections.

Descriptives Statistics: Mean ± SD						
Effects of CCK-4 Injections	Control FP n=21	Control LP n=21		PMDD FP n=18	PMDD LP n=18	
	CCK-4	Placebo	CCK-4	CCK-4	Placebo	CCK-4
Anxiety/ Fear/Appre-hension	1.0± 1.3	0.2± 0.5	1.1± 1.3	1.7± 1.1	0.2± 0.4	2.3± 1.5
PSS SI score	18.4± 11.1	2± 2.6	18.7± 11.1	21.6± 11.1	3.6± 4.8	25.4± 14.9
Duration of panic	119± 43	NA	108± 39	139± 53	NA	139± 51
Number of panic symptoms	8.9± 3.5	1.3± 1.5	8.6± 3.8	9.2± 3.7	2.6± 2.5	10.3± 4.4
Δ HR (bpm)	30± 11	6± 6	29± 13	29± 14	6± 10	35± 14
Δ SBP (mm Hg)	17± 11	7± 4	16± 11	16± 9	6± 6	16± 9
Δ DBP (mm Hg)	12± 9	4± 7	8± 9	11± 7	6± 10	11± 7

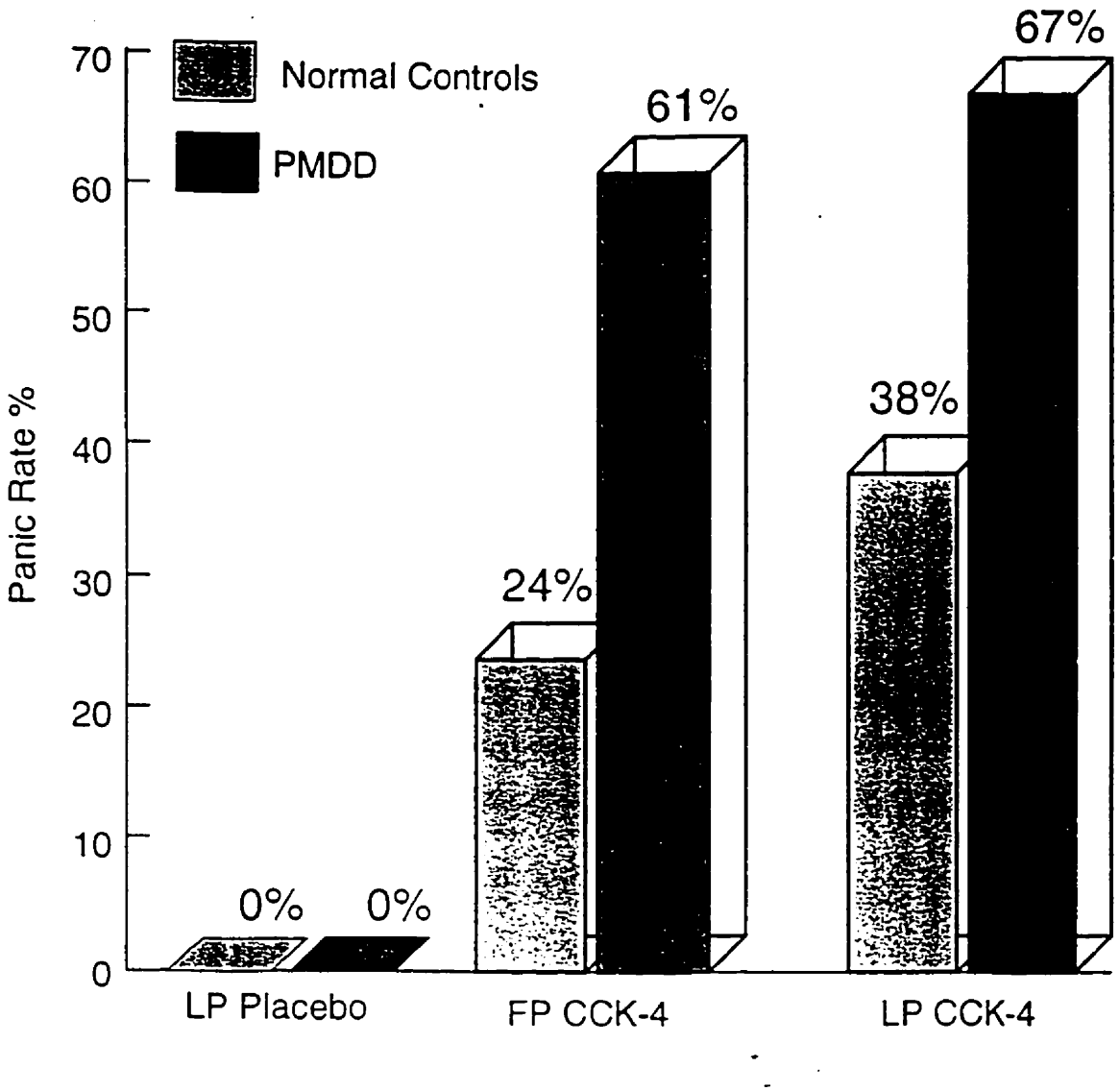

Figure 11. Rate of panic attacks in response to placebo and CCK-4 injections; FP: follicular phase; LP: luteal phase.

Table 6. Comparison Between Placebo and CCK-4 Injection During the Luteal Phase (LP).

	ANOVA						
	Anxiety	PSS SI score	Duration	number of symptoms	HR	SBP	DBP
Diagnosis	F=5.96 p=0.020	F=3.02 p=0.091	F=1.87 p=0.101	F=2.09 p=0.098	F=0.88 p=0.355	F=0.10 p=0.757	F=1.33 p=0.256
Treatment (PI vs CCK-4)	F=38.89 p<0.001	F=105.57 p<0.001	F=115.96 p<0.001	F=174.99 p<0.001	F=144.25 p<0.001	F=33.42 p<0.001	F=8.36 p=0.007
Diagnosis xTreatment	F=7.45 p=0.010	F=1.57 p=0.218	F=2.23 p=0.145	F=0.09 p=0.772	F=2.04 p=0.162	F=0.01 p=0.915	F=0.07 p=0.791
Sequence (LP V4 vs LP V5)	F<0.01 p=0.974	F=2.47 p=0.125	F=0.85 p=0.364	F=2.67 p=0.111	F=2.89 p=0.099	F=1.29 p=0.264	F=4.15 p=0.049
Diagnosis x Sequence	F=1.33 p=0.257	F=1.32 p=0.259	F=5.16 p=0.029	F=1.72 p=0.198	F=0.78 p=0.384	F=0.09 p=0.772	F=0.58 p=0.450
Treatment x Sequence	F=0.04 p=0.842	F=2.87 p=0.099	F=0.87 p=0.358	F=3.07 p=0.089	F=0.47 p=0.497	F=2.58 p=0.118	F=<0.001 p=0.984
Diagnosis x Treatment x Sequence	F=0.24 p=0.628	F=0.03 p=0.859	F=1.80 p=0.188	F=0.01 p=0.923	F=0.18 p=0.670	F=1.14 p=0.294	F<0.01 p=0.991

Table 7. Comparison Between the Two CCK-4 Injections.

	ANOVA							
	Anxiety	PSS SI score	Duration	number of symptoms	HR	SBP	DBP	Basal plasma CCK.Li
Diagnosis	F=7.32 p=0.011	F=1.69 p=0.202	F=4.16 p=0.049	F=0.65 p=0.424	F=0.28 p=0.560	F=0.09 p=0.767	F=0.10 p=0.756	F=0.03 p=0.875
Phase (FP vs LP)	F=3.33 p=0.077	F=2.75 p=0.106	F=0.42 p=0.520	F=1.08 p=0.305	F=1.09 p=0.304	F=0.23 p=0.638	F=2.40 p=0.131	F=0.38 p=0.541
Visit (V4 vs V5)	F=0.86 p=0.359	F=5.41 p=0.026	F=3.46 p=0.071	F=2.16 p=0.150	F=0.52 p=0.474	F=2.26 p=0.141	F=3.00 p=0.092	F=4.11 p=0.05
Sequences (FP/LP vs LP/FP)	F=0.15 p=0.697	F=1.12 p=0.297	F=0.11 p=0.737	F=2.35 p=0.135	F=1.63 p=0.210	F=0.66 p=0.442	F=0.73 p=0.398	F<0.001 p=0.981
Diagnosis x Phase	F=1.45 p=0.236	F=1.71 p=0.199	F=0.37 p=0.546	F=2.57 p=0.118	F=3.37 p=0.075	F=0.05 p=0.828	F=3.00 p=0.092	F=0.05 p=0.820
Diagnosis x Visit	F=0.01 p=0.918	F=1.32 p=0.258	F=1.02 p=0.320	F=0.02 p=0.883	F=0.82 p=0.371	F=0.74 p=0.397	F=0.69 p=0.413	F<0.01 p=0.944
Diagnosis x Sequence	F=1.26 p=0.268	F=0.18 p=0.676	F=0.20 p=0.661	F=0.94 p=0.339	F=0.01 p=0.941	F=0.09 p=0.770	F=0.05 p=0.820	F=0.02 p=0.903

Table 8. Basal Plasma CCK-LI for Every Diagnosis (PMDD and Controls), in Every Phase (FP and LP) for Visit 4 and 5 (V4, V5), for Every Sequence (Order of CCK-4 Injections FP/LP vs. LP/FP).

	Controls				PMDD			
	FP		LP		FP		LP	
	V4 CCK-4 (FP/LP) n=12	V5 CCK-4 (LP/FP) n=9	V4 CCK-4 (LP/FP) n=9	V5 CCK-4 (FP/LP) n=12	V4 CCK-4 (FP/LP) n=9	V5 CCK-4 (LP/FP) n=9	V4 CCK-4 (LP/FP) n=9	V5 CCK-4 (FP/LP) n=9
CCK Plasma Levels pg/ml	10.60 ± 9.8.00	7.99 ± 7.43	10.27 ± 10.03	7.29 ± 3.52	10.74 ± 8.00	7.87 ± 4.63	9.34 ± 5.96	7.01 ± 3.67

CHAPTER NINE

CHOLECYSTOKININ AND ANXIETY

CHAPTER NINE

CHOLECYSTOKININ AND ANXIETY

9.1 Introduction

The anxiogenic potential of cholecystokinin was first demonstrated two decades ago in a study designed to evaluate satiety in sheep (Della-Fera and Baile, 1979). Based on the serendipitous discovery that administration of CCK analogs elicited a fear or panic-like response in these animals, a number of researchers began investigating the relationship between cholecystokinin and anxiety. Although there is currently a large body of evidence which suggests a interaction between the cholecystokinergic system and anxiety, the exact mechanism of cholecystokinin action remains enigmatic.

9.2 Animal Studies

The anxiogenic effects of cholecystokinin were first illustrated in 1979 when pentagastrin, a synthetic pentapeptide analog of CCK-4, was infused into the lateral ventricle of sheep (Della-Fera and Baile, 1979). Instead of the anticipated satiety response, the sheep exhibited a fear response which included foot-stomping and vocalization. Five years later, the anxiogenic properties of CCK were clearly demonstrated when CCK-8 was injected into the amygdala of rats. Both the sulfated and nonsulfated forms of the octapeptide induced arousal and fear motivation in the animals (Fekete *et al.*, 1984). Since this initial demonstration by Fekete and colleagues, the

majority of researchers have used rodents to investigate the link between cholecystokinin and anxiety. A few recent studies, however, have provided provocative results through the use of non-human primate models.

Rodents

A large number of rodent studies have focused on the degree to which manipulation of the cholecystokinergic system affects exploratory behaviour. Reduced exploratory behaviour in rodents is typically used as an indicator of increased anxiety. Researchers have demonstrated that subcutaneous administration of CCK-4 to rats elicited an anxiogenic response, represented by decreased exploratory behaviour in a maze paradigm (Harro and Vasar, 1991; Koks *et al.*, 1999). The fact that CCK-4 is active at CCK-B receptor sites and almost inactive at CCK-A sites suggests that CCK-B receptors may specifically be involved in the regulation of emotionality (Harro and Vasar, 1991). It has been postulated that activation of CCK-A receptor sites mediates increased exploratory behaviour, while CCK-B activation causes a decrease in this behaviour (Männistö *et al.*, 1994). However, experiments with CCK-A receptor knockout rats (OLETF rats) revealed that exploratory behaviour is significantly reduced in these animals (Kobayashi *et al.*, 1996). The authors postulated that these findings may involve an interaction between CCK-A receptors and decreased locomotor activity via the dopaminergic system (Kobayashi *et al.*, 1996). The complex interaction between the cholecystokinergic and dopaminergic systems is detailed in Chapter Four, Section 4.2.

A study which specifically compared the effects of CCK-4 and sulfated CCK-8 used a variety of behavioural paradigms to assess anxiety in rats (Rex *et al.*, 1994). Rex and co-workers determined that in each paradigm, an acute intraperitoneal injection of CCK-4 induced anxiety in rats, whereas CCK-8s was ineffective. The authors suggested that CCK-8s did not produce anxiogenic behaviour because of the confounding mixed CCKA/B receptor properties of the octapeptide. However, other researchers have found that systemic treatment with CCK-8s decreased exploratory behaviour in rats (Vasar *et al.*, 1994). This discrepancy may, in part, be reconciled by the existence of multiple CCK-B receptor subtypes. Derrien and co-workers (1994) demonstrated that at least two different types of CCK-B receptors are present in the rat brain. These subtypes may be responsive to different doses of cholecystokinin agonists and, in addition, may mediate different behaviours depending on the type of behavioural paradigm used.

Intracerebroventricular infusion of the CCK-B agonist pentagastrin has also been shown to potentiate the acoustic startle response in rats, another index of anxiety in rats (Frankland *et al.*, 1996). In a follow-up study, Frankland and colleagues demonstrated that infusion of pentagastrin into the amygdala of rats enhanced the startle reflex, whereas injections into the striatum or nucleus accumbens were ineffective (Frankland *et al.*, 1997). These results suggested that amygdala CCK-B receptor mechanisms are involved in the potentiation of the startle response. Interestingly, the amygdala is a region of the brain which plays an important role in the mediation of fear and anxiety.

In a recent study, antisense oligodeoxynucleotides complementary to the start coding region of rat cholecystokinin precursor were intracerebroventricularly infused into

rats. Compared to rats infused with vehicle and scramble sequence oligodeoxynucleotide control, CCK-antisense exogenous administration for 3 days significantly reduced anxiety behaviour in rats as observed by a maze paradigm (Cohen *et al.*, 1998).

In addition to the exogenous administration of cholecystokinin agonists and antagonists, the role of the endogenous cholecystokinergic system has also been investigated. One particularly innovative study evaluated cholecystokinin levels in rats subjected to an anxiety-provoking stimulus. As discussed in Chapter Three, Section 3.2, Pavlasevic and co-workers (1993) measured CCK levels in rat brains after exposure to a cloth with the scent of a feline predator. Contact with this "cat-cloth" caused an increase in CCK-4 levels in the olfactory bulb, frontal cortex, central cortex, dorsal striatum, ventral striatum, central amygdala and the solitary tract nucleus. Increased concentrations of CCK-8 were observed in the ventral striatum. Analysis of plasma CCK-8 did not reveal a significant difference between rats exposed to the scented cloth and those not exposed. Unfortunately, due to the limitations of the methodology, cholecystokinin tetrapeptide levels in plasma could not be assessed .

The endogenous cholecystokinergic response to stress has also been examined by using decapitation of conspecifics as the stressful stimuli. Rats were exposed for one hour to the sounds and odour of other rats being sacrificed. Although levels of cholecystokinin peptides were inconclusive, the researchers found a consistent upregulation of CCK-B receptor binding in the cerebral cortex of stressed animals (Harro *et al.*, 1996). Nevo and colleagues (1996) demonstrated that levels CCK-like material were increased in the frontal cortex of rats subjected to stressful stimuli, including

exposure to diethyl ether and a 30-minute restraint. Other studies revealed that CCK-like immunoreactivity is increased in the hypothalamus of rats exposed to acute foot shock (Siegal *et al.*, 1987) and in the periaqueductal grey of rats restrained for 1-minute (Rosen *et al.*, 1992). The release of cholecystokinin in the central nervous system induced by anxiety or stress is described in Chapter Three, Section 3.2.

Non-Human Primate Models

A limited number of studies have described the effects of CCK agonists and antagonists in non-human primates. Intravenous administration of CCK-4 to African green monkeys elicited behaviours such as agitation, restlessness, vigilance, and at peak intensity, immobilization or freezing (Ervin *et al.*, 1991; Palmour *et al.*, 1992). These behaviours are consistent with a fear or panic response in this species. Further, the reaction of the individual animal was correlated to its social hierarchy and to the baseline behavioural characteristics of the monkey. In general, the intensity of the fear response was greater in animals that exhibited a greater baseline anxiety level as compared to their less uptight counterparts. For example, in monkeys that had a naturally calm disposition, CCK-4 administration caused only mild restlessness (Ervin *et al.*, 1991; Palmour *et al.*, 1992). The anxiogenic effects of CCK-4 could be blocked by L-262,691, a specific CCK-B antagonist, indicating that CCK-B receptors mediate the panicogenic effects of the tetrapeptide (Palmour *et al.*, 1991; 1992).

9.3 Anxiety in Humans

Pathological anxiety in humans may be manifested in a variety of different behaviours. In the DSM-IV (American Psychiatric Association, 1994), major clinical anxiety is categorized into twelve different conditions, including social phobia, post-traumatic stress disorder, obsessive-compulsive disorder, generalized anxiety disorder and panic disorder with and without agoraphobia. The most extensive research into the role of cholecystokinin in anxiety disorders has focused on panic disorder. Much of the early data on CCK and anxiety was derived from research on patients with panic disorder. In fact, this clinical human research preceded much of the research conducted with animal models (Bradwejn *et al.*, 1992).

Panic Disorder

According to the DSM-IV, panic disorder is characterized by the occurrence of recurrent, unexpected panic attacks followed by at least one month of persistent worry about having another attack, concern about the possible implications of the attacks, or significant behavioural changes related to the attacks (Table 9). A panic attack is a discrete episode of intense fear, apprehension or terror which is often associated with symptoms such as a sense of impending doom, chest discomfort, shortness of breath and a fear of going crazy or losing control. In patients with panic disorder, these panic attacks are spontaneous or uncued; the attacks occur "out of the blue" or without any type of situational trigger. Patients who experience both panic disorder and agoraphobia are

separately classified by the DSM-IV as having panic disorder accompanied by anxiety about, or avoidance of, places or situations in which help may not be available, or where escape in the event of a panic attack may be difficult or embarrassing. According to the DSM-IV, the lifetime prevalence of panic disorder, with and without agoraphobia, is estimated at between 1.5% and 3%. Approximately one-third to one-half of people diagnosed with panic disorder have agoraphobia. Panic disorder with agoraphobia is diagnosed three times as often in women as in men, and panic disorder alone is observed in twice as many women as men (DSM-IV, 1994). Some evidence suggests that panic disorder and panic disorder with agoraphobia show familial aggregation (Crowe *et al.*, 1983).

Interest in the relationship between anxiety and cholecystokinin in humans stems from the ground-breaking research of Bradwejn and de Montigny. In 1984, the two scientists demonstrated that benzodiazepenes, a class of anti-anxiety drugs (reviewed in Chapter Four, Section 4.5), specifically antagonized CCK-8s-induced excitation of rat hippocampal pyramidal neurons. The observation that anxiolytic drugs could antagonize the excitatory action of cholecystokinin suggested that CCK possessed anxiogenic properties and that cholecystokinin played a critical role in the pathology of anxiety disorders (Bradwejn and de Montigny, 1984). To expand on this preclinical data, the same team began experimenting with cholecystokinin peptides as potential anxiogenic agents in human subjects. In 1989, de Montigny demonstrated that CCK-4 induced panic-like symptoms when injected into healthy volunteers (de Montigny, 1989). Bradwejn and colleagues then embarked upon a series of carefully designed and

innovative experiments that characterized the panicogenic effects of CCK-4 administration on patients with panic disorder and control subjects (Bradwejn *et al.*, 1990; 1991; 1992a; 1992b; 1994; Jerabek *et al.*, 1999; Koszycki *et al.*, 1998; Shlik *et al.*, 1997).

The Bradwejn team showed that intravenous administration of CCK-4 to patients who suffered from panic disorder induced panic attacks in the laboratory which were strikingly similar to the spontaneous panic attacks they experienced during the course of their illness (Bradwejn *et al.*, 1990). Administration of CCK-4 to patients with panic disorder and healthy volunteers revealed a more intense behavioural response in the patient group (Bradwejn *et al.*, 1991). As described earlier, monkeys that exhibited greater baseline anxiety were also more sensitive to CCK-4 administration (Ervin *et al.*, 1991; Palmour *et al.*, 1991; 1992). Further studies revealed that the panicogenic effects of CCK-4 in both healthy subjects and in panic patients were consistent, reproducible and dose-dependent (Bradwejn *et al.*, 1991; 1992a; 1992b; Jerabek *et al.*, 1999; Koszycki *et al.*, 1998; Shlik *et al.*, 1997). A detailed description of the cardiovascular effects of CCK-4 administration is provided in Chapter Ten, Section 10.2. In addition to CCK-4, pentagastrin has also been shown to elicit panic attacks in control subjects and patients with panic disorder. As with CCK-4, the patient group was more sensitive than the control group to the panicogenic effects of pentagastrin (Abelson and Nesse, 1994; van Megen *et al.*, 1994; 1996b).

To investigate the receptors involved in the mediation of the panicogenic properties of CCK-4, Bradwejn and co-workers examined the effects of various specific

CCK receptor antagonists. For instance, L-365,260, a specific CCK-B antagonist, was shown to antagonize the panicogenic effects of CCK-4, suggesting that these effects are mediated by the CCK-B receptor (Bradwejn *et al.*, 1994). One study, however, reported that a six-week course of CI-988, a selective CCK-B receptor antagonist, was not superior to placebo in reducing panic attacks in patients with panic disorder. However, the authors speculated that poor pharmacokinetic characteristics of CI-988 may have attributed to the lack of efficacy (Pande *et al.*, 1999).

In another study, Bradwejn and Koszycki (1994) discovered that in patients with panic disorder, chronic treatment with imipramine, a serotonin reuptake inhibitor, reduced the number, duration and intensity of panic symptoms induced by CCK-4 administration. This observation suggests an interactive role of the cholecystokinergic and serotonergic system in anxiety. Further discussion regarding the relationship between these two systems is provided in Chapter Four, Section 4.3.

A number of researchers have also been interested in the role of the endogenous cholecystokinergic system in panic disorder. One explanation of the enhanced sensitivity of patients with panic disorder to the effects of CCK-4 and pentagastrin administration may be that these patients have a dysfunctional cholecystokinergic system. One of the major goals of the research presented in this dissertation was to develop a sensitive assay which could accurately measure CCK levels in human subjects, an assay which could eventually be used to assess the endogenous cholecystokinin system in patient populations. Currently, such collaborations are underway with Dr. Bradwejn, as well as with a number of other researchers who are using this assay to examine cholecystokinin

levels in a variety of patient groups. For instance, Lydiard and colleagues demonstrated that cerebrospinal fluid concentrations of CCK-8 were reduced in patients with panic disorder as compared to healthy controls (Lydiard *et al.*, 1992). The authors suggested that the unexpected lower CCK-8 levels were a means of compensation for either an enhanced receptor sensitivity or for a decreased number of CCK receptors in panic patients. Alternatively, the authors speculated that CCK-8 levels may be reduced to compensate for increased activity of CCK-4 in the central nervous system. Unfortunately, at that time, an assay was not available to accurately measure tetrapeptide levels. Currently, the use of our assay is being explored as a means for evaluating the hypothesis that the CCK-4:CCK-8 ratio is aberrant in patients with panic disorder.

Other approaches to examining the endogenous cholecystokinergic system in panic disorder include the measurement of CCK-8 levels in lymphocytes. One such study revealed that CCK-8 levels were lower in patients with panic disorder than in healthy volunteers. After a 30-day treatment with alprazolam, an anti-anxiety medication, the frequency and severity of panic attacks decreased in the patients. CCK-8 levels, however, were not significantly different after treatment. Although longer drug trials are needed, these findings indicated that aberrant CCK levels in patients may be a trait marker rather than a state marker (Brambilla *et al.*, 1993).

Recently, investigators in the field of molecular psychiatry explored possible associations of panic disorder with polymorphisms in the genes for the CCK-A receptor, CCK-B receptor and preprohormone CCK. Wang and colleagues (1998) reported the possible association of a cholecystokinin promotor polymorphism with panic disorder.

More recently, Kennedy and collaborators, including Dr. Bradwejn, studied polymorphisms in the CCK preprohormone and receptor genes in ninety-nine patients with panic disorder and controls matched for gender and ethnicity. The CCK preprohormone polymorphism and the CCK-A receptor polymorphism revealed no association with panic disorder. However, for the CCK-B receptor gene polymorphism, patients with panic disorder showed a significant association, suggesting that gene variation in the CCK-B receptor may contribute to the neurobiology of panic disorder (Kennedy *et al.*, 1999).

There is a substantial body of evidence which implicates the cholecystokinergic system in anxiety. Although the exact mechanisms of cholecystokinin action are yet unknown, recent advances in molecular psychiatry, along with the availability of highly selective CCK agonists and antagonists, will be useful agents in preclinical and clinical studies of anxiety. Additionally, the development of our assay system (Merani *et al.*, 1997) will aid in assessing the contribution of endogenous cholecystokinin dysfunction in pathological anxiety. Together, these novel tools will shed light on the neurobiology of anxiety and on the mechanisms of action underlying panic disorder.

Table 9. Diagnostic Criteria for Panic Disorder. Adapted from the Diagnostic and Statistical Manual (American Psychiatric Association, 1994).

A. Both (1) and (2)

(1) recurrent unexpected Panic Attacks

Criteria for Panic Attack:

A discrete period of intense fear or discomfort, in which four (or more) of the following symptoms developed abruptly and reached a peak within 10 minutes:

1. palpitations, pounding heart, or accelerated heart rate
2. sweating
3. trembling or shaking
4. sensations of shortness of breath or smothering
5. feeling of choking
6. chest pain or discomfort
7. nausea or abdominal distress
8. feeling dizzy, unsteady, lightheaded, or faint
9. derealization (feelings of unreality) or depersonalization (being detached from oneself)
10. fear of losing control or going crazy
11. fear of dying
12. paresthesias (numbness or tingling sensations)
13. chills or hot flushes

(2) at least one of the attacks has been followed by 1 month (or more) of one (or more) of the following:

a) persistent concern about having additional attacks

b) worry about the implications of the attack or its consequences (e.g., losing control, having a heart attack, "going crazy")

c) a significant change in behaviour related to the attacks

B. Absence of Agoraphobia

C. The Panic Attacks are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hyperthyroidism).

D. The Panic Attacks are not better accounted for by another mental disorder, such as Social Phobia (e.g., occurring on exposure to feared social situations), Specific Phobia (e.g., on exposure to a specific phobic situation), Obsessive-Compulsive Disorder (e.g., on exposure to dirt in someone with an obsession about contamination), Posttraumatic Stress Disorder (e.g., in response to stimuli associated with a severe stressor), or Separation Anxiety Disorder (e.g., in response to being away from home or relatives).

9.4 Cholecystokinin and Caffeine

Caffeine, or 1,3,7-trimethylxanthine, is found in a variety of foods and beverages (Table 10) and is considered to be the most extensively consumed stimulant in North America (Gilbert 1976; Nehlig 199). It is well-known that caffeine consumption makes certain individuals "jittery" or nervous. Because caffeine exhibits anxiogenic properties, researchers have recently focused on caffeine's potential to induce panic attacks. Additionally, as enumerated below, a few studies have examined whether patients with anxiety disorders are particularly sensitive to caffeine by administering caffeine to healthy volunteers and anxiety patients. This "caffeine challenge" paradigm serves as useful model for examining the mechanisms involved in the pathogenesis of anxiety.

The exact mechanism by which caffeine induces anxiety or panic remains unknown. At high concentrations, caffeine blocks the metabolism of cAMP by inhibiting phosphodiesterase. However, the principal mechanism of action is thought to occur via antagonism of central adenosine receptors. Caffeine's role as an anxiogenic compound likely stems from this latter mechanism, as the concentrations required to block adenosine receptors is much less than that required to inhibit degradation of cAMP (Krystal *et al.*, 1996).

A few studies have reported an interaction between caffeine and the cholecystokinergic system (Douglas *et al.*, 1990; Schiffmann and Vanderhaeghen , 1993). Preliminary results from our laboratory, described below, indicate that caffeine, administered orally, increases plasma cholecystokinin concentrations. Moreover, we observed substantial variation in post-caffeine cholecystokinin levels among individuals.

Anxiogenic Effects of Caffeine

The anxiogenic effects of caffeine have been demonstrated in both animals and humans. Baldwin and colleagues reported that intraperitoneal (i.p.) administration of caffeine produced behavioural effects thought to be related to anxiety in rats, as exhibited in various standard behavioural paradigms such as the social interaction test and plus-maze test (Baldwin *et al.*, 1989). Other studies demonstrate that these behavioural effects of caffeine are reproducible and dose-dependent. For example, using a variety of rodent behavioural paradigms, Bhattacharya and co-workers demonstrated that caffeine, at concentrations of 10, 25 and 50 mg/kg, i.p., produced a dose-related profile of behavioural changes indicative of anxiogenic activity in rats (Bhattacharya *et al.*, 1997).

In an investigation of the behavioural effects of caffeine in humans, Uhde and co-workers found that, at high doses (i.e. 720 mg), caffeine was able to induce panic attacks in healthy control subjects (Uhde *et al.*, 1984a). Moreover, the same researchers demonstrated that patients with panic disorder were more sensitive to the anxiogenic effects of caffeine than healthy control subjects (Uhde *et al.*, 1984b). Upon administration of 480 mg oral caffeine to 24 healthy volunteers and 14 patients with panic disorder, Uhde and co-workers reported that 9 panic patients and none of the control subjects experienced a panic attack after caffeine ingestion (Uhde *et al.*, 1984b). A similar study examined the effects of oral administration of caffeine (10 mg/kg) on behavioural ratings and somatic symptoms in 17 healthy subjects and 21 patients with panic disorder (Charney *et al.*, 1985). Caffeine produced significantly greater increases in subject-rated anxiety, nervousness, fear, restlessness, palpitations, nausea and tremors

in the patient group as compared with control subjects. Further, 71% of the panic patients reported that the symptoms caused by the caffeine ingestion were similar to those experienced during panic attacks (Charney *et al.*, 1985).

Another indication that patients with panic disorder are more sensitive to caffeine stems from a retrospective analysis on caffeine consumption conducted by Boulenger and co-workers (1984). The study revealed that 67% of patients with panic disorder, but only 20% of healthy subjects, had discontinued caffeine consumption due to unpleasant side-effects such as nervousness, anxiety, tension or insomnia (Boulenger *et al.*, 1984). Moreover, panic patients reported higher ratings of anxiety after drinking one cup of coffee, as compared to control subjects (Boulenger *et al.*, 1984). As a consequence , some researchers and clinicians view caffeine as an exacerbating factor in panic disorder, and have recommended that patients with panic disorder avoid caffeine altogether (Bruce and Lader, 1989; Roy-Byrne and Uhde, 1988).

The mechanism by which caffeine produces anxiogenic effects remains unknown. Some studies have supported the inference that caffeine may induce anxiety by blocking adenosine receptors (Apfeldorf and Shear, 1993; Snyder and Sklar, 1984). Caffeine is known to enhance taste sensitivity to a variety of substances, a sensitivity which can be reversed by a topical administration of adenosine (Schiffman *et al.*, 1985). Apfeldorf and Shear reported that caffeine potentiation of taste was greater in patients with panic disorder than in control subjects. The authors speculated that abnormal adenosine function may be involved in the etiology of panic disorder (Apfeldorf and Shear, 1993). It has also been suggested that caffeine inhibits the modulating effect of certain purines

on benzodiazepene receptors, because of the structural similarity of caffeinc and purines such as hypoxanthine, inosine, adenine and adenosine (Orlikov and Ryzov, 1991).

Potential Mechanisms of Caffeine Action: Role Of Cholecystokinin

A very limited number of studies have explored the link between caffeine and the cholecystokinergic system. Using *in situ* hybridization, Schiffmann and Vanderhaeghen (1993) demonstrated that chronic caffeine administration to rats resulted in increased expression of CCK mRNA in the striatum. The authors speculated that caffeine, acting via the A_2 adenosine receptor, induced dopamine depletion, which in turn up-regulated expression of cholecystokinin mRNA. A detailed description of the relationship between cholecystokinergic and dopaminergic systems is found in Chapter Four, Section 4.2 .

The mutual ability of caffeine and cholecystokinin to stimulate pancreatic enzyme secretion and gallbladder contraction, as well as the fact that both substances are provocation agents in anxiety, has led to the speculation that the anxiogenic effects of coffee may be mediated by the release of CCK (Douglas *et al.*, 1990). In humans, coffee consumption, without additives such as sugar or cream, has been shown to cause an increase in plasma cholecystokinin. However, the postulated involvement of caffeine in this study was confounded by the fact that an equal volume of decaffeinated coffee also caused a similar elevation of CCK. The temperature and osmolarity of the beverage and/or gastric distention due to liquid consumption were excluded as candidates

responsible for the cholecystokinin increase, as a control solution of sodium chloride was ineffective in releasing CCK (Douglas *et al.*, 1990).

Utility of the Caffeine Challenge

The possibility that a caffeine challenge may serve as a safer and better tolerated alternative to a CCK-4 challenge in investigating the endogenous cholecystokinergic system in certain individuals is worthy of further investigation. As described in Section 9.3, Bradwejn and colleagues have conducted numerous experiments in which CCK-4 was administered intravenously to healthy controls and patients with panic disorder (Bradwejn *et al.*, 1990; 1991; 1992a; 1992b; 1994; Jerabek *et al.*, 1999; Koszycki *et al.*, 1998; Shlik *et al.*, 1997). In a reproducible and dose-dependent manner, CCK-4 induced panic attacks in both groups, with panic patients showing an enhanced sensitivity to the challenge (Bradwejn *et al.*, 1990).

To date, it remains unclear whether the heightened sensitivity observed in panic patients is an indicator of genetic vulnerability (i.e. a trait marker), or alternatively, a consequence of the disorder (i.e. a state marker). One way to distinguish between these two alternatives is to administer CCK-4 to first-degree *unaffected* relatives of panic patients. An exaggerated response to CCK-4 injections in this group would implicate a genetic dysfunction, possibly of the cholecystokinergic system. Ironically, the very likelihood that this theory is in fact tenable underlies the reluctance of the scientific community to test this hypothesis. The Bradwejn team, for example, is concerned that

administering a panicogen as potent as CCK-4 might cause panic attacks in the predisposed group (i.e. the first-degree relatives), and that these panic attacks might actually trigger the onset of full-fledged panic disorder in this previously unaffected group (Bradwejn, personal communication). Consequently, for ethical reasons, researchers have been hesitant to administer CCK-4 to unaffected first-degree relatives. However, less potent challenges of the cholecystokinergic system, such as caffeine, would serve as an excellent compromise to using CCK-4 administration.

A Pilot Study: Preliminary Findings

Cholecystokinin is an important gastrointestinal hormone, and as a result, many researchers have examined the effects of various nutrients on cholecystokinin release. As discussed in Chapter Three, Section 3.1, a number of studies have reported that plasma cholecystokinin levels increase postprandially. The objective of our first pilot study was to develop a milkshake, which could be orally ingested and would increase plasma cholecystokinin levels in human subjects. Moreover, our aim was to develop a challenge that would be potent enough to increase cholecystokinin levels by a detectable fraction in our novel assay system, but would not be so powerful as to overwhelm the cholecystokinergic system physiologically. In other words, we wanted a challenge which would reveal subtle individual variations in plasma cholecystokinin levels, with the anticipation that this variation could eventually be correlated to factors such an individual's baseline anxiety, psychiatric history or caffeine consumption.

The ingredients used in our initial study included whole milk, ice-cream and 200 mg caffeine. Twenty-five grams of fat were present in this challenge. A baseline blood sample was taken 5 minutes prior to ingestion of the milkshake and at 10, 20, 30, 45 and 60 minutes post-ingestion. To avoid potential confounding effects of other nutrients, each subject maintained an overnight fast of 12 hours prior to the study. As represented in Fig. 12, the ingestion of the milkshake caused a 4-8 fold increase in plasma cholecystokinin-like immunoreactivity. Because the milkshake contained at least 3 known stimulators of cholecystokinin release —caffeine, fat and protein— it was not possible to determine the degree to which each component affected cholecystokinin levels.

Based on our findings from the milkshake study, we devised a second study designed to measure plasma cholecystokinin levels in response to a 200 mg oral dose of caffeine only (Fig. 13). We anticipated that caffeine would increase cholecystokinin levels based on a previous study that we conducted in African green monkeys. In that study, 10 monkeys were intravenously administered either placebo (saline) or 100 mg caffeine. Each monkey was subjected to both treatments, separated by a period of at least two weeks. Analysis of CCK-LI in cerebrospinal fluid revealed that cholecystokinin levels increased 2 fold post-caffeine administration, as compared to post-placebo injection (Fig. 14). In the pilot study with humans, we anticipated that the sole administration of caffeine would cause an increase in plasma cholecystokinin levels, but that this increase would be less-pronounced than that observed with the milkshake, which contained three potent stimulators of cholecystokinin release. In the oral caffeine study,

a baseline blood sample was taken 5 minutes prior to ingestion of an oral dose of caffeine, and at 10, 20 and 30 minutes post-ingestion. As in the prior study, each subject had fasted for 12 hours prior to the study. The data from this experiment revealed that a 200 mg oral caffeine challenge caused a 2-4 fold increase in plasma cholecystokinin-like immunoreactivity (Fig. 13).

By combining the results from both the milkshake and the caffeine pilot studies, it appears that caffeine may have accounted for a 2-4 fold increase in CCK levels in the milkshake study, while the combined effects of the other components, most likely fat and protein, were responsible for the remaining increase in cholecystokinin concentrations.

Although preliminary, we observed substantial variation in post-caffeine cholecystokinin levels among individuals (Figs. 12 and 13). This suggests that certain individuals may be more susceptible to the physiological and behavioural effects of caffeine. This susceptibility may be a function of factors such as the individual's baseline anxiety and history of caffeine consumption. In fact, the data from the two pilot studies suggest a correlation between baseline cholecystokinin concentration and caffeine consumption. Subjects who reported a low usage of caffeine, typically less than 250 mg caffeine per week, had lower levels of baseline cholecystokinin levels than subjects who were heavy caffeine users, typically consuming greater than 750 mg caffeine per week. Although further studies are needed to verify these findings, these initial results suggest that caffeine usage may affect plasma cholecystokinin levels.

Interestingly, other studies have also reported differences between heavy caffeine users and caffeine-naive subjects. For example, Dager and co-workers (1999) evaluated

behavioural and physiological parameters in 9 heavy caffeine users and 9 caffeine-intolerant individuals. The caffeine-intolerant individuals, but not the regular caffeine users, experienced marked psychological and physiological distress in response to caffeine ingestion. Further, increases in global and regionally specific brain lactate were observed only among the caffeine-intolerant subjects .

Conclusions and Critical Analysis

In summary, our initial findings demonstrate that caffeine ingestion increases plasma cholecystokinin levels 2 - 4 fold from baseline fasted levels in healthy human subjects. Additionally, fasted cholecystokinin levels may be related to an individual's average caffeine consumption. Although further studies are needed to confirm these preliminary results, the observed individualdifferences in response to caffeine suggest that caffeine-induced anxiety may occur via a cholecystokinin-mediated pathway, and that certain individuals may be more susceptible to the physiological and behavioural effects of caffeine. Future studies should include a determination of individual levels of CCK-4, CCK-8s and CCK-8ns in each sample. In our pilot study, the volume of blood taken precluded us from conducting a thorough analysis of individual CCK concentrations.

Future studies in the field of cholecystokinin and caffeine research should also include a comparison of the post-caffeine cholecystokinin plasma profiles in both healthy individuals and patients with panic disorder. As discussed above, it is known that

patients with panic disorder are more susceptible to the anxiogenic effects of caffeine as compared to healthy individuals (Charney *et al.*, 1985; Uhde *et al.*, 1984b). Studies that focus on caffeine, cholecystokinin and panic disorder may shed light on the complex neurobiological interactions that contribute to the enhanced sensitivity of panic patients to caffeine.

Finally, in order to determine whether differential susceptibility to caffeine is related to an individual's history of caffeine consumption, studies should include individuals who heavily consume caffeine as well individuals who are "caffeine-naïve." To evaluate whether an individual's cholecystokinergic response to caffeine is dependent on his or her anxiety level, standard psychological tests should be given to all participants. Psychological parameters should be measured both prior to caffeine consumption and at various time points thereafter. Finally, if caffeine reproducibly and safely increases cholecystokinin levels without triggering full-blown panic attacks or other psychological trauma, caffeine should be employed as the drug of choice in studies which aim to challenge the cholecystokinergic system in first-degree unaffected relatives of patients with panic disorder. These studies would determine whether a dysfunctional cholecystokinergic system is a marker of genetic vulnerability in panic disorder without subjecting phenotypically healthy individuals to the risk of triggering panic disorder.

Table 10. Caffeine levels in popular beverages and chocolate. Adapted from Consumer Reports On Health (Sept., 1997).

Item	Caffeine (mg)
Coffee	
Espresso, 2 oz	120
Regular, brewed, 6 oz	103
Instant, 6 oz	57
Instant, decaf, 6 oz	2
Tea	
Black, 6 oz	53
Green, 6 oz	32
Iced tea, instant, 12 oz	46
Soft drinks, 12 oz	
Jolt Cola	72
Sundrop	63
Kick	58
Mountain Dew	55
Mello Yello, Surge	53
Coca-Cola Classic	47
Sunkist Sparkling Lemonade	41
Sunkist Orange	40
Squirt Ruby Red	39
Pepsi	37
A&W Cream Soda	28
Barq's	23
Slice Cola	11
Water, caffeine enhanced, 12 oz	
Java Water	71
Krank2O	70
Water Joe	46
Aqua Java	43
Chocolate	
Baking chocolate, unsweetened, 1oz	58
Hershey Special Dark bar, 1.45 oz	31
Other bars, average, 1.55 oz	11

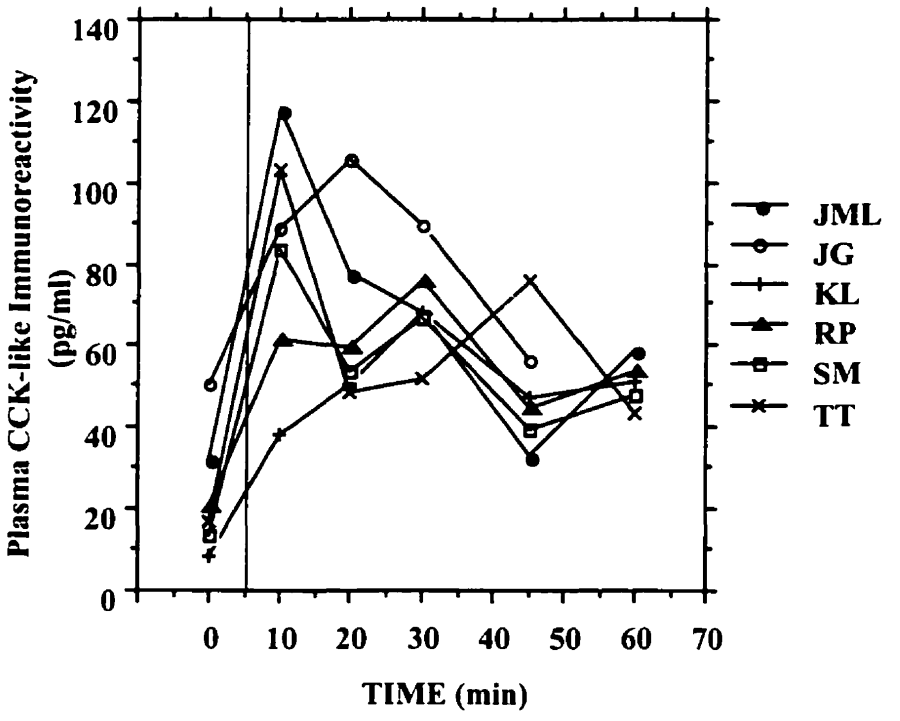

Figure 12. Plasma cholecystokinin-like immunoreactivity in 6 healthy volunteers pre- and post-ingestion of a caffeinated milkshake, containing 200 mg caffeine. A baseline blood sample was taken at Time = 0 min., prior to ingestion of the milkshake at T = 5 min. Blood was also drawn at T = 10, 20, 30, 45 and 60 min. post-ingestion.

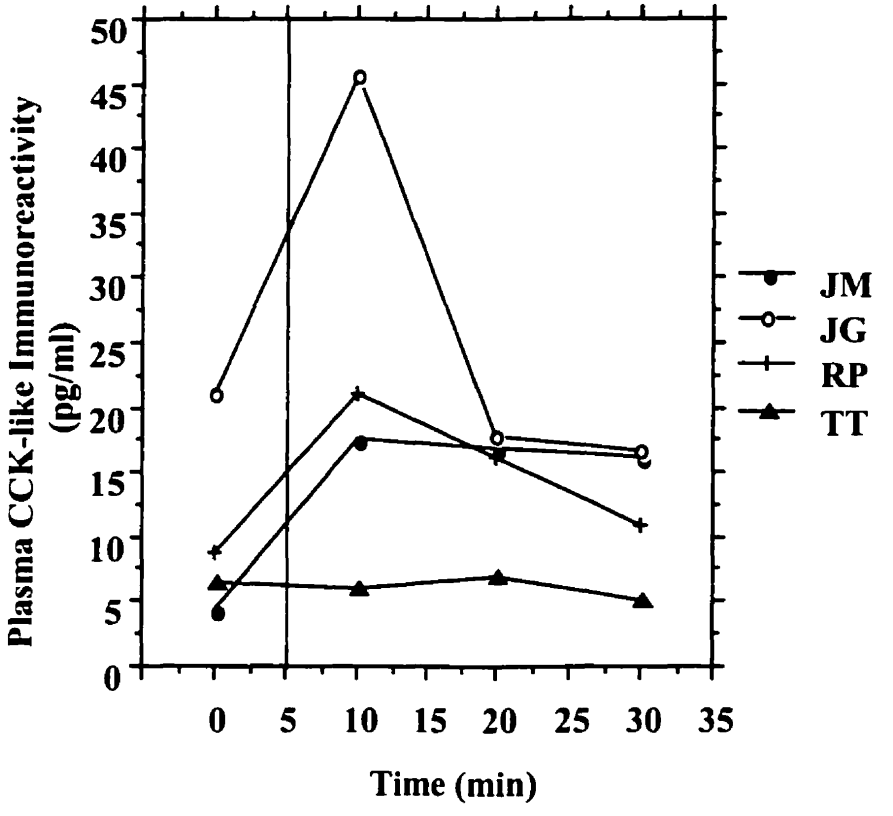

Figure 13. Plasma cholecystokinin-like immunoreactivity in 4 healthy volunteers pre- and post-ingestion of 200 mg oral caffeine. A baseline blood sample was taken at Time = 0 min., prior to ingestion of caffeine at T = 5 min. Blood was also drawn at T = 10, 20 and 30 min. post-ingestion.

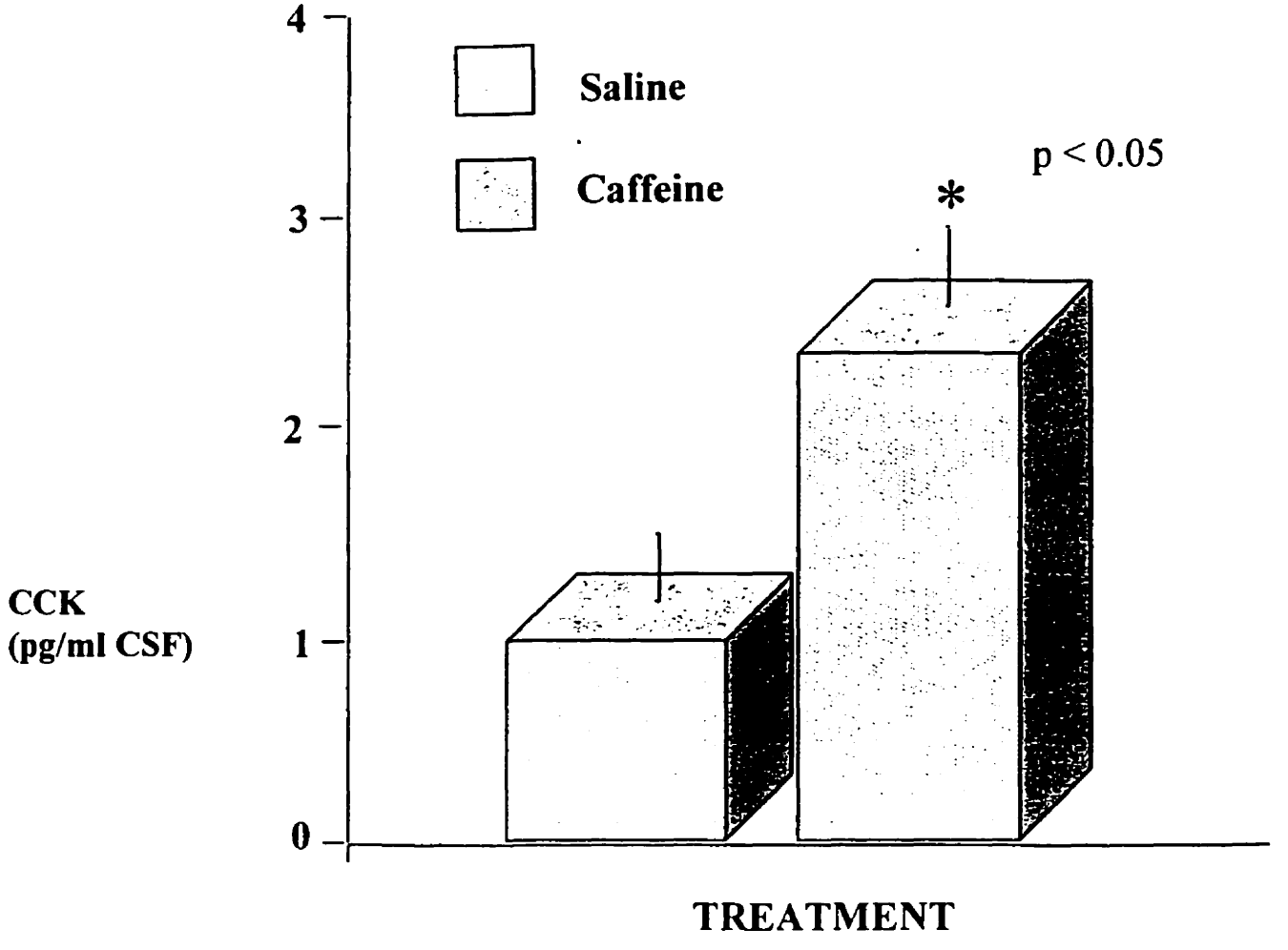

Figure 14. Cerebrospinal fluid (CSF) cholecystokinin-like immunoreactivity in 10 African green monkeys, post-saline and post-caffeine administration.

9.5 Cholecystokinin and Serotonin Interactions in the Regulation of Anxiety

Serotonin, or 5-hydroxytryptamine (5-HT), interacts with multiple brain 5-HT receptors to modulate a wide range of behavioural processes (Cowen, 1991). The serotonergic system has long been implicated in stress and anxiety (Iversen, 1984). Stimulation of the 5-HT system generally produces an anxiogenic effect. Likewise, serotonin antagonists result in the attenuation of fear and anxiety (Raiteri *et al.*, 1993; Rex *et al.*, 1994). Antagonists of the 5-HT_3 receptor subtype have been particularly useful in reducing the stress response in animal models of anxiety. In these pre-clinical studies, 5-HT_3 receptor antagonists, such as ondansetron, have proven to be anxiolytic (Vasar *et al.*, 1993). For instance, these antagonists have increased social interaction of singly housed rats and reduced the aversive response of mice in the black-white box (Costall and Naylor, 1992). In addition, a number of clinical studies have demonstrated the efficacy of 5-HT_3 antagonists in treating anxiety in human subjects (Costall and Naylor, 1992; Romach *et al.*, 1998) .

A number of studies have investigated the interaction between the cholecystokinergic and serotonergic systems. The extensive co-localization and functional relationship between the two systems have been described earlier in this dissertation (Chapter Four, Section 4.3). Many researchers have speculated that an interaction between serotonin and CCK is involved in the regulation of emotional behaviour (Paudice and Raiteri, 1991; Vasar *et al.*, 1993). In addition to the co-localization of CCK and serotonin in the brain, evidence suggests that each neurotransmitter can reciprocally modulate the release, metabolism and binding of the

other (see Chapter Four, Section 4.3). Paudice and Raiteri (1991) have demonstrated that serotonin enhanced the depolarization-evoked release of CCK-like immunoreactivity (CCK-LI) in the rat cerebral cortex and nucleus accumbens. Likewise, cholecystokinin has been shown to regulate serotonin release. Cholecystokinin release could be blocked by the 5-HT_3 antagonist ondansetron, whereas a 5-HT_1/5-HT_2 receptor antagonist was ineffective (Paudice and Raiteri, 1991). Thus, the authors concluded that serotonin-mediated release of cholecystokinin in the rat brain is potentiated via the activation of 5-HT_3 receptors. The same team of researchers demonstrated that ondansetron inhibited the depolarization-evoked release of CCK-LI in the rat frontal cortex (Raiteri *et al.*, 1993). Rex and co-workers (1994) reported that administration of BOC-CCK-4, a CCK-B receptor agonist, enhanced the release of serotonin in the frontal cortex of rats exposed to an elevated plus maze, an anxiogenic paradigm frequently used to assess anxious behaviour. Vasar and colleagues (1993) demonstrated that systemic administration with caerulein, a non-selective CCK agonist, suppressed the exploratory behaviour of rats in the elevated plus maze. Interestingly, pretreatment of rats with ondansetron completely reversed the anti-exploratory effect of caerulein, implicating the interaction of cholecystokinin and the 5-HT_3 system in anxiety.

The substantial evidence which implicated the interaction of cholecystokinin and serotonin in the regulation of anxiety prompted our investigation into the putative mechanisms involved (Dépôt *et al.*, 1998). We examined the effect of treatment with ondansetron, a specific 5-HT_3 antagonist, on endogenous and exogenous CCK levels in human plasma.

Ondansetron

The aim of our investigation was to determine whether acute and chronic treatment with ondansetron affected cholecystokinin plasma levels or disposition after intravenous administration of exogenous CCK-4. Since previous studies had implicated the 5-HT_3 pathway in the modulation of the cholecystokinergic system, we decided to use ondansetron as it is a well-characterized, specific 5-HT_3 antagonist. A detailed description of the methodology employed in our study is provided in Dépôt *et al.*, 1998. Briefly, subjects were given injections of CCK-4 on two separate occasions. Prior to the first cholecystokinin injection, subjects were given 2 mg ondansetron 60 minutes before CCK-4 was administered. Subjects then continued taking ondansetron daily for 28 days. After this four-week treatment, the second dose of CCK-4 was injected to observe the effects of chronic ondansetron treatment on cholecystokinin. Blood samples were taken in a manner consistent with the methodology of Merani *et al.* (1997), in order for subsequent determination of cholecystokinin plasma concentrations. Plasma levels of cholecystokinin-like immunoreactivity post-CCK-4 injection were evaluated as tetrapeptide, since the injected dose of 50 μg (or ~20 000 pg/ml plasma) was approximately 2 000 times the average endogenous concentration of circulating cholecystokinin in human plasma (~10 pg/ml plasma) (Merani *et al.*, 1997).

Basal levels of plasma cholecystokinin, assessed one hour post placebo or ondansetron administration, were similar between the placebo and treatment groups. Following a single dose of ondansetron, the increase in plasma CCK levels from baseline (deltaCCK) was not significantly different between the placebo and ondansetron groups.

A similar elimination rate constant (CCKz) was also observed between the two groups (Dépôt *et al.*, 1998).

After chronic administration of ondansetron (i.e. 4-week treatment), deltaCCK was significantly higher in the ondansetron group as compared to the placebo group. Further, there was a trend towards a decrease in basal plasma cholecystokinin levels and CCKz after chronic ondansetron treatment (Dépôt *et al.*, 1998, unpublished data).

These findings suggest that multiple oral doses of ondansetron affect the disposition of endogenous CCK as demonstrated by an increase in plasma CCK concentration from baseline as compared to placebo. Moreover, the distinct trend towards a decrease in the basal plasma CCK levels and a delay in CCK removal after intravenous CCK-4 administration supports the theory that chronic ondansetron treatment influences pharmacokinetic parameters of cholecystokinin.

In summary, our investigation into the relationship between cholecystokinin and ondansetron, a specific 5-HT_3 antagonist, supports the theory that the serotonergic and cholecystokinergic systems interact to modulate anxious behaviour via a 5-HT_3 receptor-mediated pathway.

CHAPTER TEN

CHOLECYSTOKININ AND THE CARDIOVASCULAR SYSTEM

CHAPTER TEN

CHOLECYSTOKININ AND THE CARDIOVASCULAR SYSTEM

10.1 Introduction

Historically, anxiety and panic disorder, because of their physical symptoms, were presumed to be a consequence of a diseased heart or nervous system. In 1871, Da Costa described a syndrome which occurred in young soldiers and consisted of chest pain, palpitations, sweating, light-headedness and shortness of breath. He attributed this "irritable heart syndrome" to a disturbance in the sympathetic nervous system (Da Costa, 1871).

Several lines of evidence implicate cholecystokinin in the regulation of the cardiovascular system. Direct evidence is derived from studies that involve the intravenous administration of cholecystokinin tetrapeptide to human subjects. These landmark experiments, described below, establish that administration of CCK-4 induces rapid and reproducible cardiovascular changes — changes that are an inherent component of the panic attacks that are caused by cholecystokinin injection (Bradwejn *et al.*, 1990; Bradwejn *et al.*, 1992c; Shlik *et al.*, 1997). Very few studies have directly investigated the role of cholecystokinin in cardiovascular pathology. These studies include the examination of CCK in hypertension (Kirouac and Ganguly, 1993b), and our own research on CCK and cardiomyopathy, details of which can be found in the manuscript entitled *Altered Cholecystokinin Binding in the Cardiomyopathic Hamster Brain* (Merani *et al.*, 2000), provided at the end of this chapter.

Indirect evidence for the involvement of cholecystokinin in cardiovascular function stems from epidemiological studies which report an association between cardiovascular disease and panic disorder (Kahn *et al*, 1990; Katerndahl, 1993; Fleet *et al.*, 1996), leading to the hypothesis that a common cholecystokinergic pathway may be involved in the etiology of both disorders.

10.2 Cardiovascular Effects of Cholecystokinin

There is a large body of evidence, discussed in detail in Chapter Nine, Section 9.3, which implicates cholecystokinin in anxiety and panic disorder (Bradwejn *et al.*, 1990; De Montigny, 1989; Harro *et al.*, 1993; Lydiard *et al.*, 1992). Numerous studies have shown that intravenous injections of CCK-4 induce panic attacks in healthy control subjects and patients with panic disorder (Bradwejn *et al.*, 1990; Bradwejn *et al.*, 1992c; Shlik *et al.*, 1997). Because CCK-4 has proven to be a valuable pharmacological tool in the investigation of the pathogenesis of panic disorder, the behavioural and cardiovascular effects of CCK-4 administration are welldocumented.

Administration of CCK Tetrapeptide

Administration of CCK tetrapeptide has been reported to elicit a pronounced cardiovascular response in both healthy control subjects and patients with panic disorder. Intravenous administration of CCK-4 has been reported to induce dyspnea, palpitations and chest pain symptoms in panic patients (Bradwejn *et al.*, 1990; Bradwejn *et al.*,

1992c). Shlik and co-workers (1997) demonstrated that maximal increases in heart rate and mean blood pressure were higher in healthy volunteers after a 50 μg intravenous dose of CCK-4 than after a placebo injection. These changes were evident between 20 and 80 seconds after injection. Marked dyspnea was also observed after cholecystokinin administration. Since bronchoconstriction was not believed to be involved, the authors concluded that dyspnea was a consequence of the action of CCK-4 on respiratory control rather than respiratory resistance (Shlik *et al.*, 1997).

Bradwejn and colleagues (1992) designed a study in which they specifically evaluated the effects of CCK-4 on cardiovascular parameters in patients with panic disorder (Table 11). A strong dose-response curve was observed between intravenous cholecystokinin administration and heart rate. Diastolic blood pressure also increased linearly with increasing doses of cholecystokinin tetrapeptide. A linear dose-response trend for systolic blood pressure was observed, but was not significant. Cardiovascular changes peaked within 2 minutes of CCK-4 injection, paralleling the behavioural changes that occurred with CCK-4 administration (Bradwejn *et al.*, 1992b).

Administration of CCK Octapeptide

A comprehensive examination of the cardiovascular effects of CCK-8s in human subjects is not available because of the severe side effects produced by the peptide. In contrast to CCK-4 injections, subjects have reported the occurrence of intense gastrointestinal symptoms, such as nausea and abdominal cramps, promptly upon

injection of CCK-8s, making it prohibitive for testing in human subjects (De Montigny, 1989). However, animal studies have determined that intravenous CCK-8s elicits a dose-dependent bradycardia and increase in mean arterial blood pressure in the rat (Gaw *et al.*, 1995). In rats bled to hemorrhagic shock, intravenous injection of CCK-8s resulted in the prompt and sustained improvement of cardiovascular function by inducing a large increase in arterial pressure and in circulating blood volume (Guarini *et al.*, 1988).

Thus, only a limited number of studies have examined the direct connection between cholecystokinin and the cardiovascular system. These include experiments that show that administration of CCK peptides causes pronounced cardiovascular changes and that CCK receptors are altered in a rat model of hypertension (described in Section 10.4). Our investigation into the role of cholecystokinin in cardiovascular disease was prompted by these direct studies, and in addition, by more indirect findings: the studies which establish that CCK plays an important role in panic disorder and the studies which demonstrate a strong association between panic disorder and cardiovascular illnesses (Section 10.3).

Table 11. A dose-ranging study of the cardiovascular effects of intravenous administration of cholecystokinin tetrapeptide in patients with panic disorder. Values reported as percent maximum increase from baseline. Adapted from Bradwejn *et al.*, 1992.

Dose of Administered CCK Tetrapeptide or Placebo	Heart Rate	Systolic Blood Pressure	Diastolic Blood Pressure
Placebo	7.4 %	8.8 %	6.2 %
10 μg	18.4 %	8.9 %	10.2 %
15 μg	20.1 %	11.6 %	10.3 %
20 μg	25.9 %	14.2 %	10.8 %
25 μg	31.7 %	20.2 %	12.2 %

10.3 Cardiovascular Risk and Panic Disorder

Indirect evidence which supports the hypothesis that cholecystokinin is involved in cardiovascular regulation is derived from epidemiological data which reveals an association between panic disorder and cardiovascular morbidity. As previously discussed in Chapter Nine, Section 9.3, the involvement of the cholecystokinin system in panic disorder is well-established (Bradwejn *et al.*, 1990; De Montigny, 1989; Lydiard *et al.*, 1992). Endogenous cholecystokinin peptide concentrations have been reported to be altered in patients with panic disorder (Lydiard *et al.*, 1992). Further, administration of exogenous CCK-4 not only causes the behavioural symptoms of panic, but simultaneously increases the heart rate and diastolic blood pressure (Bradwejn *et al.*, 1990; Bradwejn *et al.*, 1992c; Shlik *et al.*, 1997). In light of these findings, one can speculate that the cardiac dysfunction that is frequently found in patients with panic disorder may be related to an aberrant cholecystokinergic system.

In addition to the cognitive elements of fear and anxiety that occur during panic attacks, panic episodes are characterized by chest pain and increased heart rate and blood pressure (Bradwejn *et al.*, 1990; Bradwejn *et al.*, 1992c). In fact, these cardiovascular changes, which mimic the symptoms of cardiovascular infarction, usually prompt patients with panic disorder to seek emergency medical attention. One study reported that 17.5% of emergency room patients complaining of chest pain could be diagnosed with panic disorder (Yingling *et al.*, 1993). In a study conducted at the Montreal Heart Institute, 441 consecutive patients consulting the emergency department with the chief complaint of

chest pain were assessed for panic symptoms. Approximately 25% of chest pain patients met criteria for panic disorder (Fleet *et al.*, 1996).

A specific association between panic disorder and idiopathic dilated cardiomyopathy has been reported. Echocardiograph screening revealed that 23% of panic disorder patients had an increased left ventricular chamber size and 20% of patients had an increased left ventricular mass as compared to control subjects (Kahn *et al*, 1990).

Chest pain is a common symptom of panic disorder. In fact, the majority of patients with panic disorder initially see a cardiologist or primary care physician as a result of their chest discomfort (Beitman *et al.*, 1987). In a study of 94 patients with chest pain and normal coronary arteries, 34% were reported as having panic disorder (Beitman *et al.*, 1989). In a study of 49 patients with coronary artery disease, none of the patients with typical angina pectoris had panic disorder, whereas 40% of patients with atypical angina pectoris had panic disorder (Basha *et al.*, 1989).

Further evidence to support the theory that cardiovascular dysregulation is present in patients with panic disorder is derived from a study which revealed that resting panic patients have reduced levels of plasma noradrenaline and abnormal heart rate variability (Middleton *et al.*, 1994). Treatment of patients by either cognitive therapy or imipramine and their subsequent clinical recovery from panic disorder has resulted in a normalization of certain cardiovascular measures such as plasma noradrenaline, heart rate variability and blood pressure response to standing (Middleton and Ashby, 1995).

Mitral Valve Prolapse and Panic Disorder

Mitral valve prolapse is the most common type of valvular heart disease in North America (McLachlan *et al.*, 1998). It is thought that the primary source of dysfunction is that the prolapsed mitral valves and their support structures show myxomatous degeneration. Affected mitral leaflets and chordae tendineae are frequently thickened, enlarged and redundant (Alpert *et al.*, 1991).

Like panic disorder, mitral valve prolapse occurs more frequently in women than in men. The prevalence in young women has been reported to range from 6% to 17% and from 2% to 4% in men (Alpert *et al.*, 1991). Although the exact genetic defect remains to be determined, evidence suggests that mitral valve prolapse is an inherited illness which may be transmitted in an autosomally dominant manner (Devereux, 1994). Symptoms of mitral valve prolapse include chest pain, palpitations and dyspnea. The disease is usually benign, and most patients require no treatment. However, significant complications may occur; these include progressive mitral regurgitation with heart failure, cardiac arrhythmias, infective endocarditis, systemic emboli and rarely sudden death (McLachlan *et al.*, 1998). High levels of circulating catecholamines have been reported in patients with mitral valve prolapse (Pasternac *et al.*, 1982).

The frequency of mitral valve prolapse in patients with panic disorder and the frequency of panic disorder in patients with mitral valve prolapse have both been examined. A wide range of frequencies has been reported in the literature. The frequency of mitral valve prolapse in patients with panic disorder has ranged from zero to 39% (Chan *et al.*, 1984; Grunhaus *et al.*, 1982). The frequency of panic disorder in

patients with mitral valve prolapse has ranged from zero to 16% (Hartman *et al.*, 1982; Mazza *et al.*, 1986). The discrepancy in the reported values has been attributed to the lack of uniform criteria used to diagnose panic disorder and particularly mitral valve prolapse (Alpert *et al.*, 1991; Katerndahl, 1993). In an attempt to review the literature and combine the results from different studies, a meta-analysis was conducted to resolve the conflicting data on the association between mitral valve prolapse and panic disorder. A significant association between the two disorders was found (Katerndahl, 1993). The most recent study to investigate the relationship between mitral valve prolapse and panic disorder compared the prevalence of mitral valve prolapse in 122 patients with panic disorder. Although the incidence of mitral valve prolapse was higher in the panic disorder group as compared to healthy controls (32.2% versus 16.7%), the difference was not significant (Hamada *et al.*, 1998).

Among the studies that have demonstrated that mitral valve prolapse and panic disorder co-occur, evidence of a causal link between the two pathologies has also been an issue of contention (Alpert *et al.*, 1991; Coplan *et al.*, 1992). Investigators have argued that the two disorders are associated by chance (Alpert *et al.*, 1991), that mitral valve prolapse causes panic disorder (Pariser *et al.*, 1978) and that panic disorder causes mitral valve prolapse (Coplan *et al.*, 1992; Gorman *et al.*, 1988). A common pathophysiological or biochemical mechanism that unites both illnesses remains unidentified (Alpert *et al.*, 1991).

One study has reported that patients with both panic disorder and mitral valve prolapse demonstrated an amelioration of the prolapse after behavioural and/or

pharmacological treatment with either benzodiazepines or tricyclic antidepressants. One plausible explanation for this finding is that autonomic overdrive in panic disorder results in a temporary deformation in the mitral valve. Thus, according to one scientist, upon alleviation of panic disorder and the attenuation of autonomic overdrive, the prolapse can actually be reversed (Coplan *et al*, 1992). Alternatively, if a common biochemical abnormality, such as cholecystokinin, is involved in the etiology of both illnesses, then normalization of a defect in the cholecystokinergic system may be one mechanism by which treatment of panic disorder can alleviate mitral valve prolapse.

10.4 Cholecystokinin and Hypertension

Changes in dopaminergic transmission and up-regulation of dopamine receptors have been observed in the ventral and dorsal striatum of the spontaneously hypertensive rat (SHR), a genetic model for human hypertension (Kirouac and Ganguly, 1993a; Linthorst *et al.*, 1990). Because of the solid relationship between the cholecystokinergic and dopaminergic systems (described in Chapter Four, Section 4.2), one research team has proposed that an interplay between CCK and dopamine contributes to the pathophysiology of hypertension (Kirouac and Ganguly, 1993b). Based on findings that cholecystokinin can modulate dopamine neurotransmission and vice versa (Freeman *et al.*, 1991;You *et al.*, 1996), Kirouac and Ganguly (1993b) speculated that changes in dopamine release or receptor binding in the spontaneously hypertensive rat are related to changes in CCK-8 neurotransmission. Upon perfusion of the rat nucleus accumbens with CCK-8s, the scientists demonstrated that CCK-8s-induced release of dopamine is greater

in the SHR than in normotensive rats (Kirouac and Ganguly, 1995a). Using *in vitro* autoradiography, the same investigators demonstrated that SHRs, as compared to normotensive rats, have an increase in CCK-8 receptor density in the lateral region of the caudate-putamen, and a decrease in CCK-8 receptor binding density in the posteromedial nucleus accumbens (Kirouac and Ganguly, 1993b). Abnormal cholecystokinin receptor densities may be a reflection of altered peptide levels. For example, reduced levels of cholecystokinin peptide may result in an up-regulation of receptor synthesis; conversely, an increased receptor density may cause a reduction in peptide levels. In a study designed to measure cholecystokinin peptide concentrations, Shulkes and co-workers (1989) found that the SHR had significantly lower levels of cholecystokinin octapeptide in the hippocampus, cortex, spinal cord and pituitary, as compared to age-matched control rats.

To investigate whether the alteration in cholecystokinin receptor density is involved in the development of hypertension, Kirouac and Ganguly (1195b) examined the temporal relationship between receptor changes and the onset of hypertension. They discovered an up-regulation of cholecystokinin receptors in the nucleus accumbens of the young pre-hypertensive SHR model, and postulated that changes in CCK-8s neurotransmission or receptor function were not secondary to an increase in arterial blood pressure but might, in fact, be involved in the pathogenesis of hypertension.

10.5 Cholecystokinin and Cardiomyopathy

Cardiomyopathy is a general term applied to conditions in which lesions are present in the myocardium of the heart. Cardiomyopathy is a significant cause of myocardial failure and may lead to severe cardiac dysfunction and death (Bishop *et al.*, 1979). Idiopathic cardiomyopathy involves progressive myocardial dysfunction. Although the precise etiology of the illness is not known, researchers have speculated that idiopathic cardiomyopathy can be caused by repeated occurrences of myocarditis caused by viral or autoimmune origins (Johnson and Palacios, 1982).

In a study designed to assess the prevalence of panic disorder in idiopathic cardiomyopathy, the diagnosis of idiopathic cardiomyopathy was assigned to patients with a dilated or hypertrophic left ventricle which could not be attributed to other illnesses. The investigators found that 29 of 35 patients (83%) had panic disorder (Kahn *et al.*, 1987). The authors suggested that autonomic mechanisms may underlie the association of cardiomyopathy and panic disorder. However, the issue as to whether panic disorder is primary to cardiomyopathy, or is a consequence of cardiac degeneration remains controversial. One potential mechanism involves the development of cardiomyopathy due to a panic-related increase in centrally mediated cardiac sympathetic tone. Panic-related increases in peripheral catecholamine levels may also be involved in the pathogenesis of cardiomyopathy. Elevated catecholamine levels have long been implicated in the etiology of cardiomyopathy. Infusion of various catecholamines has been shown to induce myocardial hypertrophy in animals (Laks *et al.*, 1973).

Catecholamine surges caused by phoeochromocytomas can cause myocarditis and cardiomyopathy in humans (VanVliet *et al.*, 1966).

The hypothesis that panic disorder may be the consequence of end-stage cardiomyopathy is another tenable theory. Congestive heart failure is associated with elevated catecholamine levels (Cohn *et al.*, 1984), which in turn, could trigger panic attacks (Kahn *et al.*, 1987). However, it is important to note that the majority of patients with panic disorder do not have end-stage cardiomyopathy.

Several lines of evidence prompted our investigation into the role of the cholecystokinergic system in cardiomyopathy. First, the co-morbid association of panic disorder and cardiomyopathy, described above, may be the consequence of an aberration in a common pathway, possibly cholecystokinin. Second, intravenous administration of CCK frequently evokes cardiovascular changes (Bradwejn *et al.*, 1990; Bradwejn *et al.*, 1992c; Shlik *et al.*, 1997). Further, alterations in brain cholecystokinin receptor density have been found in animal models for other disorders of the cardiovascular system, such as hypertension (Kirouac and Ganguly, 1993b).

To determine whether cholecystokinin is involved in the pathology of cardiomyopathy, we examined brain CCK receptor binding in the cardiomyopathic (CMO) hamster. The CMO hamster, an animal model of cardiomyopathy, is an inbred strain of the golden Syrian hamster with an inherited form of myocardial degeneration and congestive heart failure. This animal model serves as a useful paradigm for the cardiac necrosis that occurs in certain human myocardial diseases (Homburger and Bajusz, 1970). The precise neuronal mechanisms involved in cardiomyopathy are not yet

established; however, there have been reports of altered neurochemical concentrations in CMO animals (Allen *et al.*, 1995; Sole *et al.*, 1978). Biochemical studies have revealed elevated levels of serotonin and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the medulla, pons and posterior hypothalamus of aged CMO hamsters as compared to control animals (Sole *et al.*, 1978). Using immunoreactive staining, Allen and co-workers (1995) demonstrated that the CMO hamster brain contained more densely stained tyrosine hydroxylase and serotonin immunoreactive axons in the lateral parabrachial nucleus.

The discovery of increased catecholaminergic and serotonergic immunoreactivity in the CMO hamster brain, together with findings that establish significant co-localization and functional interactions of CCK with dopamine and serotonin (Chapter Four, Sections 4.2 and 4.3), warranted an experiment which evaluated the role of cholecystokinin in cardiomyopathy.

The main objective of our investigation was to compare the cholecystokinin receptor density in the cardiomyopathic and Syrian hamster brain. We employed quantitative *in vitro* autoradiography using ^{125}I-CCK-8ns as tracer (Merani *et al.*, 1998). Although quantitative autoradiography has been used by other investigators to characterize neural cholecystokinin binding in the Syrian hamster (Miceli *et al.*, 1989), our study was the first to describe CCK binding in the CMO model. Since the cardiomyopathic hamster shows myocardial degeneration in progressive stages, three different age groups were used. CCK binding was examined in 25-, 125- and 225-day old cardiomyopathic animals and age-matched controls. These ages were chosen to

represent the pre-necrotic, necrotic and compensated stages of cardiomyopathy respectively (Li and Rouleau, 1993). A detailed description of the technique, as well as the corresponding results are described in the following manuscript-in-submission entitled *Altered Cholecystokinin Binding in the Cardiomyopathic Hamster Brain* (Merani *et al.*, 2000).

10.6 Manuscript—Altered Cholecystokinin Binding in the Cardiomyopathic Hamster Brain

Altered Cholecystokinin Binding in the Cardiomyopathic Hamster Brain

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ABSTRACT

Quantitative in vitro autoradiography was employed to examine neuronal cholecystokinin (CCK) receptor density in the cardiomyopathic (CMO) and Syrian hamster brain. ^{125}I-CCK-8 binding was examined in coronal and saggital brain sections of age and sex-matched cardiomyopathic and control hamsters (25, 125 and 225 days old), using unlabelled nonsulfated CCK-8, devazepide and PD-135,158 as competitors. General binding profiles revealed high density labeling in the cortex and olfactory bulb, while moderate binding was observed in the brain stem and hypothalamus. Little or no binding was observed in the thalamus and cerebellum. In the cardiomyopathic hamster brain, CCK binding was significantly elevated in the olfactory bulb, frontal cortex and occipital cortex as compared to control animals. In addition, a positive correlation between age and CCK binding in the olfactory bulb was observed in both cardiomyopathic and control hamsters. These results suggest that brain cholecystokinergic function may be significantly altered in cardiomyopathy.

Key Words: ***Cholecystokinin; CCK; Autoradiography; Receptor; Cardiomyopathy; CMO; Hamster***

INTRODUCTION

Cholecystokinin (CCK), first isolated in the gastrointestinal system, is now considered one of the most abundant peptides in the mammalian central nervous system (Beinfeld *et al.*, 1981). CCK is a putative neurotransmitter and neuromodulator which is involved in a wide range of physiological and behavioural processes (Bradwejn *et al.*, 1992b; Crawley and Corwin, 1994). Two main types of cholecystokinin receptors have been characterized. CCK-A receptors exist primarily in peripheral tissue and discrete brain regions, while CCK-B receptors are widely distributed throughout the brain (Moran *et al.*, 1986). The differential pharmacology of the two cholecystokinin receptors led to the original identification of the CCK-A and CCK-B receptor subtypes (Innis and Snyder, 1980). Subsequent studies demonstrated that the CCK-A receptor has a 500-1000 fold higher affinity for sulfated CCK analogues as compared to nonsulfated counterparts, whereas the CCK-B receptor has only a 3-10 fold higher affinity for sulfated peptides. The CCK-A receptor may also be recognized by its high affinity for the synthetic antagonist L-364,718 (devazepide). Alternatively, the CCK-B receptor possesses a high affinity for the antagonist L-365,260 and PD-135, 158 (Innis and Snyder, 1980).

Cholecystokinin has been implicated in various psychiatric illnesses, particularly anxiety disorders (Bradwejn *et al.*, 1992b; Harro *et al.*, 1993). Intravenous administration of exogenous CCK has been shown to induce panic attacks in healthy volunteers and patients with panic disorder, with the latter group demonstrating an

enhanced sensitivity to the challenge (Bradwejn *et al.*, 1991; 1992a). Panic disorder has been associated with idiopathic cardiomyopathy (Kahn *et al.*, 1987) and may be accompanied by additional cardiovascular abnormalities such as reduced heart rate variability and lower plasma noradrenaline (Middleton *et al.*, 1994), reduced cardiac left ventricular hypertrophy and chamber dilation (Kahn *et al.*, 1990) and mitral valve prolapse (Alpert *et al.*, 1991). The potential involvement of CCK in the co-morbid association of panic disorder and cardiomyopathy, together with the finding that CCK administration frequently evokes cardiovascular changes (Bradwejn *et al.*, 1992a), prompted our investigation of neuronal CCK receptor binding in the cardiomyopathic (CMO) hamster, an animal model of cardiomyopathy.

The CMO hamster is an inbred strain of the Syrian hamster with an inherited form of myocardial degeneration and congestive heart failure that serves as a paradigm for the cardiac necrosis that occurs in certain human myocardial diseases (Homburger and Bajusz, 1970). Although the exact neuronal mechanisms in cardiomyopathy are not yet established, recent findings indicate increased catecholaminergic and serotonergic immunoreactivity in the CMO hamster brain (Allen *et al.*, 1995). The co-localization and functional interactions of CCK with dopamine and serotonin (Crawley and Corwin, 1994; Hökfelt *et al.*, 1980; Raiteri *et al.*, 1993), as well as recent reports of neuronal CCK receptor alterations in other cardiovascular pathologies (Kirouac and Ganguly, 1993; 1995), further indicated a role for CCK in cardiomyopathy.

The primary objective of this study was to employ quantitative *in vitro* autoradiography with ^{125}I-labeled nonsulfated CCK-8 (^{125}I-CCK-8ns) to examine

neuronal CCK receptors in the cardiomyopathic and Syrian hamster. Quantitative autoradiography has previously been utilized to characterize CCK binding in the normal Syrian hamster brain (Miceli *et al.*, 1989), but this is the first report to examine cholecystokinin binding in the cardiomyopathic animal. Since the CMO hamster shows myocardial deterioration in progressive stages, CCK receptor binding was examined in 25-, 125- and 225-day old cardiomyopathic animals and age-matched controls.

MATERIALS AND METHODS

Materials

Nonsulfated CCK octapeptide (CCK-8ns) was obtained from Peptides International, Louisville, KY; ^{125}I was from Amersham, Oakville, ON. Bovine serum albumin (BSA), aprotinin, bacitracin and DTT were from Sigma Chemical Company, St. Louis, MO; KCl and $MgCl_2$ were obtained from Fisher, Montreal, PQ; Tris-(hydroxymethyl) aminomethane and NaCl were purchased from ACP Inc., Montreal, PQ; L-364,718 (devazepide) and PD-135,158 were generous gifts from Dr. Franco Vaccarino, Clarke Institute, Toronto, ON.

Animals & Slide Preparation

Twenty-four male cardiomyopathic (BIO 14.6) and control Syrian hamsters (Canadian Hybrid Farms, Nova Scotia, Canada), age 25, 125 and 225 days, were chosen to represent the pre-necrotic, necrotic and compensated stages of cardiomyopathy respectively (Li and Rouleau, 1993). The cardiomyopathic (CMO) hamsters (200-230

days) used were of the UM-X7.1 myopathic line established by cross-breeding diseased animals from the BIO 14.6 Golden Syrian hamster strain (BioResearch Institute, Cambridge, Mass., USA) with unrelated healthy hamsters. These animals exhibit the same pathological changes in heart and skeletal muscles originally found in their progenitors. Their main advantage resides in the fact that the disease is rather homogenous, with a predictable clinicopathological course starting with cardiac lesions at 30 days of age, and developing moderate to severe heart failure between 200 and 300 days. At 200 days, cardiomyopathic hamsters have generalized edema and pleural and abdominal effusion, altered liver size, color and firmness and an increase of heart volume. Age-matched normal Golden Syrian hamsters (*Mesocricetus auratus*) were used as a control (Charles River, St. Constant, Quebec, Canada). The animals were housed in plastic cages at room temperature with 60% humidity, in a 12 h light-dark cycle and had free access to water and regular pellet chow (Purina, Richmond, IN, USA). Hamsters were decapitated and brains immediately extracted and frozen in an isopentane-dry ice bath. Tissues were stored at -80°C until sectioning. Coronal and saggital sections (10 µm) were cut using a cryostat microtome, thaw-mounted onto gelatin-coated glass slides and dried overnight at 4°C in a dessicator. Slides were stored at -80°C until use.

Autoradiography

CCK-8ns was iodinated as previously described method for Bolton-Hunter CCK-4 (Merani *et al.*, 1997). Autoradiography was performed using a modified version

of that described in Niehoff (1989). Briefly, thawed sections of hamster brain were preincubated in buffer containing 130 mM NaCl, 5 mM $MgCl_2$, 4.7 mM KCl, 0.2% BSA in 50 mM Tris/HCl, pH 7.4, for 20 min. at room temperature. Slides were then transferred to incubation buffer containing 130 mM NaCl, 5 mM $MgCl_2$, 4.7 mM KCl, 1mM DTT, 0.025% bacitracin and aprotinin in 50 mM Tris/HCl, pH 6.5, in the presence of 50 pM ^{125}I CCK-8ns for 90 min. at room temperature. Slides were washed 3 times for 2 minutes in 50 mM Tris buffer, pH 7.4 at 4°C, and immersed in ice-cold distilled H_2O for 10 seconds. Slides were dried by a stream of cool air prior to exposure to a phosphor-sensitive cassette for 72 hrs. The cassette was scanned and visualized by Phosphorimager. Specific brain regions were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Non-specific binding was determined in adjacent regions by displacement with unlabeled CCK-8ns (10^{-6} M). Eight measurements for total and eight measurements for nonspecific binding were averaged, and nonspecific binding was subtracted from total binding to yield valves for specific binding. Receptor subtype specificity was assessed by using CCK A (devazepide) and CCK B (PD 135,158) receptor antagonists.

Data Analysis

Receptor binding data were fitted with the aid of nonlinear regression analysis to obtain displacement curves and IC_{50} values, using LIGAND and GraphPad prism (Version 3.0, San Diego, California) computer program. Statistical evaluation was

performed with a multiple analysis of variance (MANOVA) model. The level of significance was set at 5% for all analyses. Results are expressed as mean ± SEM.

RESULTS

^{125}I-CCK-8ns exhibited high brain-specific binding in all ages of control and CMO hamsters as illustrated on the colour phosphorimager scans (Fig.15). Specific binding to cholecystokinin receptors was confirmed by displacement with unlabeled CCK-8ns (10^{-6} M) (Fig.15). Complete displacement of ^{125}I-CCK-8ns was achieved with the antagonist PD-135,158 confirming that the CCK-B receptor subtype mediated cholecystokinin binding (results not shown). Minimal displacement occurred with the CCK-A receptor antagonist devazepide (results not shown). These results are consistent with reports that indicate that the majority of CCK binding sites in mammalian brain are CCK-B receptors (Crawley and Corwin, 1994; Miceli and Steiner, 1989; Middleton *et al.*, 1994). Statistical analysis of ^{125}I-CCK-8ns binding by repeated measure ANOVA with age as a between group factor ($p < 0.0028$), brain area as a within-group factor ($p < 0.001$), and hamster strain (control vs. CMO) as a blocking factor ($p < 0.001$) showed that the binding was affected by all these features.

As presented in Fig.16, the ^{125}I-CCK-8ns binding concentrations were highest in occipital cortex, olfactory bulb and frontal cortex. Moderate binding was observed in the brain stem and hypothalamus. Low binding was present in the thalamus and cerebellum.

Analyses of ^{125}I-CCK-8ns binding data are presented in Fig.16. In the olfactory bulb and frontal cortex, changes in binding as an effect of age seem to be related to

receptor density as opposed to receptor affinity, as indicated by the non-parallel nature of the curves. In the occipital cortex, no effect of age is observed in CMO animals. In the occipital cortex of control animals, however, parallel curves suggest a higher EC_{50} at 225 days, indicating that binding affinity may be reduced in older animals. A specific comparison of binding in the occipital cortex between CMO and control animals at different ages (Fig.17) indicates that observed differences between the diseased and control hamsters are based on binding density rather than differential affinities. This is confirmed by the EC_{50} values presented in Table 12.

Analysis of the binding data also suggest the presence of two binding states: a high-affinity and low-affinity state (Table 12). Although further investigation and analysis is needed to confirm this finding, it is likely that B1 reflects the CCK receptor in its "activated" state, while B2 reflects the pool of inactive receptors. In the occipital cortex, significant differences exist at every age in B2 levels between control and CMO hamsters.

On the basis of all measurements, ANOVA analysis showed higher density ^{125}I-CCK-8ns binding sites in cardiomyopathic hamsters than in normal hamsters. However, this was statistically significant in hamsters at the age of 125 days, showing necrotic symptoms in the heart ($p < 0.018$), and hamsters with advanced cardiomyopathy at the age of 250 days ($p < 0.05$) but not in hamsters before onset of cardiac lesion aged 25 days ($p < 0.15$). An increase of specific CCK binding with age in the olfactory bulb ($p < 0.05$) was observed in both cardiomyopathic and control brain.

In cardiomyopathic hamsters, CCK binding was significantly elevated in the olfactory bulb ($p < 0.05$), frontal cortex ($p = 0.016$) and occipital cortex ($p = 0.023$) as compared to control animals. A trend toward increased CCK binding in CMO strain was observed in the hypothalamus ($p = 0.085$). No strain effect was present for the ^{125}I-CCK-8ns binding sites in the cerebellum, thalamus and brain stem.

DISCUSSION

The present study demonstrates that CCK receptor binding is altered in the olfactory bulb, frontal cortex and occipital cortex of the cardiomyopathic hamster as compared to control animals. This alteration primarily involves increased binding at the CCK-B receptor subtype, as demonstrated by nearly complete displacement with PD-135,158, a specific CCK-B receptor antagonist. These findings are consistent with other studies that have reported an altered cholecystokinergic system in aged animals (Harro and Oreland, 1992). For instance, cholecystokinin peptide has been shown to be a more potent satiation agent in older rats as compared to younger animals when administered exogenously (Salorio *et al.*, 1994). An altered cholecystokinin receptor binding profile has also been observed in the brains of older animals (Harro and Oreland, 1992). Further investigation is needed to determine the precise role of olfactory cholecystokinin receptor binding in the aging process.

Several lines of evidence implicate cholecystokinin in the regulation of the cardiovascular system. Direct evidence is derived from studies which involve the

intravenous administration of cholecystokinin tetrapeptide to human subjects. These landmark experiments have demonstrated that CCK-4 induces rapid and reproducible cardiovascular changes, an inherent component of the panic attacks as caused by cholecystokinin injection (Bradwejn *et al.*, 1991; 1992a). Animal studies have determined that intravenous CCK-8s elicits a dose-dependent bradycardia and increase in mean arterial blood pressure in the rat (Gaw *et al.*, 1995). In rats bled to hemorrhagic shock, intravenous injection of CCK-8s resulted in the prompt and sustained improvement of cardiovascular function by inducing a large increase in arterial pressure and in circulating blood volume (Guarini *et al.*, 1988). Indirect evidence for the involvement of cholecystokinin in cardiovascular function stems from epidemiological studies which report an association between cardiovascular disease and panic disorder (Alpert *et al.*, 1991; Kahn *et al.*, 1987; Kahn *et al.*, 1990; Katerndahl, 1993), lending support to the hypothesis that a common cholecystokinergic pathway may be involved in the etiology of both disorders. Despite abundant evidence which indicates that CCK is involved in the modulation of the cardiovascular system, only a limited number of studies have investigated the role of cholecystokinergic system in cardiovascular pathology. These studies include the examination of CCK receptor density in hypertension (Kirouac and Ganguly, 1993; 1995) and our own current research in cardiomyopathy, as outlined in this study.

The precise neuronal mechanisms involved in cardiomyopathy are not yet established; however, there have been reports of altered neurochemical concentrations in CMO animals (Allen *et al.*, 1995; Sole *et al.*, 1978). Elevated levels of serotonin and

its major metabolite, 5-hydroxyindoleacetic acid, have been noted in the medulla, pons and posterior hypothalamus of CMO hamsters as compared to control animals (Sole *et al.*, 1978). More recently, Allen and co-workers (1995) demonstrated that the CMO hamster brain contained more densely stained tyrosine hydroxylase and serotonin immunoreactive axons in the lateral parabrachial nucleus (Allen *et al.*, 1995). Because the cholecystokinergic system is intricately involved in the co-modulation of dopamine and serotonin (Hökfelt *et al.*, 1980; Raiteri *et al.*, 1993), we can speculate that a dysfunctional cholecystokinergic system may play an important role in the neuropathology of cardiomyopathy.

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References for this manuscript are integrated in the References Section provided at the end of this dissertation.

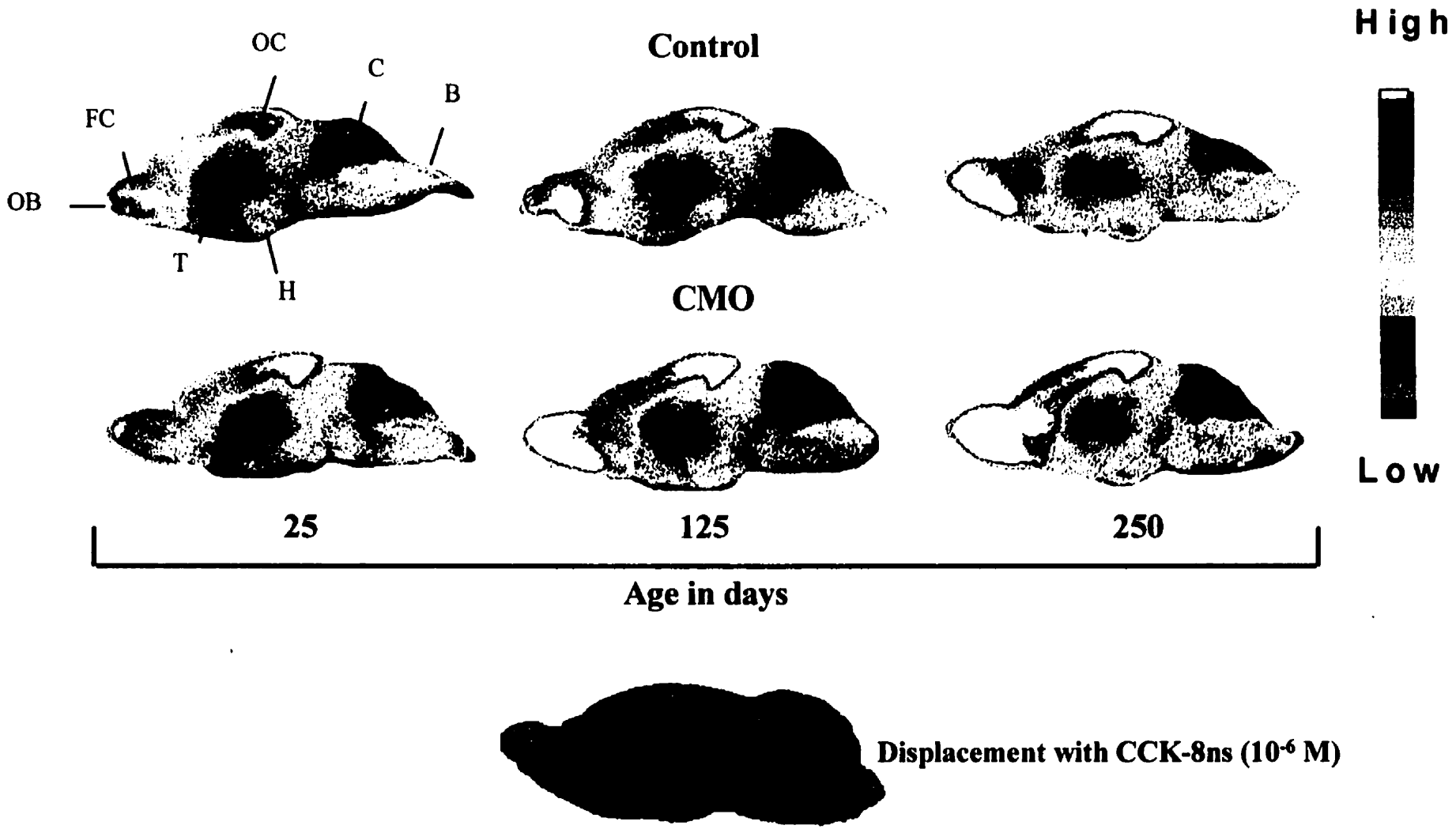

Figure 15. Autoradiographic visualization of ^{125}I Cholecystokinin octapeptide binding in brain regions of control and cardiomyopathic (CMO) hamsters at 25, 125 and 225 days. OB-Olfactory Bulb; FC-Frontal Cortex; OC-Occipital Cortex; C-Cerebellum; B-Brainstem; H-Hypothalamus; T-Thalamus.

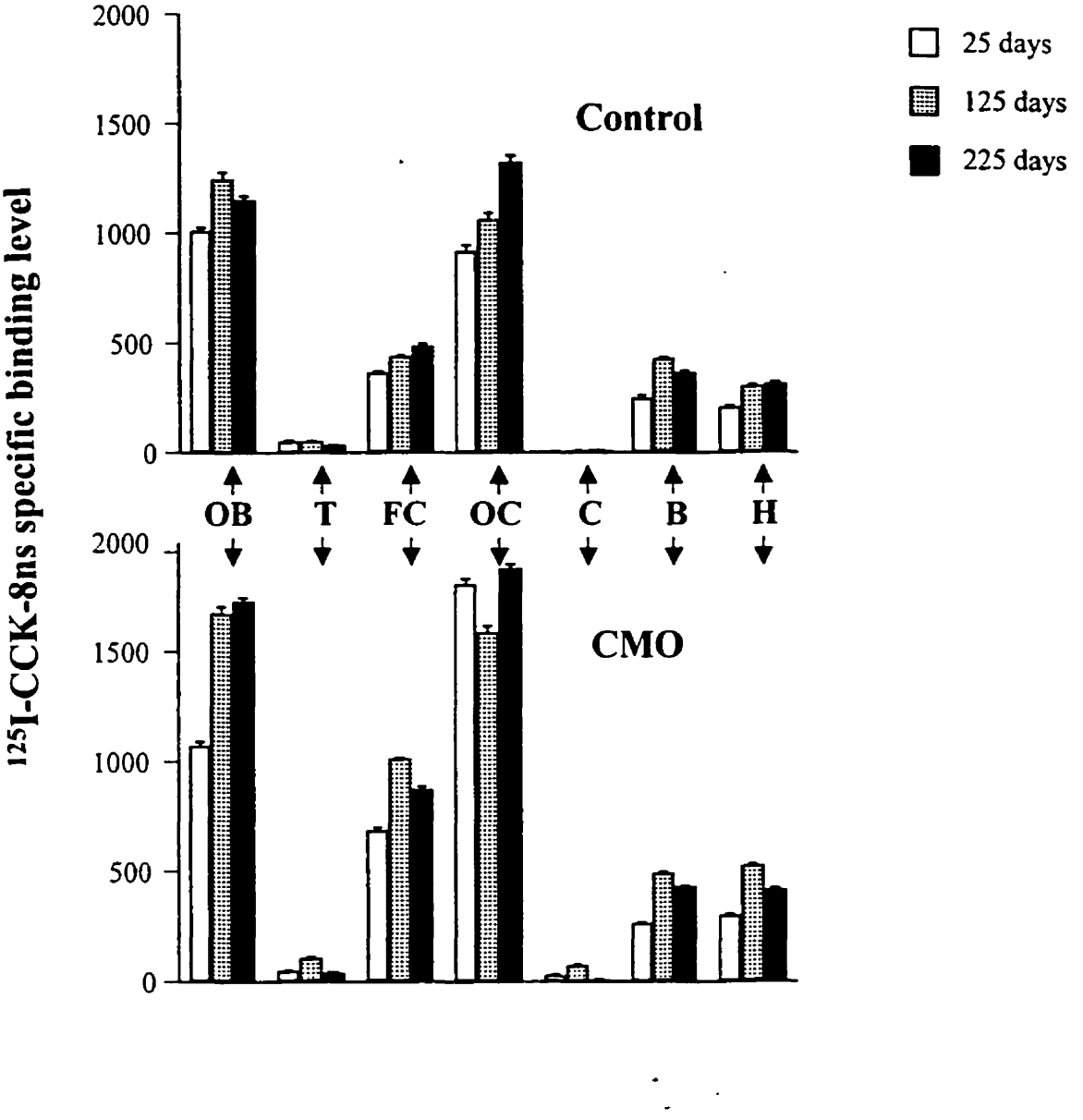

Figure 16. Specific binding levels of ^{125}I Cholecystokinin octapeptide in brain regions of control and cardiomyopathic (CMO) hamsters at 25, 125 and 225 days. OB-Olfactory Bulb; T-Thalamus; FC-Frontal Cortex; OC-Occipital Cortex; C-Cerebellum; B-Brainstem; H-Hypothalamus.

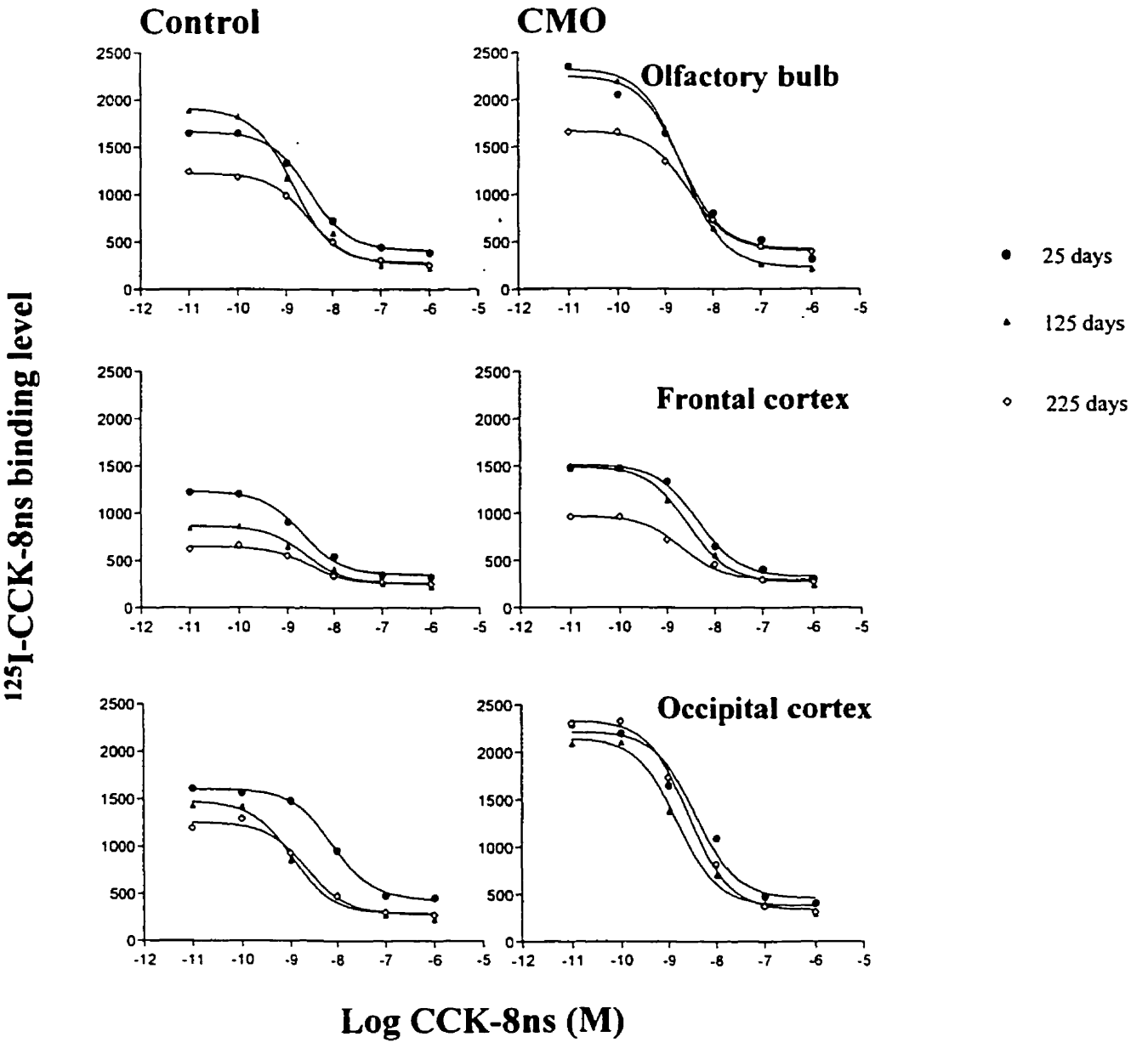

Figure 17. ^{125}I-CCK-8ns binding levels in the olfactory bulb, frontal cortex and occipital cortex of the control and cardiomyopathic (CMO) hamster at 25, 125 and 225 days.

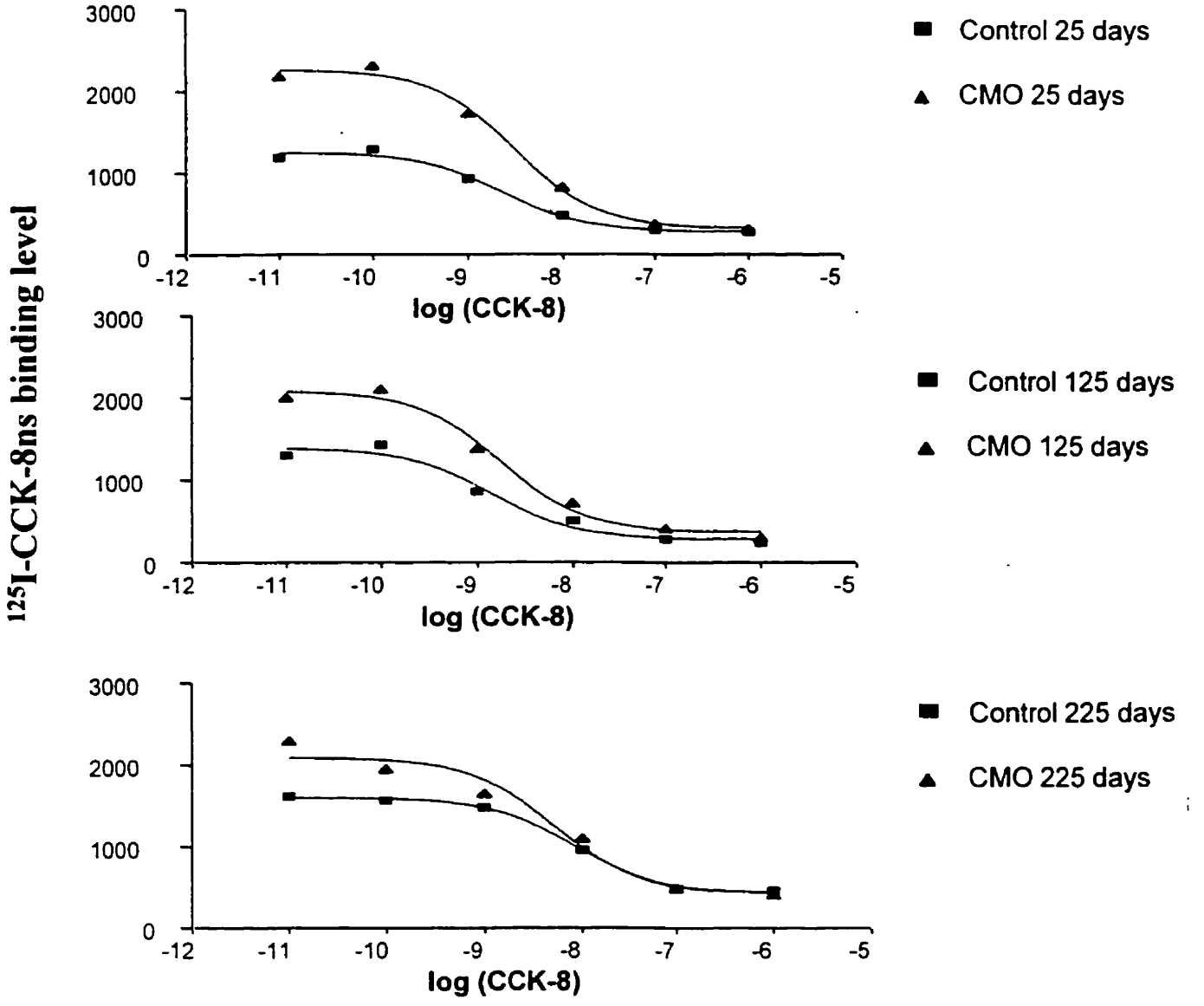

Figure 18. ^{125}I-CCK-8ns binding levels in the occipital cortex of the control and cardiomyopathic (CMO) hamster at 25, 125 and 225 days.

Table 12. Inhibition of ^{125}I Cholecystokinin binding by CCK-8ns in the occipital cortex of the control and cardiomyopathic (CMO) hamster brain. B1 and B2 (expressed in fmol/mg) represent high and low affinity binding levels respectively.

Age & Strain	B1	SE	p-value	B2	SE	p	EC_{50} (nM)	SE	p-value
Control 25 days	281.60	40.6	t=0.56	1255.00	44.46	t=12.69	2.24	0.13	t=0.79
CMO 25 days	325.20	65.79	p=0.59	2270.00	66.52	p<.001	3.01	0.10	p=0.45
Control 125 days	281.80	69.00	t=0.85	1388.00	83.67	t=5.68	1.40	0.20	t=0.38
CMO 125 days	369.60	77.41	p=0.43	2085.00	89.67	p=.001	1.74	0.14	p=0.71
Control 225 days	428.60	22.73	t=0.001	1603.00	18.27	t=3.93	0.81	0.05	t=0.87
CMO 225 days	429.90	137.70	p=0.99	2091.00	122.60	p<.01	5.01	0.23	p=0.42

10.7 Conclusions and Critical Analysis

Our investigation into the role of the cholecystokinergic system in cardiomyopathy revealed that neuronal cholecystokinin receptor density was altered in certain regions of the cardiomyopathic hamster brain. These initial observations suggest that cholecystokinergic dysregulation may play a role in cardiomyopathy. More comprehensive kinetic analysis, including binding and saturation data is required to substantiate these preliminary findings.

Further studies should also include an analysis of cholecystokinin peptide levels in normal and cardiomyopathic hamsters. Although the volume of plasma currently required by our assay precludes a determination of individual cholecystokinin peptide levels in single animals, blood from a homogenous population of hamsters could certainly be pooled to conduct adequate measurements. Data on cholecystokinin peptide levels would facilitate investigations into the peripheral or central nature of the cholecystokinergic dysfunction in cardiomyopathy. Additionally, peptide data would provide basic information as to the endogenous regulation of the cholecystokinin receptor, for example, whether receptor up-regulation or down-regulation is correlated with CCK peptide levels.

Finally, because the ultimate aim of our investigation into the role of cholecystokinin in cardiomyopathy is to ameliorate the disease condition in the human population, plasma cholecystokinin levels in human patients with cardiomyopathy should be evaluated. This type of study would not be difficult to undertake as large numbers of cardiomyopathic patients are already being diagnosed, monitored and

treated in hundreds of hospitals. Obtaining blood samples from these patients would not pose an undue burden for most patients or institutions. Indeed, if it is shown that the cholecystokinergic system contributes to cardiomyopathy, treatment strategies targeting cholecystokinin could rapidly be developed to impede the progression of the disease and to help improve the quality of life of a large number of affected individuals.

CHAPTER ELEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER ELEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

Cholecystokinin is an important gastrointestinal and neuropeptide that is involved in the regulation of physiological and behavioural processes. Aberrations of the cholecystokinergic system have been implicated in a variety of pathologies, including eating disorders, premenstrual dysphoric disorder, anxiety and cardiomyopathy. One useful strategy to investigate the role of cholecystokinin in these and other illnesses is to compare cholecystokinin levels in affected individuals with levels in healthy control subjects. However, prior to the developments reported in this dissertation, an adequately sensitive assay for specific cholecystokinin peptides did not exist. Accordingly, we constructed a novel assay system that could quantify the different forms of cholecystokinin found in human plasma, cerebrospinal fluid and other tissues. Our assay system, which involved the extraction of CCK fragments using reverse phase chromatography, the separation of peptides by high performance liquid chromatography and the detection and quantification of peptides with a double-antibody radioimmunoassay, is capable of detecting and discriminating among physiological concentrations of CCK-4, CCK-8s and CCK-8.

The first application of our cholecystokinin assay system entailed the quantification of plasma cholecystokinin in healthy human subjects. It was essential to first establish a profile of cholecystokinin levels in healthy subjects before embarking on

any studies which investigated cholecystokinin levels in disease. Using our assay system, we determined mean CCK-like immunoreactivity (CCK-LI) and levels of CCK-4, CCK-8s and CCK-8ns in 12 healthy subjects. The ability to measure each individual peptide represented a significant advance in the field of cholecystokinin research because it offered scientists an instrument to determine whether aberrant CCK ratios, particularly the CCK-4:CCK-8 ratio, are involved in the etiology of certain pathologies.

Although our assay system permits identification of individual peptide levels, future refinements to the assay should include techniques which are better able to concentrate plasma samples. At present, a significant volume of blood is needed to measure levels of individual cholecystokinin peptides. This limitation makes it difficult to obtain multiple samples at time short intervals, thus impeding detailed evaluation of time-course studies. However, given the low levels of cholecystokinin peptides present in baseline plasma samples, such improvements may simply be methodologically infeasible.

Further, although we found no effect of gender on plasma cholecystokinin levels, it would be advantageous for future studies to evaluate cholecystokinin levels in a larger population size and across a broader age range. This would be useful in establishing a more precise profile of cholecystokinin peptides in healthy human plasma. Moreover, a larger and more diverse study might also uncover any possible effects of race and age on cholecystokinin.

After developing the assay system for cholecystokinin, we collaborated with a number of international investigators for the joint purpose of examining CCK levels in

various psychiatric illnesses. In a preliminary study in women with the eating disorder bulimia nervosa, we discovered that fasted plasma CCK-LI levels were nearly five times lower in bulimic women as compared to healthy control subjects. At the post-binge stage, however, CCK levels in bulimics normalized to levels comparable to that of control women postprandially. These findings indicate that cholecystokinin may specifically contribute to the overeating phase in bulimia nervosa.

Because our study with bulimia nervosa was only a pilot study, an evaluation of a larger population of bulimic and healthy patients is essential. It would also be interesting to compare CCK levels in male bulimic subjects with levels in female bulimics. This may shed light not only on the biological underpinnings of bulimia in males, but also may help determine why females are at a much higher risk for bulimia than males.

Further, only total cholecystokinin-like immunoreactivity was measured in our bulimic study. For the reasons described above, it would be useful to analyze individual CCK peptide levels in plasma. Finally, it would valuable to conduct a parallel study in patients with anorexia nervosa. Because anorexia is a disease in which patients do not take in enough calories and bulimia is an illness in which patient ingest an excessive number of calories, one would predict that anorexic patients might have elevated levels of cholecystokinin, a well-established satiety hormone. It would also be intriguing to examine whether, after eating, cholecystokinin levels in anorectics also normalized.

More generally, studies which examine the role of cholecystokinin postprandially in healthy individuals or in patients with eating disorders could be useful in elucidating the role of cholecystokinin with respect to satiety. Such research may lead to therapeutic

strategies for obesity which specifically target cholecystokinin peptides or receptors. Accordingly, cholecystokinin, or nutrients which specifically increase cholecystokinin levels, may prove to be the next generation of appetite suppressants.

In addition to investigating bulimia nervosa, we also used our assay system to measure CCK levels in females with premenstrual dysphoric disorder (PMDD). In this study, we determined that PMDD subjects and healthy female volunteers had similar CCK-LI levels. We also discovered that plasma CCK levels were elevated in all subjects, irrespective of diagnosis, during their initial visit to the study site. These observations suggest that anticipatory anxiety may significantly increase plasma cholecystokinin levels, and must be accounted for as a confounding variable in studies that seek to measure plasma CCK levels. Finally, we found that plasma cholecystokinin levels did not vary with menstrual phase. This is a crucial discovery for researchers who were uncertain about the potential confounding effects of menstrual phase in their interpretation of cholecystokinin data, as well for those scientists who might not have otherwise included female subjects in their studies for fear that menstrual phase would distort cholecystokinin levels.

Because we measured only total cholecystokinin-like immunoreactivity in the PMDD study, it would be valuable to evaluate individual peptide levels in each subject. We were unable to do this in the reported study because the volume of plasma was insufficient: each person's blood sample had to be divided among various clinics in order to measure other hormones such as estrogen and progesterone. Although differences in total CCK-LI were not observed between PMDD and control women, this does not

preclude the finding that an altered CCK ratio is present in premenstrual dysphoric disorder. Evidence of altered CCK-4:CCK-8 values would only be uncovered if each peptide was quantified separately.

In another set of studies, we found that an oral dose of caffeine increased plasma CCK-LI levels 2 - 4 fold from baseline fasted levels in healthy human subjects. We observed substantial variation in post-caffeine cholecystokinin levels among individuals. Individual differences in response to caffeine suggest that caffeine-induced anxiety may occur via a cholecystokinin-mediated pathway, and that certain individuals may be more susceptible to the physiological and behavioural effects of caffeine.

In order to determine whether differential susceptibility to caffeine is related to an individual's history of caffeine consumption, a similar study should be performed which includes a larger number subjects, some of whom are heavy users of caffeine and some of whom are "caffeine-naïve." Further, to assess whether an individual's cholecystokinergic response to caffeine is dependent on his or her anxiety level, this study should be repeated in conjunction with standard psychological tests. For example, tests that measure anxiety levels should be given to each subject prior to caffeine consumption and at various time points thereafter. Accordingly, one could then determine whether cholecystokinin levels are correlated to an individual's baseline and post-caffeine anxiety level. Such studies would be useful in establishing a link between cholecystokinin and anxiety, as well as in providing a biological justification for discouraging caffeine consumption in patients with anxiety disorders. Finally, if caffeine reproducibly and safely increases cholecystokinin levels without triggering full-blown panic attacks, researchers should consider using

caffeine to challenge the cholecystokinergic system in first-degree unaffected relatives of patients with panic disorder. These studies would offer insight into whether a dysfunctional cholecystokinergic system is a marker of genetic vulnerability in panic disorder.

In another investigation into the role of the cholecystokinergic system in anxiety, we used our CCK assay to determine the effects of ondansetron, a 5-HT_3 serotonin receptor antagonist, on cholecystokinin levels in plasma. We found that chronic treatment with ondansetron affects the endogenous CCK and pharmacokinetic parameters of injected CCK tetrapeptide. These findings indicate that the cholecystokinergic and serotonergic systems may interact to modulate anxious behaviour via a 5-HT_3 receptor-mediated pathway.

An interesting experiment which would expand upon our findings in the ondansetron study consists of an evaluation of endogenous plasma cholecystokinin levels in two groups of patients with panic disorder — those treated with ondansetron and a placebo group. Because clinical studies have demonstrated that 5-HT_3 antagonists are effective in treating anxiety disorders, this type of study would help determine the extent to which the efficacy of serotonergic drugs is mediated by cholecystokinin. A similar study could also be performed after treatment with other types of pharmacological treatment, such as benzodiazepines. Finally, in the ondansetron study, basal plasma cholecystokinin levels were measured immediately prior to intravenous CCK-4 administration. From our study with subjects with premenstrual dysphoric disorder, we discovered that anticipatory anxiety, due to, for example, the perceived stress of receiving

an injection of CCK-4, could elevate plasma CCK levels. Accordingly, future studies should attempt to control for this anticipatory anxiety by measuring CCK levels at times where stress is minimal; for example, when subjects are comfortable with the clinical setting and on days when they are cognizant that they will not be receiving an injection of CCK-4.

In our investigation into the role of the cholecystokinergic system in cardiomyopathy, we found that neuronal cholecystokinin receptor density was altered in certain regions of the cardiomyopathic hamster brain. These finding suggest that cholecystokinergic dysregulation may play a role in cardiomyopathy. A valuable corollary to these findings would be an evaluation of cholecystokinin levels in the plasma and neural tissue of CMO hamsters. This may provide information as to the peripheral or central nature of the cholecystokinergic dysfunction, and additionally provide some basic information about the endogenous regulation of the receptor, for example, whether receptor levels are correlated with CCK levels. In addition, it would be intriguing to obtain data on plasma cholecystokinin levels in human patients with cardiomyopathy, in order to determine whether levels are altered in this disease.

Finally, investigations into the underpinnings of psychopathological or physiological illness in which a neurotransmitter dysfunction is suspected, should explore interactions between the key neurotransmitter systems, including the cholecystokinergic system. Current evidence suggests that some of these illnesses may not be associated with a single disorder in any one neurotransmitter, but rather an abnormality in the mutual modulation, or "cross-talk", between two or more of these systems.

In conclusion, the research presented in this dissertation has culminated in the development of an assay system for cholecystokinin peptides. In addition, this novel assay, along with other established techniques, has been used to investigate the role of the cholecystokinergic system in both normal physiology and pathology. These studies represent an important contribution to the field of cholecystokinin research by providing insight into the biology, clinical features and prognosis of the diseases studied as part of this doctoral research. Finally, it is hoped that these studies, together with the combined efforts of future cholecystokinin researchers, will ultimately result in prevention and treatment strategies of those disorders whose etiology is based in a dysfunctional cholecystokinergic system.

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